

**FARNESYLTRANSFERASE: GENE EXPRESSION IN PLANTS
AND ROLE IN PLANT DEVELOPMENT**

by

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Farnesyltransferase: Gene Expression in Plants and Role in Plant Development

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ABSTRACT

Protein farnesyltransferase (FTase, E. C. 2.5.1.21) post-translationally modifies regulatory proteins involved in controlling cell growth, division, and differentiation. Recently, a cDNA clone (*PsFTb*) encoding a pea (*Pisum sativum*) FTase subunit was isolated. Initial studies led to the hypothesis that FTase plays a role in the regulation of plant cell division.

To gain insight into FTase function in plants, a detailed study of the expression pattern of FTase genes was carried out. A cDNA (*NgFTb*) encoding the subunit of tobacco FTase was cloned from a *Nicotiana glutinosa* cDNA library to initiate studies in tobacco. In tobacco BY-2 suspension culture, levels of *NgFTb* mRNA and FTase activity transiently increased at the early log phase of cell growth and rapidly declined before cells entered stationary phase. These data, along with inhibitor studies in the BY-2 system, support our hypothesis. To understand the expression and regulation of pea FTase subunit genes, 5'-upstream sequences of both pea FTase subunit genes (*PsFTb* and *PsFTa*) were cloned from a pea genomic library. The 5'-upstream sequence (~2 kb) of *PsFTa* was fused to GUS (-glucuronidase) and GFP (green fluorescent protein) reporter genes and introduced into tobacco plants. This 2 kb upstream region appears insufficient to provide *PsFTa* promoter function. On the other hand, 3.2 kb of *PsFTb* 5'-upstream sequence expressed as a *PsFTb*:GUS construct is fully functional in transgenic tobacco plants. GUS expression was most prominent in actively growing cells supporting FTase involvement in plant cell cycle control. GUS activity was also found in mature and imbibed embryos but not premature embryos, consistent with the role of FTase in abscisic acid (ABA) signaling. An unexpected pattern of GUS activity, not correlated with dividing cells or ABA signaling, was also observed in the transgenic plants. GUS activity was detected in vascular bundles adjacent to actively-growing tissues and in regions that connect two organs, *e.g.*, junctions between stems and leaf petioles, cotyledons and hypocotyls, roots and hypocotyls. Auxin promotes *PsFTb* expression while light and sucrose inhibit expression. These spatial and temporal expression patterns strongly suggest that FTase has a broader role associated with regulation of nutrient transportation or allocation in plants.

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DEDICATION

This dissertation is dedicated to my parents,
Changying Jing and Jingxuan Zhou

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CHAPTER I.
LITERATURE REVIEW

I.1. INTRODUCTION

Posttranslational modifications modulate the conformation and function of many proteins that are involved in a variety of essential biological processes. Several types of modification are well known, including phosphorylation, glycosylation, acylation, and methylation (Casey *et al.*, 1992; 1994; Schafer and Rine, 1992). Protein prenylation, a novel posttranslational modification, involves the covalent attachment of either a 15-carbon farnesyl or a 20-carbon geranylgeranyl isoprenoid to the carboxy-terminal cysteine residue(s) of target proteins via a thioether bond. (Hancock *et al.*, 1989; 1991a; 1991b).

Although the first report of protein prenylation came from studies of specific fungal mating factors in the late 1970s (Kamiya *et al.*, 1978), the overall significance of protein prenylation in controlling cellular processes was not recognized until recently. The first indication of an important role for protein prenylation came from the studies of inhibitors that block the isoprenoid pathway in mammalian cells (Schmidt and Glomset, 1982; Fairbanks *et al.*, 1984). Subsequent discovery of prenylated proteins in mammals led to extensive investigation of this unique posttranslational modification (Beck *et al.*, 1988; Wolda and Glomset, 1988; reviewed in Zhang and Casey, 1996).

In the past few years, tremendous progress has been made in characterization of the enzymes involved in protein prenylation and their target proteins. Protein prenylation is found in yeast, mammals, and plants, and thus, is likely to be ubiquitous in eukaryotes. Known prenylated proteins include yeast mating pheromones, small GTP binding proteins (*e.g.*, the Ras superfamily), the γ -subunit of the heterotrimeric G protein, nuclear lamins (lamin B and prelamin A), cGMP phosphodiesterase, and rhodopsin kinase (reviewed in Clarke, 1992; Marshall, 1993; Cates *et al.*, 1997). Many of these proteins are involved in the regulation of cellular functions, *e.g.*, cellular signaling, intracellular trafficking, cytoskeletal organization, cell growth and polarity, viral replication and protein folding/assembly. Thus, protein prenylation has a central role in controlling diverse cellular processes in eukaryotic cells ([Figure I.1](#)).

I.1.1. Protein prenylation and the isoprenoid pathway

Isoprenyl moieties transferred to proteins are derived from the isoprenoid pathway which is also called the mevalonic acid (MVA) pathway, named after the product of the first committed step in the pathway. This pathway leads to the production of a large number of important compounds ([Figure I.2](#)). To date, more than 22,000 compounds have been identified in this diverse family of isoprenoids (Conolly and Hill, 1992; Bach, 1995). Farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP) are two intermediate isoprenoid compounds known to be transferred to proteins. Other biological important isoprenoid molecules include cholesterol and other sterols, carotenoids, sesquiterpenes, α -tocopherol and dolichols. The basic structural unit of all the isoprenoids is isoprene, a five carbon building block.

In early steps of the MVA pathway, sequential condensation of three acetyl-CoA units generates 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA). HMG-CoA is converted to mevalonate, a 6-carbon molecule, in an irreversible reaction catalyzed by HMG-CoA reductase (HMGR). Because of its irreversible nature, this reaction is a rate-limiting step in the isoprenoid pathway (Goldstein and Brown, 1990; Chappell, 1995). It has been shown, at least in mammalian systems, that this is the major regulatory point for biosynthesis of cholesterol, an end product of the pathway. The precursor of all isoprenoids, isopentenyl pyrophosphate (IPP), is formed by two

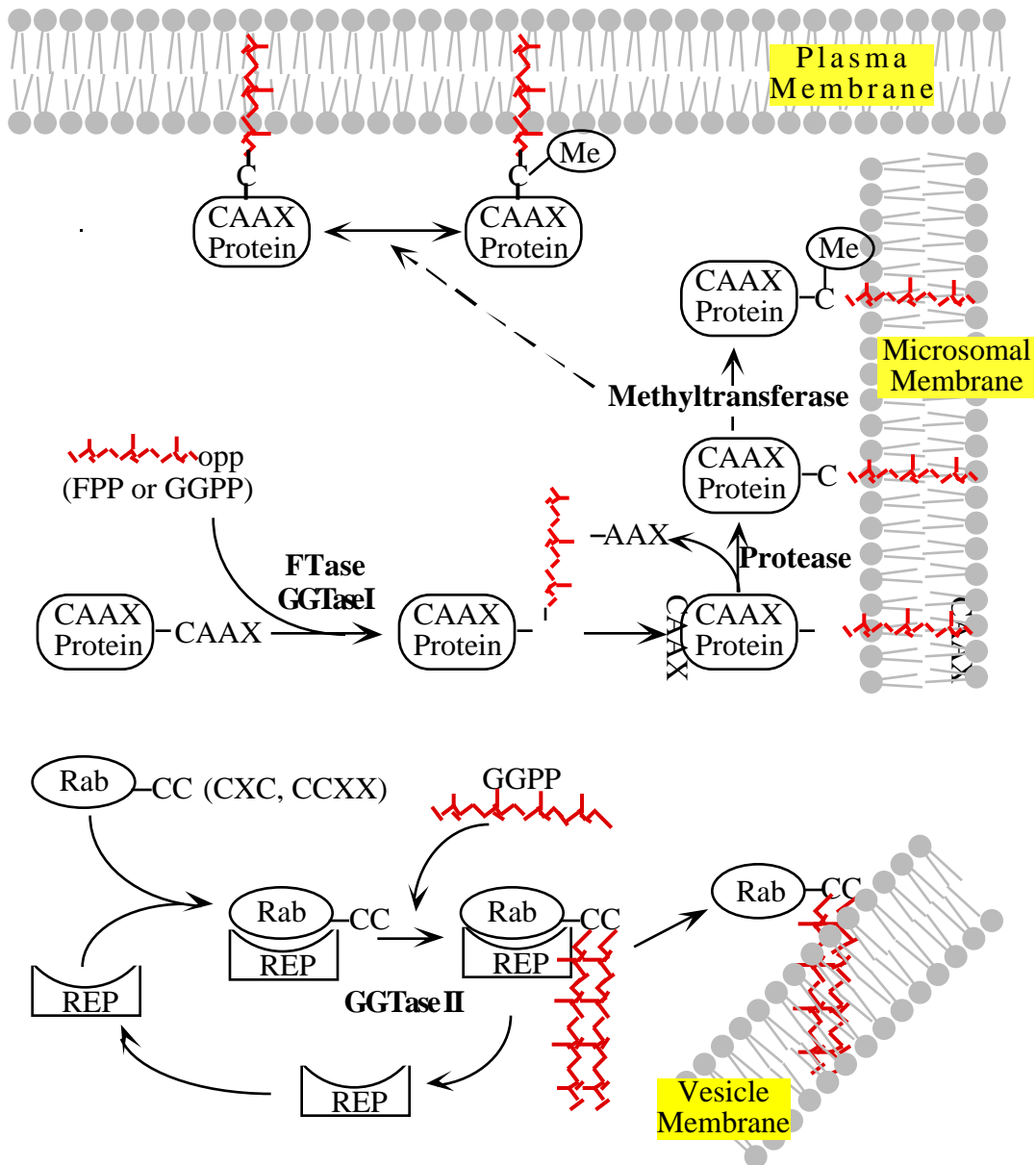


Figure I.1. Overview of protein prenylation in cells
(modified from Zhang and Casey, 1996)

FPP, farnesyl-pyrophosphate; GGPP, geranylgeranyl-pyrophosphate; C, cysteine; A, aliphatic amino acid; FTase, farnesyltransferase; GGTase, geranylgeranyltransferase; Me, methyl moiety; REP, Rab escort protein.

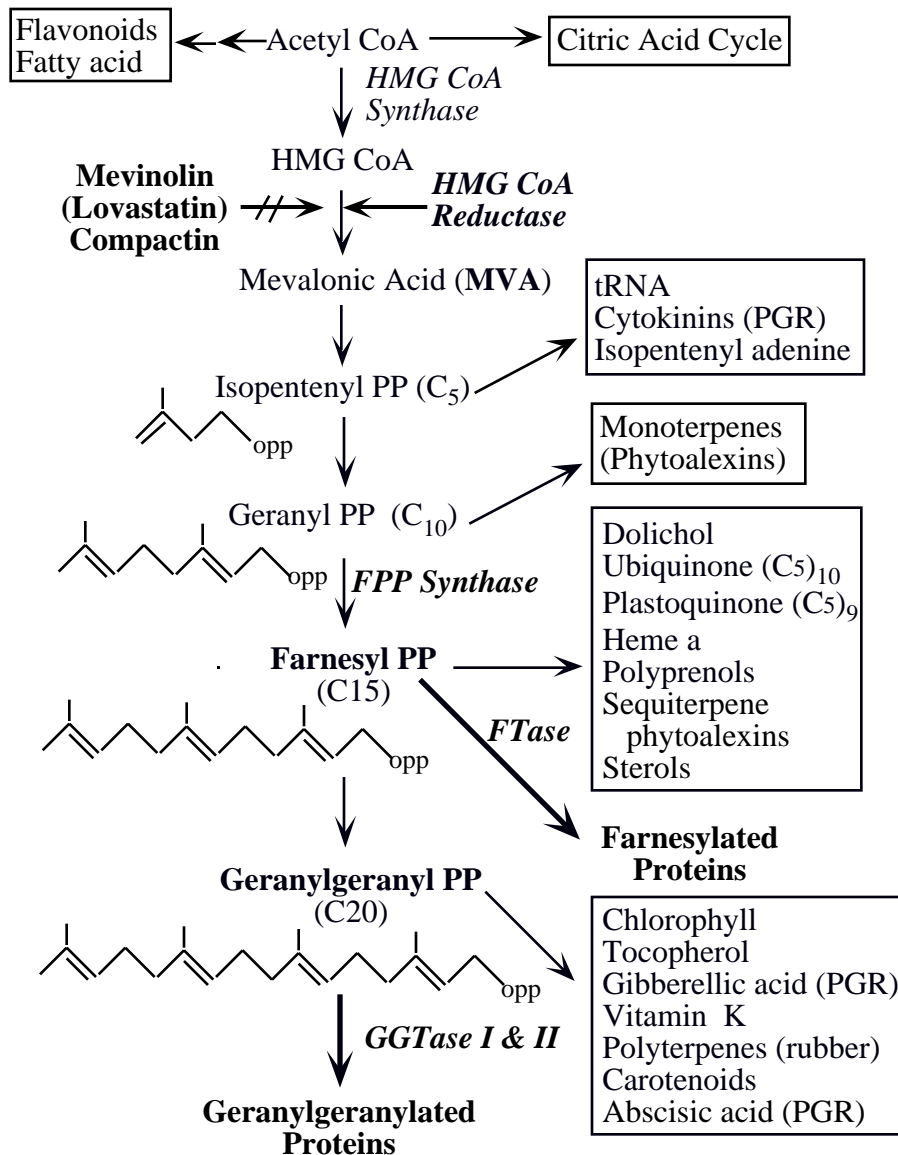


Figure I.2. An outline of isoprenoid (MVA) pathway.

The key intermediates in the prenylation of proteins are shown in bold.

HMG CoA: 3-hydroxy-3-methylglutaryl coenzyme A; PP: pyrophosphate; PGR: plant growth regulator.

successive phosphorylations of mevalonate with ATP followed by an ATP-dependent dehydration and decarboxylation. The polymerization of one IPP and one DMAPP (dimethylallyl pyrophosphate, an isomer of IPP) in a head-to-tail fashion produces 10-carbon geranyl pyrophosphate (GPP). The addition of another IPP molecule to GPP generates a farnesyl pyrophosphate (FPP). Besides acting as a substrate for protein farnesylation, FPP is the precursor for sterols, dolichol, and ubiquinone and is produced by a single enzyme FPP synthase. It is believed that FPP synthase is also responsible for the synthesis of GPP (Chappell, 1995). Studies on purified FPP synthase showed more than 99% of the product generated is FPP, while only a trace amount is GGPP (geranylgeranyl-pyrophosphate) (Sinensky and Lutz, 1992). Additional polymerization reactions give rise to GGPP and other isoprenoids. GGPP is used in protein geranylgeranylation, another form of protein prenylation. Also, many end products of the MVA pathway, such as gibberellins, abscisic acid and carotenoids, are derived from GGPP. These polymerization reactions, unlike the more general nucleophilic condensations in other biosynthetic pathways, are electrophilic in nature. They involve the attack of a carbocation (*i.e.*, loss of pyrophosphate to give an electron-deficient carbon atom) of one substrate molecule to an electron-rich atom of a double bond on the other IPP molecule (Chappell, 1995).

Plant isoprenoids comprise the largest number and diversity within all the isoprenoids. For instance, more than 470 isoprenoid derivatives have been identified in tobacco alone (Wahlberg and Enzell, 1987). There are many unique and structurally diverse metabolites in the plant isoprenoid pathway, such as monoterpene, cytokinins, gibberellins, and abscisic acid. These products function in photosynthesis, respiration, membrane architecture, regulation of growth and development, and defense against pathogen invasion (reviewed in Chappell, 1995). It is therefore reasonable that the isoprenoid pathway in plants is much more intricate than that in other eukaryotes. There are many more branch pathways in the later steps involved in the production of diverse end products. Consistent with the diverse functions of the plant MVA pathway, many plants have a HMGR gene family in contrast to the single HMGR gene found in animals. New evidence even indicates that plants have an alternative pathway for IPP synthesis, which resembles the bacterial isoprenoid biosynthetic pathway (Rohmer Pathway) (Rohmer *et al.*, 1993; Bach, 1995). Nevertheless, early steps in the isoprenoid pathway leading to the synthesis of FPP and GGPP commonly exist in all eukaryotes.

I.1.2. Protein prenylation and prenyltransferases

Three distinct protein prenyltransferases, classified into two functional classes, are known to catalyze protein prenylation. All are heterodimeric enzymes composed of an α and a β subunit. The enzymes have been extensively studied at the gene and protein levels in yeast and mammalian systems. Each prenyltransferase modifies proteins with a unique C-terminal motif (Table I.1) (Kohl *et al.*, 1991; Moores *et al.*, 1991; Chen *et al.*, 1991a; 1991b).

The first functional class, termed the CAAX (C, cysteine; A, aliphatic amino acids; X, any amino acid except leucine or phenylalanine) motif prenyltransferases, includes protein farnesyltransferase (FTase) and protein geranylgeranyltransferase type I (GGTase I). Genetic and biochemical analyses revealed that FTase and GGTase I share an identical β subunit, whereas the α subunit is unique for each enzyme (Reiss *et al.*, 1991b; Seabra *et al.*, 1991; Zhang *et al.*, 1994). FTase transfers a farnesyl molecule from FPP to the cysteine residue of proteins with CAAX at their carboxyl termini. If X is a leucine or phenylalanine, the protein will be recognized by GGTase I and a geranylgeranyl group from GGPP will be attached to the cysteine residue

Table I.1. Classes of Known Protein Prenyltransferases

Enzyme	Substrate		Subunits	Corresponding Genes				
	C-terminal Sequences	Isoprenoid		Yeast	Mammals (rat brain)	Plant		
						Pea	Arabidopsis	Tomato
FTase	CAAX (X: M,S,C,A,Q)	FPP	RAM2	F/GG	PsFTa*	?	LeFTa**	
			RAM1	F	PsFTb*	ERA1	LeFTb**	
GGTase I	CAAX (X: L, F)	GGPP	RAM2	F/GG	?	?	?	
			CDC43	GG	?	?	?	
GGTase II	CC, CXC, CCXX, etc.	GGPP	BET4		?	?	?	
			BET2		?	?	?	
			Component B	?	REP	?	?	?

Note: modified from Casey and Seabra, 1996.

Abbreviations: see Figure I.1.

*: used in this study.

** : Gruissem *et al.*, unpublished data.

(Vorburger *et al.*, 1989; Casey *et al.*, 1991; Moores *et al.*, 1991; Reiss *et al.*, 1991b). Under physiological conditions, CAAX prenylation is followed by proteolysis of the last three amino acids (AAX) and subsequent methylation of the C-terminal prenylated cysteine residue (Gutierrez *et al.*, 1989) (Figure I.3).

The other class of protein prenyltransferases is GGTase II. It is also called Rab geranylgeranyl-transferase since it modifies Rab proteins (Ras-related GTPases). Distinct from FTase and GGTase I, GGTase II requires a third component, REP (Rab Escort Protein) factor, for its activity (Seabra *et al.*, 1992). The heterodimer is considered the actual enzymatic component, whereas REP functions by forming a complex with Rab substrate proteins and escorting the complex to the catalytic dimer of GGTase II (Andres *et al.*, 1993). Target proteins for GGTase II have the carboxy-terminal sequence CC, CCXX or CXC. An unidentified internal sequence is also required in target proteins for this kind of geranylgeranylation (Seabra *et al.*, 1992). The geranylgeranyl group from GGPP is transferred to both cysteines at the C-terminus. GGTase II-catalyzed reactions are mechanistically different from that for the proteins containing CAAX. In addition, proteins with the CXC motif are methylated at the C-terminal prenylcysteine, whereas CC- and CCXX-containing proteins are not (Farnsworth *et al.*, 1991; Khosravi-Far *et al.*, 1991; Kinsella and Maltese, 1992).

I.2. FARNESYLATION AND FARNESYLTRANSFERASE

Although protein geranylgeranylation is the most common form of prenylation found in nature, protein farnesylation has been the major focus of protein prenylation studies. The first evidence for protein prenylation came from farnesylation of certain fungal mating factors (*e.g.*, rhodotorucine A from *Rhodospiridium toruloides* and **a**-mating factor from *Saccharomyces cerevisiae*) (Kamiya *et al.*, 1978; Ishibashi *et al.*, 1984; Anderegg *et al.*, 1988). Nuclear lamin B, the first prenylated protein identified in mammals, is modified by a farnesyl isoprenoid (Wolda and Glomset, 1988; Beck *et al.*, 1988). However, the discovery that Ras proteins are farnesylated attracted more attention to the study of farnesylation. Farnesylation is required for the activities of the oncogenic forms of these proteins (Casey *et al.*, 1989; Hancock *et al.*, 1989). Consequently, protein farnesyltransferase is so far the most extensively studied enzyme among the three protein prenyltransferases.

I.2.1. Enzymology of protein farnesyltransferase (FTase)

In animals and fungi, genetic and biochemical approaches have contributed enormously to our understanding of FTase at both the gene and protein levels. It has been found that FTase is a soluble heterodimeric enzyme in both systems and is composed of α and β subunits (Casey *et al.*, 1994). Pioneering research using *Saccharomyces cerevisiae* led to the discovery that the *RAM1/DPRI* gene encodes the β subunit of the yeast FTase (Powers *et al.*, 1986; Fujiyama *et al.*, 1987). The gene was identified based on its involvement in **a**-factor processing and as a suppressor of G protein function (Schafer *et al.*, 1990). A second gene known as *RAM2* was found to encode the α subunit of yeast FTase (Goodman *et al.*, 1990; He *et al.*, 1991). The two subunits form a stable complex and both are required for catalytic activity. Mutations in *RAM1/DPRI* or *RAM2* abolish FTase activity. Yeast *RAM1/DPRI* mutants are defective in cell

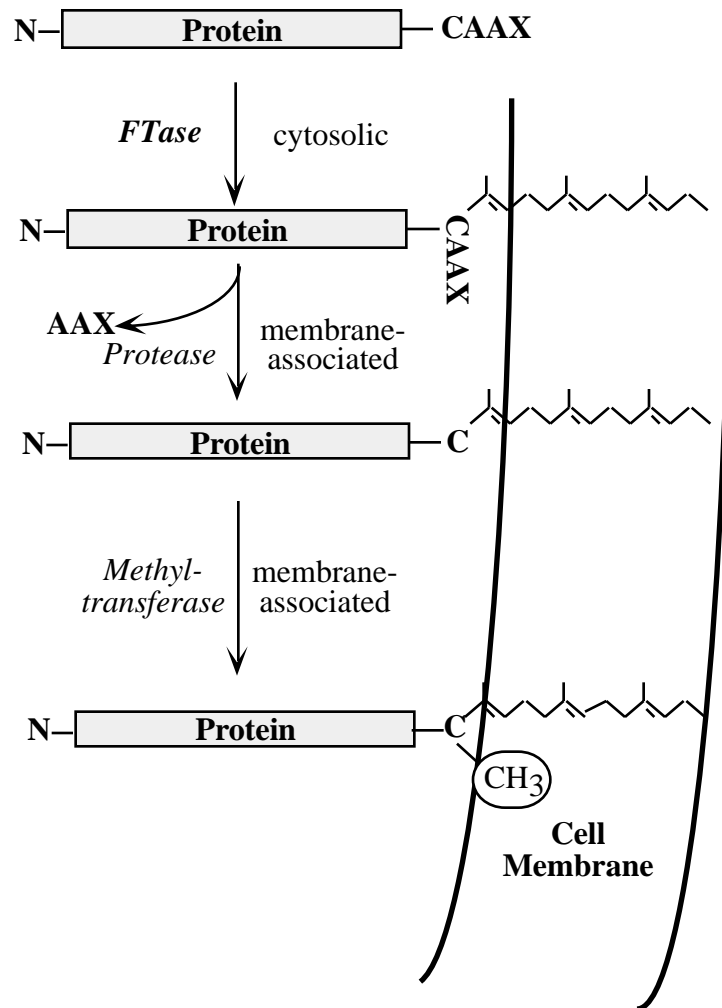


Figure I.3. Steps in processing of the carboxy-terminus of farnesylated proteins.

Abbreviations: see Figure I.1.

division and mating; mutations in *RAM2* are lethal. Co-expression of *RAM1/DPRI* and *RAM2* in *Escherichia coli* produces FTase activity that can farnesylate the yeast **a** factor peptide and Ras protein substrates *in vitro* (Goodman *et al.*, 1988; 1990; Schafer *et al.*, 1990; He *et al.*, 1991).

The first mammalian FTase was identified from rat brain cytosol (Reiss *et al.*, 1990). Like the yeast counterpart, it has two subunits consisting of two nonidentical peptides, 48 kD_{F/GG} and 46 kD_F. The nomenclature of_{F/GG} is chosen since the_{F/GG} subunit is shared by FTase and GGTase I. These two subunits exhibit 30% and 37% amino acid identity to the_F and_{F/GG} subunits of yeast FTase, respectively. Similar to the yeast system, co-expression of mammalian FTase subunits in HEK 293 cells, *E. coli*, and baculovirus-transfected Sf9 cells yields a functional enzyme (Chen *et al.*, 1991b; Chen *et al.*, 1993; Omer *et al.*, 1993). In addition to the rat FTase proteins, cDNA clones encoding mammalian FTase have been isolated from bovine and human libraries (Kohl *et al.*, 1991; Omer *et al.*, 1993).

All FTases identified so far are zinc metalloenzymes (Casey and Seabra, 1996). Mg²⁺ and Zn²⁺ are required for their activities. The Zn²⁺ is a component of the enzyme itself and is required for binding of the substrate peptide but not the isoprenoid moiety (Moomaw and Casey, 1992; Reiss *et al.*, 1992). Measurement of zinc content of recombinant FTase confirmed that there is one mole of zinc per mole of enzyme (Chen *et al.*, 1993). It remains to be elucidated if zinc plays a structural role or is directly involved in catalysis. One speculation for the catalytic role of zinc is that it could activate the sulfhydryl of the cysteine residue on the target protein and make it more nucleophilic, similar to the “metalloactivation of cysteine” mechanism demonstrated in a DNA repair enzyme *Ada* (Myers *et al.*, 1995). In contrast, the strict requirement for very high amounts (millimolar) of Mg²⁺ for full FTase activity suggests it is probably not an integral component of FTase. The Mg²⁺ ion may contribute to the binding of the pyrophosphate moiety of the FPP (Moomaw and Casey, 1992; Reiss *et al.*, 1992).

A surprising property of FTase is its ability to recognize short peptides. Simple tetrapeptides consisting of only the CAAX motif sequence were found to be as effective as substrates as the parental proteins from which they were derived (Reiss *et al.*, 1990). This unique property has proven useful in studying FTase and has led to the identification of a class of potent FTase inhibitors--CAAX peptide analogs (discussed below) (Tamanoi, 1993; Kohl *et al.*, 1995).

FTase can bind target proteins or FPP independently (Reiss *et al.*, 1991b). Binding of FPP is of such high affinity that the complex can be isolated by gel filtration. However, it was shown that no covalent adduct forms since intact FPP is released upon enzyme denaturation (Reiss *et al.*, 1991b). *In vitro* experiments showed that protein and peptide substrates can be cross-linked to the_{F/GG} subunit (Reiss *et al.*, 1991b; Ying *et al.*, 1994) as well as a photoactivated FPP analog (Omer *et al.*, 1993). These results suggest that both substrates are predominately bound to the_{F/GG} subunit of FTase. However, since divalent affinity-labeled short peptide substrates were cross-linked to both the_F and_{F/GG} subunits of FTase upon photoactivation, the actual binding site for the peptide substrate may reside near the interface of the two subunits (Reiss *et al.*, 1991b; 1992).

Steady-state kinetic studies suggest that FTase proceeds through an ordered, sequential mechanism with FPP binding first (Gomez *et al.*, 1993; Pompliano *et al.*, 1993; Yokoyama *et al.*, 1995). The binding of FPP to FTase is a two-step process, with the second step presumably involving a conformational change in the FTase-FPP complex. FTase-FPP rapidly reacts with the protein substrate to form the product (Dolence *et al.*, 1995). The release of the product is believed

to be a rate-limiting step in catalysis. Although kinetic analysis indicates that FTase has distinct binding sites for its two substrates, direct evidence for the formation of a ternary complex is lacking.

Molecular approaches have been used to study the structure and function of FTase. Comparison of available gene sequence data on mammalian and yeast FTase subunits reveals that there are five tandem repeats, in which four residues, asparagine, arginine, glutamate, and tryptophan, are highly conserved (Boguski *et al.*, 1992; Feng and Kung, 1993). Mutations of the four residues in the mammalian FTase subunit gene impair enzyme activity, but do not affect the formation of heterodimer complexes. Moreover, other mutation and deletion experiments in mammalian systems have led to the identification of additional key amino acids in the subunit that are required for FTase activity (Andres *et al.*, 1993; Omer *et al.*, 1993). These data demonstrate an important role for the subunit in enzyme catalysis.

The subunit of FTase may also be involved in the regulation of FTase enzyme activity. Using the yeast two-hybrid method for identifying protein-protein interactions and the transforming growth factor- (TGF-) type I receptor as bait, a gene encoding an subunit of FTase was isolated from a human fetal brain library (Wang *et al.*, 1996). The gene product interacted specifically with the type I receptors of TGF- and activin. The N-terminal 81 amino acids of the subunit, which were important for the activity of FTase in mammalian cells, were also critical for the binding of receptors (Andres *et al.*, 1993). It was suggested that the specific binding of the TGF- type I receptor to the FTase subunit may regulate FTase activity (Wang *et al.*, 1996)

Point mutations showed that the conserved residues of mammalian FTase subunit are also important for enzyme function. Mutations in the conserved subunit residues corresponding to known yeast mutants (*e.g.*, D200, G249, G349) impair FTase activity (Omer *et al.*, 1993). However, the mutant proteins still form heterodimers with the subunit, suggesting the binding sites of two subunits in subunit are separated from the catalytic regions.

Although the binding sites of FPP and the target protein are indicated in FTase subunit (Reiss *et al.*, 1991b; 1992), the precise mechanism for substrate binding still needs to be determined. Fundamental questions, such as what is the function of each subunit and how the two subunits interact each other also remain to be answered. More detailed mutational analyses should provide a better understanding of the mechanism of FTase catalysis and regulation.

I.2.2. Farnesylation signals

The fact that the CAAX tetrapeptides can act as substrates for farnesylation suggests that only these four C-terminal residues form the recognition site in the protein substrate. This property of FTase facilitates precise delineation of optimal sequences within this peptide required to direct farnesylation (Moores *et al.*, 1991; Reiss *et al.*, 1991a; 1991b).

Amino acid substitution of CAAX in tetrapeptides as well as native proteins for farnesylation revealed that the cysteine of the CAAX motif was the most critical residue for farnesylation. Replacement with a serine residue, or movement of the cysteine to other positions, completely abolished the farnesylation of the protein or tetrapeptides (Reiss *et al.*, 1991a; 1991b). X in the C-terminus is the second most important residue in the CAAX motif. This residue determines if a farnesyl or a geranylgeranyl moiety is attached to target proteins. For farnesylation, the preferred residues are methionine, alanine, cysteine, serine, and glutamine, but not leucine or phenylalanine. While many amino acids with basic or aromatic side chains can be tolerated in the first A position, the preferred second A (nearest to the C-terminal) residues are the aliphatic amino acids (*e.g.*, isoleucine). Substitution with an aromatic residue (*e.g.*, phenylalanine or tyrosine) at the second A markedly reduces the binding affinity (Goldstein *et al.*, 1991; Chen *et al.*, 1993).

I.2.3. Farnesylated proteins and their biological functions

All farnesylated proteins identified so far contain the C-terminal CAAX signal (Schafer and Rine, 1992). These proteins represent a diverse group of proteins in yeast and mammalian systems, including yeast mating pheromones, nuclear lamins, Ras small GTP binding proteins, heterotrimeric large G-proteins, phosphatases, cGMP phosphodiesterase, rhodopsin kinase, and a peroxisomal protein (from rat) of unknown function termed PxF (Table I.2) (Anant *et al.*, 1992; Inglese *et al.*, 1992; James *et al.*, 1994b; Cates *et al.*, 1997).

Yeast mating pheromones

Yeast mating pheromones (also referred to as yeast mating factors) are diffusible substances released by haploid fungal cells to trigger cell cycle arrest and cell fusion to form diploid zygotes. The fusion is restricted to two haploid fungal cells with opposite mating type (**a** and α). The first identified prenylated protein belongs to the mating pheromone family (Kamiya *et al.*, 1978). The most extensively characterized mating pheromone is **a**-factor of yeast.

Yeast (*S. cerevisiae*) has two **a**-factor genes, *MFA1* and *MFA2*. The products of *MFA1* and *MFA2* (36 and 38 amino acids, respectively) both have the C-terminal consensus sequence CAAX and undergo a series of modifications before being secreted as active **a**-factors. The modification processes, independent of the classic secretory pathway, include farnesylation of the cysteine residue at the C-terminal CAAX, removal of a 21 amino acid *pro* sequence at the amino terminus, cleavage of the AAX, and methylation of the newly generated carboxyl terminus (Anderegg *et al.*, 1988).

In vitro studies of the processing of **a**-factor demonstrated that the four steps of modification occur in a defined order (Schafer *et al.*, 1989; 1990; Hrycyna and Clark, 1990; Ashby *et al.*, 1992). Conserved within most farnesylated proteins, farnesylation is a precondition for the subsequent proteolytic cleavage and carboxy-terminal methylation. Furthermore, accumulation of the **a**-factor precursor containing the *pro* sequence in mevalonate-starved cells suggested that farnesylation is required for the proteolytic removal of the amino terminus (Schafer *et al.*, 1989). Since the precursor forms of **a**-factors accumulate in the cytosol, farnesylation and proteolysis appear to be essential for secretion of the pheromones. On the other hand, because non-methylated **a**-factors are readily secreted, methylation may not be required for secretion (Schafer *et al.*, 1989; Schafer and Rine, 1992). In addition, the farnesyl isoprenoid is important for the ability of the **a**-

Table I.2. Prenylated proteins in yeast and mammalian cells

Protein	C-terminal Sequence	Modification
<u>Fungal mating pheromones</u>		
<i>Saccharomyces cerevisiae</i> a -factor	DPACVIA	farnesylation
<i>Tremella brasiliensis</i> (A-9291-I)		farnesylation
<i>Tremella mesenterica</i> (A-10)		farnesylation
<i>Rhodospiridium toruloides</i> Rhodotorucine A	RNGCTVA	farnesylation
<u>Nuclear lamins</u>		
chicken prelamin A	PQGCSIM	farnesylation
human prelamin A	PQNCSIM	farnesylation
human lamin B	NRSCAIM	farnesylation
chicken lamin B1	ERSCVVM	farnesylation
chicken lamin B2	SRGCLVM	prenylation*
Chinese hamster ovary lamin B	NKSCAIM	prenylation*
<u>Ras related small G-proteins</u>		
human rap1A	KKSCLLL	geranylgeranylation
human rap1B	KSSCQLL	geranylgeranylation
human rap2	CSACNIQ	prenylation*
human rac 1	KRKCLLL	geranylgeranylation
human rac2	KRACSLL	geranylgeranylation
human rab2	GGGCC	geranylgeranylation
human/bovine rab3A (smg p25A)	HQDCAC	geranylgeranylation
human rab5	RNQCCSN	geranylgeranylation
RhoB	CINCKVL	prenylation*

Table I.2. Prenylated proteins in yeast and mammalian cells (continued)

Protein	C-terminal Sequence	Modification
<u>Ras proteins</u>		
human/mouse H-ras	SCKCVLS	farnesylation
human K-ras-4B	KTKCVIM	farnesylation
human N-ras	GLPCVVM	farnesylation
<i>Saccharomyces cerevisiae</i> RAS1	SGGCCIIIC	farnesylation
<i>Saccharomyces cerevisiae</i> RAS2	SGGCCIIS	farnesylation
<u>Heterotrimeric large G-proteins</u>		
<i>Saccharomyces cerevisiae</i> STE18 (subunit)	SVCCTLM	farnesylation
bovine transduction (subunit)	KGGCVIS	farnesylation
bovine brain G-protein (subunit)	KFFCAIL	geranylgeranylation
<u>Other proteins</u>		
Rhodopsine kinase	KSGMCVLS	farnesylation
cGMP phosphodiesterase (subunit)	ASKSCCIQ	farnesylation
YDJ1 (yeast dnaJ)	EGVQCASQ	farnesylation
PxF	CLIM	farnesylation**

Note: modified from Cox and Der, 1994; Clarke, 1992.

* can be farnesylated or geranylgeranylated *in vitro* (Clarke, 1992).

** from James *et al.*, 1994b.

factor to induce growth arrest and morphological alteration of yeast cells of the mating type (Anderegg *et al.*, 1988).

However, other pheromones are modified differently, *e.g.*, rhodotorucine A from the basidiomycetous jelly fungus *Rhodospiridium toruloides*, and tremerogen A-10 from the jelly fungus *Tremella mesenterica*. Rhodotorucine A is an 11-amino acid peptide derived from its much larger pheromone precursors. These precursors, encoded by three genes, contain three to five tandem copies of the rhodotorucine A peptide separated by a four-amino acid motif, (C)-T-V-A/S-K. The maturation of rhodotorucine A consists of cleavage of the precursors into multiple pheromone peptides followed by processing similar to **a**-factor precursor. In contrast to **a**-factor, rhodotorucine A is not methylated at its carboxy-terminal cysteine (Akada *et al.*, 1989).

Tremmerogen A-10 is synthesized as a large precursor of an unknown primary sequence. The active pheromone is a 10-amino acid peptide in farnesylated and carboxy-methylated form. Different from the common farnesylation, the farnesyl group contains an extra alcohol group (Sakagami *et al.*, 1981). Not only compactin (an inhibitor of HMGR to block the isoprenoid pathway), but also tunicamycin and monensin (inhibitors of glycosylation and Golgi transport, respectively), inhibit the maturation of tremmerogen A-10. This data indicates that the tremmerogen A-10 precursor is glycosylated and processed in the Golgi (Miyakawa *et al.*, 1985). Since farnesylation is an early modification step and may occur in the cytoplasm, it may play a role in targeting the tremmerogen A-10 to the endoplasmic reticulum.

In addition to examples discussed above, a wide variety of evolutionarily divergent fungal species secrete prenylated pheromones. For example, tremmerogen -13 from jelly fungus *Tremella mesenterica* has a covalently linked farnesyl moiety, M-factor from the fission yeast, *Saccharomyces pombe*, appears to have the same processing as **a**-factor, and A-9291-I from *Tremella brasiliensis* contains an oxidized farnesyl group (Davey, 1992; Caldwell *et al.*, 1995). Prenylated pheromones therefore may be ubiquitous among the fungi.

Nuclear lamins

Nuclear lamins are a class of proteins that are a major component of the karyoskeleton. They are believed to form intermediate filaments that associate with the inner surface of the nuclear membrane and mediate nuclear breakdown and reformation during cell mitosis (Moir *et al.*, 1995). There are three types of lamin proteins in eukaryotes: A-, B-, and C-type. Whereas A- and C-type lamins are found in soluble form during mitosis, B-type lamins are always associated with the nuclear membrane (Franke, 1987).

Nuclear lamin was the first prenylated protein discovered in mammalian cells. Shortly after farnesylated yeast mating factors were identified, studies by other investigators showed that in fibroblasts high concentration of compactin, an inhibitor of HMGR, can block passage through the cell cycle and alter cell morphology (Quesney-Huneus *et al.*, 1979; Habenicht *et al.*, 1980). Both effects could be prevented by the addition of mevalonic acid but not cholesterol, an end product of the mammalian isoprenoid pathway. Furthermore, independent experiments showed that a product of mevalonate metabolism other than cholesterol was required for the cells to enter S phase (Schmidt and Glomset, 1982; Fairbanks *et al.*, 1984). Subsequently, the search for the required product revealed that metabolites of mevalonic acid are incorporated into a group of proteins (Schmidt *et al.*, 1984; Maltese and Sheridan, 1987). One of the proteins was identified as the

nuclear envelop-protein lamin (Beck *et al.*, 1988; Wolda and Glomset, 1988), and the metabolite species was shown to be a farnesyl molecule (Farnsworth *et al.*, 1989).

It is now known that mammalian lamin and prelamins A contain a C-terminal CAAX sequence, and undergo posttranslational processing involving farnesylation. The modifications are essential for the biological activities of lamins in the mitotic control of membrane assembly (Lutz *et al.*, 1992). The modification processes of lamin resemble that of Ras proteins. The first step is to attach a farnesyl moiety to the cysteine of the CAAX, followed by removal of the three C-terminal amino acids (AAX) (Wolda and Glomset, 1988; Gutierrez *et al.*, 1989; Vorburger *et al.*, 1989). Evidence also shows that lamin is carboxy-methylated. Although the methylation appears to be transient, it actually may have a role in regulating the disassembly of the nucleus during mitosis (Chelsky *et al.*, 1987).

The processing of lamin A is more complex and requires proteolytic cleavage from a precursor protein, prelamins A. Farnesylation of prelamins A at its cysteine residue of the CAAX motif is necessary before further removal of the 18 carboxy-terminal amino acids, including the farnesylated CAAX sequence. The mature form of lamin A, assembled into the nuclear lamina, is thus not farnesylated (Beck *et al.*, 1990). Further studies by Lutz and his colleagues (1992) suggested that the farnesylation of prelamins A occurs in the nucleus instead of cytosol. They observed that unfarnesylated prelamins A accumulated in nuclear inclusions in mevalonate-starved cells. Upon addition of mevalonate, the accumulated molecules were rapidly processed and assembled into the nuclear lamina. The cleavage of 18 C-terminal amino acids from prelamins A to release mature lamin A implies that the C-terminal sequence of the lamin A precursor is not required for the assembly of lamin A into the lamina. The farnesylated lipopeptide released from the cleavage may itself have a biological function (Lutz *et al.*, 1992).

Ras proteins

The Ras and Ras-related proteins are ubiquitous in eukaryotes. They are small GTP binding proteins (20-35 kD) comprised of an ever-expanding tribe of small GTPases termed the Ras superfamily. Most known prenylated proteins are members of this superfamily.

On the basis of amino acid sequence similarity, over 80 Ras-related proteins have been divided into four subfamilies: Ras, Rho (Rho and Rac), Rab/Ypt, and Ran (Feig, 1994; Sano and Ohashi, 1995). They are involved in the control of a wide variety of cellular functions, for instance, cytoskeletal organization of polymerized actin to produce stress fibers (Rho) and membrane muffling (Rac) (Ridley and Hall., 1992), regulation of intracellular vesicular transport (Rab/Ypt) (Novick and Brennwald, 1993), mediation of protein import into the nucleus (Ran) (Moore and Blobel, 1993), and control of the cell cycle (Ras) and cell polarity (Rho) (Khosravi-Far and Der, 1994; Khosravi-Far *et al.*, 1995). Although geranylgeranylation is the predominant form for the rest of Ras-related proteins including Rho and Rab/Ypt families, farnesylation is required for the functional Ras proteins. To date, Ras proteins are the best characterized class of prenylated proteins and served as model systems in studying protein prenylation.

The Ras proteins are also referred to as p21^{ras} since their molecular weights are about 21 kD. Mammalian cells express four closely related isoforms: N-, H-, K-rasA, and K-rasB, each of which can function as an oncogene following mutational activation. They are normally synthesized on soluble cytoplasmic ribosomes and associated with the inner surface of the plasma membrane

independent of the secretory pathway (Schafer and Rine, 1992; Boguski and McCormick, 1993). Like most of the GTP binding proteins, Ras proteins act as molecular switches, which are turned on and off by cycling between the active GTP-bound and the inactive GDP-bound states. The regulation of Ras is an early step in the signal transduction pathways for growth factors and other agonists that promote cell proliferation and cell differentiation. Upon activation, these proteins transmit signals from tyrosine kinases at the plasma membrane to downstream protein kinase cascades. These cascades, involving Raf (a family of cytosolic serine/threonine kinases) and mitogen-activated protein (MAP) kinases, deliver signals to the cell nucleus and ultimately lead to the activation of gene transcription (Figure I.4) (Moodie and Wolfman, 1994). Oncogenic Ras proteins, usually resulting from point mutations in the GTP binding domain of normal Ras proteins, bind GTP constitutively and therefore chronically transduce a signal to promote cell differentiation and division.

The finding that oncogenic forms of Ras proteins lose the ability to transform cells in the absence of farnesylation has highlighted the importance of this modification to biological activity of the target proteins. It also accounts for much of the current interest in protein prenylation. In independent studies, Hancock *et al.* (1991a) and Kato *et al.* (1992) reported that farnesylation was critical for the function of both normal and oncogenic Ras. Mutants of Ras proteins that lacked the cysteine or the AAX residues of the CAAX motif did not undergo farnesylation. The mutant proteins remained cytosolic and completely biologically inactive. Specific inhibitors of HMGR (*e.g.*, compactin and lovastatin), which prevent prenylation by blocking the isoprenoid pathway, caused accumulation of unprocessed, cytosolic Ras proteins and abolished their biological activity. Manumycin, a specific inhibitor for FTase, suppressed the phenotype induced by an activated *ras* mutation in *Caenorhabditis elegans* (Hara and Han, 1995) and inhibited cell growth of a human hepatoma cell line (Hep G2) (Nagase *et al.*, 1996). Thus, the highly hydrophobic farnesyl moiety is important for membrane association, a prerequisite for biological activity (discussed further in section I.2.4).

The function of Ras proteins in regulating signal transduction pathways that control cell proliferation and differentiation is quite conserved among many eukaryotes, like yeast and mammals. This feature also makes the Ras proteins a primary focus in the study of many basic biological processes (Cox and Der, 1992; Moodie and Wolfman, 1994; Marshall, 1996). Ras proteins from yeast can transform mammalian cells (DeFeo-Jones *et al.*, 1985). A yeast mutant strain lacking Ras activity can be rescued by addition of mammalian Ras proteins (DeFeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985). One common feature of both the *Drosophila melanogaster* and *C. elegans* Ras proteins is their involvement in regulation of specific developmental stages. In a variety of mammalian cells, Ras proteins regulate cell differentiation, progression from G1 to S phase, and immunological responses (Kato *et al.*, 1992; McCormick, 1995). In all these different systems, both the mechanism of Ras regulation and the Ras-mediated MAP kinase cascade are conserved. Surprisingly, Ras homologs have not yet been identified in plants, although isoprenylated proteins with molecular weights similar to Ras have been detected and several Ras-related proteins have been isolated (discussed further in section I.3.2).

Heterotrimeric GTP binding proteins (G-proteins)

The heterotrimeric GTP binding proteins (G-proteins) are members of the ever-growing GTPase superfamily. Acting as key elements in signal transduction pathways, G-proteins pass signals from transmembrane receptors to intracellular second messengers, resulting in the regulation of a variety of enzymes (Neer, 1995), activation of ion channels (Krapivinsky *et al.*, 1995), and mediation of vesicular transport (Helms, 1995).

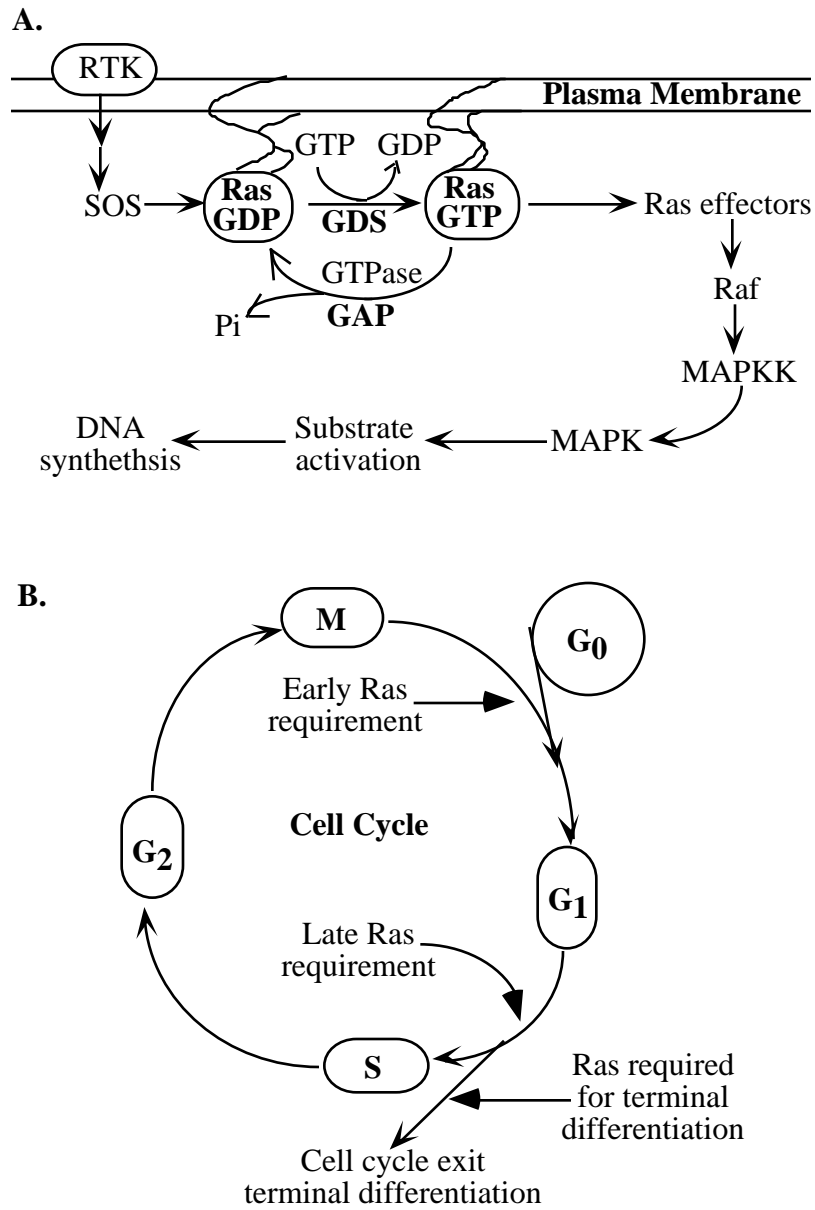


Figure I.4. Schematic diagram of the roles of Ras proteins involved in signal transduction pathways and cell cycle control (modified from Moodie and Wolfman, 1994).

A. Ras-dependent activation of MAPK cascade.

RTK, receptor tyrosine kinase; GDS, guanine-nucleotide dissociation stimulator- protein; SOS, CDC25 homolog; GAP, GTPase-activating protein; MAPK, mitogen activation protein kinase; MAPKK, MAPK kinase.

B. A model of Ras signaling requirements at defined points of the cell cycle.

M, mitosis; S, DNA synthesis; G, gap.

Comprised of α , β , and γ subunits, G-proteins are generally localized to the inner face of the plasma membrane. To date, twenty different α subunits, five β subunits, and twelve γ subunits have been identified from both vertebrates and invertebrates. The α subunit binds and hydrolyzes GTP. The β and γ subunits form a dimer that serves as a functional unit (Neer, 1994).

The G-protein α subunits contain the CAAX motif and are subject to prenylation. The α subunit of bovine transducin, the G-protein involved in visual signal transduction, contains a farnesyl group covalently linked to the C-terminal cysteine residue (Fukuda *et al.*, 1990; Lai *et al.*, 1990). It can also be modified by proteolysis and carboxy-methylation. In addition, STE18, the α subunit of the G-protein involved in yeast mating, requires prenyl modification, probably farnesylation, for membrane localization and functional activity (Finegold *et al.*, 1990). Mutations in the relevant cysteine residue of α subunits produce proteins that are not prenylated and remain cytosolic. Moreover, inhibition of mevalonate synthesis in cells results in redistribution of a substantial fraction of G from the membrane to the cytoplasmic fraction (Muntz *et al.*, 1992). Interestingly, the unprenylated G can still bind to the $\beta\gamma$ subunit to form G dimer. However, the G complex was unable to function in interaction with either the β subunit or with a form of adenylyl cyclase that is regulated by G (Iniguez-Llihi *et al.*, 1992).

The farnesylation of bovine transducin is of particular interest because several other proteins involved in visual signal transduction in the rod outer segment membrane are prenylated. For instance, the α subunit of cGMP phosphodiesterase is farnesylated and carboxy-methylated (Anant *et al.*, 1992), while the β subunit is geranylgeranylated. In addition, rhodopsin kinase, which is involved in the attenuation of rhodopsin activation, is also farnesylated and carboxy-methylated (Inglese *et al.*, 1992). The cGMP phosphodiesterase responds to activated transducin by degrading the second messenger, cGMP. Rhodopsin itself has a distinct type of prenyl modification and is also palmitoylated, a modification common to many prenylated proteins (O'Brien and Zatz, 1984). The involvement of several prenylated proteins in a single signal transduction pathway suggests that this modification may mediate colocalization and interaction among these proteins.

At least two G-protein α subunits contain C-terminal sequences (CGLF) that resemble the CAAX motif. However, neither of these proteins is prenylated *in vivo* (Maltese and Sheridan, 1990; Sanford *et al.*, 1991). In contrast, Ras mutant proteins with this CGLF can be prenylated *in vivo*, indicating that some internal sequence feature may also have a role in determining this specific modification (Kato *et al.*, 1992).

I.2.4. Functional significance of protein farnesylation

Membrane association

By analogy with other known lipid modifications of proteins such as fatty acyl and glycosphospholipid anchors, it is attractive to speculate that farnesyl moieties also function as a hydrophobic membrane anchor (Glomset *et al.*, 1990). Indeed, all of the known farnesylated

proteins are, at least partially, associated with cell membranes. This membrane association is essential for the biological activities of these regulatory proteins. For instance, the farnesylation of lamin is required for its assembly into the nuclear lamina (Holtz *et al.*, 1989); the oncogenic Ras proteins have the ability to transform cells only when they are farnesylated and associated with the inner face of the plasma membrane (Cox and Der, 1992). Studies of Ras proteins have demonstrated that both a mutation of the cysteine residue which is the site for attachment of a farnesyl group and inhibition of isoprenoid biosynthesis (*i.e.*, HMGR inhibitors) abolish farnesylation and result in soluble proteins (Hancock *et al.*, 1989; Casey *et al.*, 1989). This finding has subsequently been confirmed with many other prenylated proteins (Glomset and Farnsworth, 1994; Zhang and Casey, 1996).

Other studies indicate that the farnesyl group, although an essential anchor, is not a sufficient condition for membrane association. For instance, an intermediate in the maturation of Ras (c-p21^{ras}) is farnesylated but found in the cytosol (Hancock *et al.*, 1989). Investigation of Ras proteins has been carried out to understand the function of each step in the posttranslational modification of the CAAX motif proteins--farnesylation, proteolysis and the final methylation. In rabbit reticulocyte lysates, K-ras was modified at different levels (farnesylated only, farnesylated plus proteolyzed, and completely processed) by adding or removing specific inhibitors. Analysis of the processing intermediates showed that 20% of the farnesylated intermediates associated with the membrane; this fraction increased to 40% after the proteolysis step, and finally reached 80% with the fully processed K-ras (Hancock *et al.*, 1991b). A parallel study of the I187TY mutant of K-ras showed similar results. The mutant protein which can be only farnesylated without any further modifications exhibited about 50% membrane association (Kato *et al.*, 1992). Furthermore, investigation of the α and β subunits of large G-proteins provided data consistent with the above observations (Fukada *et al.*, 1994; Parish *et al.*, 1995). The unmethylated α dimer of the retinal G-protein transducin had a much reduced membrane association. The efficiency of activating its downstream regulator was greatly reduced as well (Parish *et al.*, 1995). These results demonstrate that, although farnesylation is critical for the membrane association of Ras and G-proteins, the proteolysis and methylation steps also contribute significantly to efficient membrane association and biological activity.

The notion that methylation has an influence on the membrane binding of prenylated proteins is further supported by biophysical evidence and computer modeling (Black, 1992; Shahinian and Silvius, 1994). Methylation of a prenylated cysteine increases its hydrophobicity by removal of the negative charge at the carboxyl terminus. This data is also consistent with the fact that geranylgeranylated proteins display fairly strong affinity for binding membranes even without methylation. In fact, the Rab proteins with CC C-terminal motifs modified by GGTase II are not methylated. The two geranylgeranyl molecules that are attached to both cysteines at the Rab carboxyl terminus are believed to provide enough hydrophobicity for effective bilayer association.

Detailed studies of Ras proteins revealed that there is a second signal needed for stable membrane association of some prenylated proteins. The second signal can be provided by additional palmitoylation (*e.g.*, H-ras and N-ras) or by a polybasic sequence upstream of the CAAX motif (*e.g.*, six consecutive Lys residue for K-rasB) (Hancock *et al.*, 1990). In addition to the features for general membrane association, other signals must exist in the prenylated proteins for their targeting to specific membranes within the cell. However, solid evidence is limited on this aspect and several general models for specific targeting have been proposed (Schafer and Rine, 1992). The highly diverse sequences near the C-terminus of Ras proteins may have a role in

specific membrane localization. One of the few clear cases is nuclear lamin B. A nuclear localization signal in lamin is responsible for targeting to the nucleus (Holtz *et al.*, 1989).

Protein-protein interactions

In addition to its role in membrane association of proteins, protein farnesylation is involved in protein-protein interactions. This role is consistent with the fact that many farnesylated proteins (*e.g.*, Ras, G) are key components in signal transduction, in which protein-protein interaction is critical.

The best demonstration of the importance of farnesylation in protein-protein interactions comes from the study of the function of normal and oncogenic Ras proteins. Mammalian protein hSOS, a homolog of yeast CDC25 and *D. melanogaster* SOS, can bind to Ras to regulate its rate of nucleotide exchange. Farnesylation of Ras is required for the formation of the K-ras/SOS complex, and the hSOS factor catalyzes guanine nucleotide exchange with a much higher efficiency on farnesylated K-ras than the unprocessed form (Orita *et al.*, 1993; Piorfiri *et al.*, 1994). Similar observations were also made in yeast. Farnesylation of yeast Ras2 dramatically improves its binding affinity for adenylyl cyclase, even when the adenylyl cyclase was in soluble form (Kuroda *et al.*, 1993).

It has been shown that farnesylation of the subunit of the G-proteins is required for the dimer to interact with the subunit (Iniguez-Lluhi *et al.*, 1992; Kisselev *et al.*, 1995). Although both farnesylated and unfarnesylated subunits can bind subunit in the cytosol, the removal of the AAX C-terminal residues after farnesylation abolishes the ability of the subunit to form a dimer with the subunit. This suggests that the dimer formation must occur prior to the completion of subunit modification processes (Casey *et al.*, 1994; Higgins and Casey, 1994).

Applications in pharmaceutical industry

Ras oncogenes are one of the most frequently identified classes of oncogenes in human cancers. Ras proteins have been found in many human cancer tissues including bladder, breast, stomach, colon, liver, lung, kidney, ovary, and pancreas (Barbacid, 1987). The finding that farnesylation is required for the function of Ras led to the awareness that, as first proposed by Schafer *et al.* (1989), inhibition of Ras farnesylation could be a novel anti-cancer approach. Ever since then, the development of protein farnesylation inhibitors as potential anti-cancer drugs has been a focus for scientists world-wide (Khosravi-Far and Der, 1994; Kohl *et al.*, 1995).

As discussed in section I.2.3, Ras proteins are key elements in signal transduction pathways associated with cell proliferation and differentiation. Oncogenic Ras proteins constitutively induce unrestricted cell growth and division. However, the oncogenic Ras loses its ability to transform cells if it is not farnesylated (Powers *et al.*, 1986). Therefore, farnesylation has become an attractive target for designing novel anti-cancer drugs (Gibbs *et al.*, 1994)

A large number of inhibitors that suppress the activity of FTase have been chemically synthesized. Based on the design template, they can be divided into 3 groups: FPP analogs,

CAAX peptide analogs, and bisubstrate analogs (reviewed in Zhang and Casey, 1996). In addition, several microbial products have been identified that block farnesylation, such as manumycin and pepticinnamin from *Streptomyces sp.* and chaetomelic acid from *Chaetomela acutiseta* (Hara *et al.*, 1993; Tamanoi, 1993).

More importantly, it was found that farnesylation inhibitors, at concentrations that inhibited the growth of tumor cells, did not affect the growth of untransformed cells (Kohl *et al.*, 1993; James *et al.*, 1993; 1994a; Carboni *et al.*, 1995). Further research showed that inhibition of the processing of Ras by a farnesylation inhibitor blocked the activation of downstream MAP kinase cascade in H-ras transformed cells and normalized the morphology of the transformed cells. In contrast, the inhibitor had no effects on the MAP kinase cascade in either untransformed cells or cells transformed with the v-src oncogene (James *et al.*, 1994a). The specificity of farnesylation inhibitors raises the greatest potential for developing more efficient anti-tumor drugs that have reduced or no general toxicity.

I.3. FARNESYLATION AND FTASE IN PLANTS

I.3.1. *In vitro* protein prenylation activities

The fundamental importance of protein prenylation in yeast and mammals has triggered analogous research in the plant kingdom. The existence of protein prenylation in plants was first indicated by several biochemical and molecular studies (Randall *et al.*, 1993; Yang *et al.*, 1993a; Zhu *et al.*, 1993a; Schmitt *et al.*, 1996). Using radioactively-labeled substrates, Randall *et al.* (1993) showed that the radioactivity from ¹⁴C-mevalonic acid was incorporated into a group of proteins in cultured tobacco cells (*N. tabacum*). Most of the labeled proteins were membrane-associated and had molecular masses between 14 and 31 kD and between 55 and 66 kD. These sizes are similar to those of mammalian Ras-like GTP binding proteins and nuclear lamins. More convincingly, it was shown that tobacco cell extracts were capable of catalyzing the transfer of radioactivity from ³H-FPP and ³H-GGPP to yeast Ras1 proteins containing either a CAIM or a CAIL carboxy-terminus *in vitro*. These results indicate that protein farnesylation and geranylgeranylation exist in tobacco cells.

Protein farnesylation and geranylgeranylation activities have also been demonstrated in cells of the plant *Atriplex nummularia* (Zhu *et al.*, 1993a). ANJ1 is a stress-induced protein in *A. nummularia* with a CAQQ motif at its carboxyl terminus. It can be farnesylated *in vitro* by cell extracts of both *A. nummularia* and *S. cerevisiae*. Sequence alignment shows that ANJ1 is a higher plant homolog of the bacterial molecular chaperone DnaJ, which is critical for bacterial growth at high temperature and for phage DNA replication. Further evidence shows that farnesylation facilitates ANJ1 membrane association. More importantly, the ability of ANJ1 to complement the normal growth of a yeast temperature sensitive mutant, *mas5*, is dependent upon its farnesylation. Protein prenylation has also been observed in pea (*P. sativum*) (Yang *et al.*, 1993a), soybean (*Glycine max*) (Biermann *et al.*, 1994), tomato (*Lycopersicon esculentum*) (Schmitt *et al.*, 1996; Yalovsky *et al.*, 1996), cotton (*Gossypium hirsutum*) (Trainin *et al.*, 1996), and *Arabidopsis thaliana* (Cutler *et al.*, 1996), suggesting that it is ubiquitous in plants.

I.3.2. Putative proteins for prenylation in plants

The existence of protein prenylation in plants is further supported by the discovery of putative protein substrates for prenylation. Over the past several years, distinct proteins that have the conserved carboxy-terminal sequence for prenylation have been found. These important proteins, such as ANJ1, Ras-related proteins (*e.g.*, Rab, Rho) and heterotrimeric G-proteins, play regulatory roles in many plant physiological processes (Terry *et al.*, 1993; Yang *et al.*, 1993b; Bednarek *et al.*, 1994; Loraine *et al.*, 1996; Yalovsky *et al.*, 1996; Trainin *et al.*, 1996).

Although Ras homologs have not been found in plants, many members of the conserved Ras superfamily have been identified in a variety of plant species using various strategies. Using polyclonal antibodies against *YptVI* (a yeast Rab protein) to screen a tomato young-fruit cDNA library, three clones encoding Rab homologs were isolated (Loraine *et al.*, 1996). The deduced translational products of all three cDNAs share more than 75% identity at the protein level to the mammalian Rab1A and all have the conserved C-terminal motif required for geranylgeranylation (*e.g.*, CC). *In vitro*, the expressed products of these cDNA clones are substrates for prenylation by GGPP but not by FPP. A mutant form lacking the C-terminal CC signal could not be modified and failed to complement the yeast *ypt1* mutant strain for growth. Interestingly, geranylgeranylation of the wild-type homolog was inhibited by addition of mutant proteins. This indicates that there may be some regions in the substrate protein besides the C-terminal region that are recognized by the tomato GGTase II.

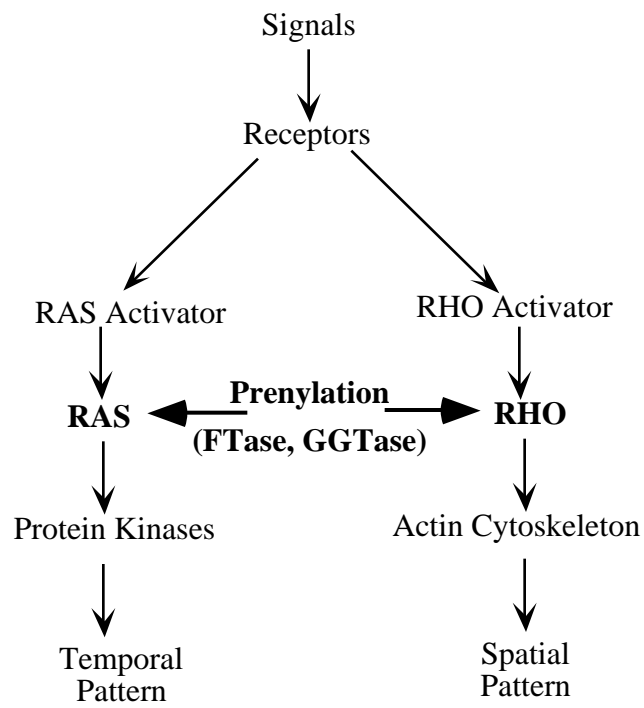
As an alternative strategy for identifying putative prenylated proteins, Biermann and colleagues used ³H-FPP and ³H-GGPP to screen a soybean cDNA expression library blotted on nylon membranes (Biermann *et al.*, 1994). In the presence of tobacco cell extracts (containing prenyltransferases), target proteins for prenylation from the expression library would be labeled with radioactive isoprenyl molecules. Using this method, cDNA clones encoding several distinct proteins were isolated. One soybean cDNA encodes a farnesylated protein highly homologous (63% amino acid identity) to ANJ1, the only plant protein known to be farnesylated *in vivo* (Zhu *et al.*, 1993a; 1993b). Deduced amino acid sequences from two other cDNA clones (GMFP2 and GMFP3) had basic regions containing six or eight repeats of a hexapeptide. The basic repeats of a consensus sequence with (E/G)(G/P)EK(P/K)K suggest that both proteins could be located in the nucleus and may bind nucleic acid. Preliminary data indicate that GMFP3 interacts with DNA although the binding does not appear to be sequence specific (Crowell *et al.*, 1995).

This *in vitro* isoprenylation strategy was used to identify additional isoprenylated plant proteins from Arabidopsis and tobacco (Randall *et al.*, 1996). These prenylation substrates include Rho-related proteins, proteins homologous to v-SNARE vesicle docking proteins, a protein with significant similarity to the Arabidopsis AIG1 protein, and a Rab1-related protein.

I.3.3. Plant FTase--molecular cloning and functional analyses

Compared to the yeast and mammalian systems, relatively little is known about the role of protein farnesylation in plants. Because of the fundamental function of FTase and farnesylation in yeast and animals, it is reasonable to speculate that they may have analogous roles in plant cell cycle control and plant development (Figure I.5).

Earlier studies using inhibitors demonstrated that the HMGR inhibitor mevinolin, when applied during the early stage of tomato fruit development, effectively arrested cell growth, resulting in retarded fruit development (Narita and Grissem, 1989; Gillaspay *et al.*, 1993). Since



Cell Growth and Division

Figure I.5. Functions of protein prenylation in cell growth and division.

mevinolin can affect protein farnesylation by blocking the biosynthesis of farnesyl pyrophosphate, it is possible that the depletion of FPP and GGPP inhibited protein prenylation in plants and led to the arrest of cell division and development. This hypothesis was supported by the results from the treatment of tobacco cell cultures with lovastatin and perillyl alcohol (Morehead *et al.*, 1995). Lovastatin is a specific inhibitor of HMGR (Maltese and Sheridan, 1990) and perillyl alcohol is a monoterpene inhibitor of protein prenylation (Gelb *et al.*, 1995). When added to cell cultures at the early growth stage (within the first two days following transfer to fresh medium), both compounds severely impaired the growth of cultured cells. The increase in FTase activity during culture growth occurred before cells entered the logarithmic phase (Morehead *et al.*, 1995). These data strongly suggest that protein prenylation is essential for the early growth stages of tobacco cultures.

To provide molecular tools to study the function of FTase in plants, Yang and colleagues (Yang *et al.*, 1993a) initiated the cloning of genes encoding FTase subunits. Using a polymerase chain reaction (PCR)-based strategy, a cDNA encoding the β subunit of pea farnesyltransferase (*PsFTb*) was cloned. *PsFTb* from pea was the first prenyltransferase gene identified in plants. Sequence comparison of the β subunits of rat and yeast FTases showed strong sequence similarity at the amino acid level (Figure I.6).

The full-length cloned *PsFTb* gene has an open reading frame encoding 419 amino acids with a estimated molecular mass of 46.8 kD. The deduced amino acid sequence of *PsFTb* showed 48% and 40% identity to the counterpart of rat and yeast FTase β subunits, respectively (Figure I.6). RNA analyses using this *PsFTb* as a probe detected the mRNA of *PsFTb* in the apical buds of pea. Further analyses of pea etiolated seedlings indicated that *PsFTb* transcript levels were down-regulated by light during seedling maturation. This data suggested light might be involved in the regulation of FTase activity by regulating *PsFTb* at the transcriptional level.

Since plants have distinct growth and development programs from those of yeast and animals, it is possible that apart from the presumed role in cell division and growth, FTase and farnesylation may have some unique features involved in plant growth and development. This notion is supported by the fact that potential targets proteins for farnesylation continue to be found in plants (Crowell *et al.*, 1995; Randall *et al.*, 1996). As discussed in section I.1.3.2, some of these proteins have distinct structures with functions that may be unique to plants.

The recent cloning and characterization of the Arabidopsis *Eral* gene has presented an important insight into potential roles of farnesylation in plant signal transduction pathways (Cutler *et al.*, 1996). The *Eral* gene encodes a homolog of FTase β subunit in Arabidopsis. It was identified in a mutant screen of Arabidopsis (ecotype wassilewskija) that exhibit an enhanced response to abscisic acid (ABA) using a T-DNA insertional mutagenesis and tagging approach. T-DNA insertion mutants of Arabidopsis seeds that are hypersensitive to ABA were identified by their ability to maintain dormancy at a very low concentration of ABA at which wild-type seeds would germinate. ABA is known to mediate arrest of plant growth and development in processes such as seed dormancy and leaf senescence (Zeevaart and Creelman, 1988). An increased degree of dormancy was observed among *eral* mutants, in contrast to the *abi* (ABA insensitive) mutants, which have reduced dormancy compared to wild-type Arabidopsis. The finding that *Eral* gene transcripts only accumulated in flower buds strongly supported the idea that the *Eral* gene may have a role in early seed development. It was proposed that farnesylation is required for some key components regulating ABA signaling in Arabidopsis.

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HAASSSF TVYCPPSSSP VWSEPLVSLRPEHARERLQD -DSVE TVTSTIEQAKVEEKIQEVFSSYK -F Consensus
1 EA SRA-----RE -TPTP VSGRD VNI S- -- QV HI VL Pea
1 LQKRVG---RS IARAKFINTALLG KRPVH VVIAH DS SRA QPLAKRE L E TD TIARR VML Yeast
1 HAASSSF TVYCPPSSSP VWSEPLVSLRPEHARERLQD -DSVE TVTSTIEQAKVEEKIQEVFSSYK -F Rat
1 HA S SFT TVYCPPSSSP VWSEPLVSLRPEHARERLQD -DSVE TVTSTIEQAKVEEKIQEVFSSYK -F Human

NHLVPRLLMLQRE -----KHFH--MLKRGLR-QLTDAVEALDASRPWLCYWLHSLELLLEP -IPD Consensus
34 ANIP-----N IAS IIKAWKAWWIIKSTIA S S- -DI Pea
62 QSMLEIYDDEKNIEPALT E HAKH V DVAFEIS PPQHTA IHSRQW L W IAN S R V N I D W L S I Yeast
65 NHLVPRLLMLQRE -----KHFH--MLKRGLR-QLTDAVEALDASRPWLCYWLHSLELLLEP -IPD Rat
65 NHLVPRLLMLQRE -----KHFH--MLKRGLR-QLTDAVEALDASRPWLCYWLHSLELLLEP -IPD Human

IVATD VQF LELCQSPKGGFGGPGQYPHLAPTVAARNALCIIGT-EEAYDXINREKLLQYLVS LK Consensus
66 DLEDNTVD LNRKUDFN GAVR HSGGDI FRIH IYVAND IITLHG- KSLASIKWV N V G FRRN Pea
128 DTKRKI LK LFTISP S G F GGGPGDLSHLA S VYAA IAL SL COMIDGCW I D R K G I V W I X Yeast
121 WHIDV VQF LELCQSPKGGFGGPGQYPHLAPTVAARNALCIIGT-EEAYDXINREKLLQYLVS LK Rat
121 IVATD VQF LELCQSPKGGFGGPGQYPHLAPTVAARNALCIIGT-EEAYDXINREKLLQYLVS LK Human

QPDG GFLNHVG-GEVDVRSAYCAASVASLLNLTPLDFEGVAEWIAR CQNV EGG IGGVPGH -EAHG Consensus
131 QPDG GFLNHVG-GEVDVRSAYCAASVASLLNLTPLDFEGVAEWIAR CQNV EGG IGGVPGH -EAHG Pea
194 EPNAGEITCLVEGEVDI IYV A S I I I L N I L T E E T A S V I N M L K N D M E G G F G C I W D E A H G Yeast
186 QPDG GFLNHVG-GEVDVRSAYCAASVASLLNLTPLDFEGVAEWIAR CQNV EGG IGGVPGH -EAHG Rat
186 QPDG GFLNHVG-GEVDVRSAYCAASVASLLNLTPLDFEGVAEWIAR CQNV EGG IGGVPGH -EAHG Human

GYTF CGLAALVILKHERSLNLKSL LQWVTSRQHRFEGGFQGR CNKLVDGCYSFWQGGLLALLHRAL Consensus
195 GYTF CGLAALVILKHERSLNLKSL LQWVTSRQHRFEGGFQGR CNKLVDGCYSFWQGGLLALLHRAL Pea
260 GYTF CGLAALVILKHERSLNLKSL LQWVTSRQHRFEGGFQGR CNKLVDGCYSFWQGGLLALLHRAL Yeast
250 GYTF CGLAALVILKHERSLNLKSL LQWVTSRQHRFEGGFQGR CNKLVDGCYSFWQGGLLALLHRAL Rat
250 GYTF CGLAALVILKHERSLNLKSL LQWVTSRQHRFEGGFQGR CNKLVDGCYSFWQGGLLALLHRAL Human

HAQGDPA L S -----GV--NSHW----- Consensus
259 I I I I E Q N A E A S Q F V T V S D A P E E K E C L D G T S S H A T S H I R H E G H N E S C S S D W K N I G V N F I S F V R Q S E Pea
323 -----AF S V G----- Yeast
316 HAQGDPA L S -----GV--NSHW----- Rat
316 HAQGDPA L S -----GV--NSHW----- Human

-HFHQQALQEYILHCCQEPAG-GLLDKPGKSRDFVHTCYCLSGLSIAQHS-S--GSGANL-----H Consensus
325 PL F A S I N L Q M L S S U E D F G R U R I G R A N H A S D V N L S G L S L C I V V N R P D S P P -----P Pea
328 Q C F K H I L D V I L H C C Q E P A G G L D K P G K S R D F V H T C Y C L S G L S I A Q H S S S T P N D S P A N I K C T P Yeast
329 -H F H Q Q A L Q E Y I L H C C Q E P A G G L D K P G K S R D F V H T C Y C L S G L S I A Q H S S S G S G A N L ----- Rat
329 -H F H Q Q A L Q E Y I L H C C Q E P A G G L D K P G K S R D F V H T C Y C L S G L S I A Q H S S S G S G A N L ----- Human

DVMVGVP SNHLQPTHPVVM IGPDKVIQATTVFLQKVPVGFEEKNDXKSHHPATD Consensus
385 K A V I G P V N I E P I A L F I A W L I V R E I N E F S I L Pea
394 I L I G - - S K I D V N P V G L P I E N R R I I H A E -----K S N L S S P S Yeast
384 D V M V G V P S N H L Q P T H P V V M I G P D K V I Q A T T V F L Q K V P V G F E E K N D X K S H H P A T D Rat
384 D V M V G V P S N H L Q P T H P V V M I G P D K V I Q A T T V F L Q K V P V G F E E K N D X K S H H P A T D Human

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Figure I.6. Comparison of amino acid sequences of FTase β subunits from pea, yeast, rat, and human.

Gene sequences are from GenBank. Their accession number are pea: L08664, yeast: S07864, rat: A40037, human: L00635. The consensus residues are indicated by black rectangles. Gaps introduced into the alignments are indicated by dashes. The regions to design primers FT1 and FT2 are underlined. MegAlign (DNASTar Inc., Madison, WI) program was used for the sequences alignment.

In spite of the aforementioned progress, many important questions, such as what are the other functions of FTase and how is farnesylation regulated during plant growth and development, remains to be answered. The cloned FTase genes in plants have provided us with useful molecular tools to begin to address these questions. Characterization of cloned FTase genes, together with the continued identification and functional analyses of genes and their products (*e.g.*, prenylation target proteins) involved in protein prenylation, should ultimately lead to a full understanding of the role of FTase and the regulation of farnesylation in plant development.

I.4. OBJECTIVES OF THIS STUDY

Based on the fundamental importance of FTase and the nature of its substrate proteins in yeast and mammals, it is anticipated that farnesylation also plays an important role in modulating plant processes involved in growth and development. The demonstration of FTase activity in plant cells and the cloning of a gene for the pea FTase subunit have established a solid foundation for further work in understanding the physiological role for farnesylation in plants.

In this dissertation research, my goal was to further characterize the structure of plant FTases and to elucidate their expression patterns during plant growth and development. This work provided new insights into the function of FTase in plants. My specific objectives were:

1) to study the relationship between FTase and cell growth and division in plants. The FTase subunit gene (*PsFTa*) was cloned from pea (*P. sativum* cv. Alaska). The coordinate and differential regulation of the two subunit genes of pea FTase were analyzed.

2) to examine the expression pattern of FTase subunit gene in tobacco cell culture system. Molecular probes specific for the subunit of tobacco FTase were identified and used for analyses of FTase mRNA in relationship to cell division and growth in tobacco suspension cultured BY-2 cells.

3) to study the regulation and tissue specificity of FTase subunit gene in plant development. The promoter regions of pea FTase subunit were cloned from a pea genomic library and used to analyze pea FT expression patterns in transgenic tobacco plants (*N. tabacum* cv. Xanthi) by *promoter:GUS* (-glucuronidase) reporter gene analysis.

4) to study the regulation of FTase subunit gene expression in transgenic tobacco and the correlation between the expression of *PsFTa* and *PsFTb* during plant development. The promoter regions of pea FTase subunit were cloned from a pea genomic library and used to analyze pea FT expression pattern in plants by *promoter:GFP* (green fluorescent protein) in tobacco and Arabidopsis (ecotype Columbia).

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CHAPTER II.
PROTEIN FARNESYLTRANSFERASE IN PLANTS:
MOLECULAR CHARACTERIZATION AND INVOLVEMENT
IN CELL CYCLE CONTROL

This chapter was published In *The Plant Cell* [Qian, D., Zhou, D., Ju, R., Cramer, C. L., and Yang, Z. (1996) Protein Farnesyltransferase in Plants: Molecular Characterization and Involvement in Cell Cycle Control. *Plant Cell* 8, 2381-2394]. The impact of this work was highlighted in “IN THIS ISSUE” of *The Plant Cell* [Taylor, C.B. (1996) Farnesylation--A Sticky Business. *Plant Cell* 8, 2151-2153].

Much of the work presented in this article was performed by Dr. Daqi Qian at Ohio State University. I have contributed to the initial cloning of pea FTase subunit gene, expression of FTase fusion genes in *E.coli* and purification of recombinant proteins, immunoassay, characterization of the BY-2 cell line growth kinetics, and RNA analyses.

CHAPTER III.

MOLECULAR CLONING OF A HOMOLOG OF THE β SUBUNIT OF PROTEIN FARNESYLTRANSFERASE FROM *NICOTIANA GLUTINOSA*

Some of the information presented in this chapter was published in abbreviated form in *Plant Physiology* (Zhou *et al.*, 1996) as a Plant Gene Registration article:

Zhou, D., Yang, Z., and Cramer, C. L. (1996) A cDNA clone encoding the β subunit of protein farnesyltransferase from *Nicotiana glutinosa*. *Plant Physiology*, 112, 1398-1399. (Accession No. 73203) (PGR96-109). The copyright of this article belongs to the American Society of Plant Physiologists.

ABSTRACT

Protein farnesylation is catalyzed by farnesyltransferase (FTase). In yeast and animal systems, it has been demonstrated that FTase consists of non-identical α and β subunits. Genes for both subunits have been cloned from yeast, rat, human, and most recently, from pea (*Pisum sativum*). Farnesylation activity has also been detected in tobacco suspension cultures. In order to gain a useful tool in analysis of FTase gene expression in the tobacco system, we initiated cloning of a FTase β subunit gene from *Nicotiana glutinosa*. A short region of tobacco β subunit was initially generated by reverse transcription-polymerase chain reaction (RT-PCR) based on highly conserved sequences of previously cloned FTase genes. This fragment was used to probe an *N. glutinosa* cDNA library. The largest cDNA fragment (*NgFTb*) cloned represents a near-full-length cDNA with total a 1449 bp encoding 446 amino acids. Sequence comparisons suggested that the cloned *NgFTb* fragment lacks about 30 bp of 5' end coding sequence including the start codon. The predicted amino acid sequence exhibits 61.0%, 61.6%, 21.8%, 38.8% identity with the pea, Arabidopsis, yeast and rat FTase β subunits, respectively. The NgFTb polypeptide has a 50 amino acid region near the carboxyl terminus that is not present in rat and yeast FTase β subunits but is found in pea and Arabidopsis homologs.

INTRODUCTION

Protein farnesyltransferase (FTase, E.C.2.5.1.21) is a heterodimeric enzyme consisting of α and β subunits (Casey and Seabra, 1996). It mediates post-translational protein modification by covalently attaching an isoprenyl moiety to a C-terminal cysteine of the target protein. The target proteins have a conserved CAAX carboxy-terminal signal. In yeast and mammalian systems, numerous studies indicate that farnesylation is essential for membrane targeting and *in vivo* activities of many regulatory proteins, including nuclear lamins, Ras and other GTP binding proteins (Casey *et al.*, 1989; Clarke 1992). Thus, protein farnesylation plays an important role in signal transduction and cell cycle control in yeast and animals.

In both yeast and mammals, the FTase β subunit is shared with another prenyltransferase—geranylgeranyltransferase I (GGTase I), which is responsible for geranylgeranylation. However, the α subunit of FTase is unique to the enzyme itself (Kohl *et al.*, 1991; Seabra *et al.*, 1991). Both α and β subunits of FTase are required for catalytic activity. The α subunit is believed to function in binding the substrate protein, whereas the β subunit may bind farnesyl pyrophosphate (Reiss *et al.*, 1991; 1992). Genes encoding FTase α and β subunits have been identified in yeast, rat, cow, and human (Goodman *et al.*, 1988; 1990; Chen *et al.*, 1991a; 1991b; Zhang *et al.*, 1994). The yeast (*Saccharomyces cerevisiae*) FTase α and β subunit genes are *DPRI/RAM1* and *RAM2*. They share 37% and 30% identities at protein level with their counterparts in rat, respectively.

Recent experiments prove that farnesylation exists in plants as well. FTase activity has been detected in tobacco suspension cultures, tomato tissues, and Arabidopsis plants (Randall *et al.*, 1993; Zhu *et al.*, 1993; Cutler *et al.*, 1996; Schmitt *et al.*, 1996). Genes encoding the β subunit of plant FTase have been cloned from pea (*Pisum sativum*; Yang *et al.*, 1993), Arabidopsis (*Arabidopsis thaliana*; Cutler *et al.*, 1996), and tomato (*Lycopersicon esculentum*) (Schmitt *et al.*, 1996). Accumulating evidence suggests a conserved role for plant FTase in mediating cell cycle progression (Randall *et al.*, 1993; Morehead *et al.*, 1995). Studies using Arabidopsis indicate that FTase is involved in abscisic acid (ABA) signal transduction (Cutler *et al.*, 1996). We are interested in further delineating the function of FTase in plant development.

Here we report the cloning and characterization of a cDNA clone encoding a tobacco homolog of FTase β subunit (NgFTb) from *N. glutinosa* by using an PCR-based strategy. The predicted amino acid sequence of the cloned cDNA shows 61.0% and 61.6% identity to the pea and Arabidopsis FTase β subunits, respectively.

RESULTS

Two degenerate oligonucleotides (FT1 and FT2) that were successfully used in cloning of the pea FTase β subunit gene (Yang *et al.*, 1993) were similarly applied to amplify tobacco sequences. The two primers were originally designed correspondent to the highly conserved regions between the yeast and rat FTase β subunit gene products (Figure III.1). Total RNA isolated from young leaves of tobacco (*Nicotiana tabacum* cv. Xanthi) was used as template for

A.

FT1 (sense) 5' - E A H G G Y T F C
 GAA GCI CAC GGI GGI TAC ACM TTC TG
 (G) (T) (T) (T)

FT2 (antisense) 5' - W F S Y C G D
 CCA AAA ICI ATA ACA ICC ATC
 (G) (G) (G) (G)

B.

1 GAAGCTCATGGTGGGTATACCTT TGTGGGTTGGCTGCATGATT Tobacco
 1 GAAGCTCATGGTGGGTATACCTT TGTGGGTTAGCTGCATGATT Pea

46 CTGATTACGAGAGCGAATCGATTGGACTTGCCAGATTATTGAT Tobacco
 46 CTGATTGGTGAGGTTAATCGCTTGGATCTGCCTCGTTTACTTGAT Pea

91 TGGGTGGTATTTAGCAAGGAGTCCGAGGTGGATTTCAGGGAGG Tobacco
 91 TGGGTGGTATTTCCGGCAAGGTAAAGAGTGTGGATTTCAGGGGAG Pea

136 ACGAATAAATTAGTCGATGGCTGCTATTC CTTTTGG Tobacco
 136 ACGAATAAATTGGTAGATGGATGCTATTC CTCGTTTGG Pea

C.

EAHGGYTFCGLAALILLXEXNRLDLPRLD Consensus

1 EAHGGYTFCGLAA MILINEANRLDLPRLID Tobacco
 1 EAHGGYTFCGLAA MILIGENRDLPLRLD Pea
 1 EAHGGYTFC ATASLAILRSMQINYEK LLE Yeast
 1 EAHGGYTFCGLAAL WILKKERS LNKSLIQ Rat

WVYFRQX-EGGFQGR TNKLYDGCYSFW Consensus

31 WVYFRQX -EGGFQGR TNKLYDGCYSFW Tobacco
 31 WVYFRQX K-EGGFQGR TNKLYDGCYSFW Pea
 31 WSSHRQ QEE EGGFQGR SNKLYDGCYSFW Yeast
 31 WTSRQ YRF EGGFQGR DNKLYDGCYSFW Rat

Figure III.1. Primers used for the PCR amplification of *N. glutinosa* cDNA and sequences comparison of the 171 bp PCR product.

- A. FT1 and FT2 degenerate primers (Yang *et al.*, 1993). Inosine (I) was used in positions of full degeneracy. The primer sites at the nucleic acid and amino acid levels are underlined in B and C, respectively.
- B. Comparison of the 171 bp PCR product with corresponding regions of FTase subunit from pea (83.6% identity). The consensus nuclear acids are indicated by black rectangles. MegAlign (DNASTar Inc., Madison, WI) program was used for the sequences alignment.
- C. Comparison of the amino acid sequences deduced from the 171 bp PCR product with its counterparts from pea, yeast, and rat. See Figure III.3 for more extensive comparison. The consensus residues are indicated by black rectangles. Gaps introduced into the alignments are indicated by dashes. MegAlign (DNASTar Inc., Madison, WI) program was used for the sequences alignment.

synthesizing cDNA by reverse transcription. Antisense primer FT2 was used to initiate the synthesis of cDNA. PCR amplification of the synthesized cDNA, using FT1 and FT2, resulted in a single DNA fragment about 171 bp in length. The PCR product was cloned into a pBluescriptII/SK vector. Sequence analysis revealed that the 171 bp PCR product contains 83.6% sequence identity with analogous region of *PsFTb* (Figure III.1B). The deduced amino acid sequence of the 171 bp fragment showed over 91% identity with the corresponding region in PsFTb (Figure III.1 C). The high homologies between this 171 bp product and *PsFTb* at both nucleotide and protein levels demonstrated that the amplified fragment indeed represents an FTase subunit-related sequence.

This 171 bp fragment was radioactively-labeled and used to screen a lambdaZap library of *N. glutinosa* cDNAs (provided by Dr. B. Baker, University of California, Berkeley; Whitham *et al.*, 1994). After three rounds of screening, seven positive clones were isolated. Two of clones were subjected to sequence analysis following *in vivo* excision of the phagemid and/or subcloning of a 1.3 kb *Xho*I fragment that cross-hybridized with the 171 bp probe. The insert of the larger clone is 1449 bp and encodes 446 amino acids of the tobacco FTase subunit. As shown in Figure III.2, the sequence, named *NgFTb*, does not contain the initiation codon. Sequence comparison with the derived amino acid sequence of the pea FTase subunit gene indicates that the 5'-end of the tobacco cDNA lacks less than 30 bp of coding sequence. The 3' non-coding region has a putative polyadenylation signal AATA(C)AG located from 1392 to 1397, and an AUUUUA motif (1346-1351) which is a common feature in many short-lived mRNAs encoding growth regulators in mammals (Brawerman, 1989).

The deduced amino acid sequence of *NgFTb* polypeptide shares 61.0%, 61.6%, 21.8%, 38.8% identity with the pea (Yang *et al.*, 1993), *Arabidopsis* (Cutler *et al.*, 1996), yeast (Goodman *et al.*, 1988) and rat (Chen *et al.*, 1991a) FTase subunits, respectively (Figure III.3). The consensus regions of the pea, yeast and rat FTase subunits exist in *NgFTb* as well.

DISCUSSION

Initial attempts to identify subunit clones from a tobacco cDNA library based on cross-hybridization with the full-length pea PsFTb cDNA (Yang *et al.*, 1993) were unsuccessful (data not shown). We therefore used an RT-PCR-based strategy as described by Yang *et al.* (1993). There are at least two reasons for the failure of the initial cloning strategy: one is the low percentage of overall homology (52.2%) between the pea and tobacco FTase subunit genes at the nucleotide level; the other is the low abundance mRNA for the FTase subunit in tobacco leaf tissues.

Analysis of the cloned *NgFTb* shows it has several stretches of amino acid sequence that are highly conserved among all cloned FTase subunit genes, including regions corresponding to the RT-PCR primers (Figure III.3). This evolutionary conservation may identify regions that have a role in catalytic activity. Sequences that are generally divergent between the pea and animal FTase subunit (near C- or N-termini) are also distinct between the tobacco and mammals. Furthermore, a Blast search of this predicted amino acid sequence of *NgFTb* through the GenBank database reveals that it has no similarity to any other prenyltransferase sequences. This result

supports that the FTase subunit has a role in binding of the protein substrates which have the FTase-specific CAAX C-terminal motif.

As expected, the cloned *NgFTb* has higher homology with the pea and Arabidopsis FTase subunit genes than with its counterparts in rat and yeast. Interestingly, NgFTb contains the 50-amino acid insertion near its C-terminus, which has been found in all cloned plant FTase subunits but is missing from the FTase subunits in yeast and animals (Figure III.3). Therefore, the unique insertion found in plant FTase subunits may confer distinct properties in regulation of plant FTase activities. Comparison of the hydrophilicity of this predicted peptide with the known FTase subunits supports the hypothesis that NgFTb is also a soluble cytosolic protein (Figure III.4).

The cloned *NgFTb* provides a useful tool to study the expression of FTase gene in both tobacco cell suspension culture and intact plant systems. Further characterization of *NgFTb* gene and its expression will provide valuable information for understanding the function of FTase and its regulation in plant cell growth, division, and differentiation.

METHODS

Polymerase chain reaction amplification

Two degenerate oligonucleotides (shown in Figure III.1A) corresponding to amino acid residues 191-199 (EAHGGYTFC) and 241-247 (DGCYSFW), respectively, of the pea FTase subunit (Yang *et al.*, 1993) were used as primers for polymerase chain reactions (PCR). Total RNA was isolated from tobacco (*N. tabacum* cv. Xanthi) young leaves as described previously (Thompson *et al.*, 1983). Antisense FT2 was used as the initial primer for reverse-transcription. PCR amplifications were performed in an 100 μ L reaction mixture containing about 50 ng cDNA, 400 pmol of each primer, and 5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus). Thirty five amplification cycles were conducted at 94°C for 1 min (denaturation), 45°C for 30 sec (annealing), and 72°C for 30 sec (extension). The ends of the amplified PCR products were filled in using *Escherichia coli* DNA polymerase I and phosphorylated using T4 polynucleotide kinase and ATP. Blunt-ended fragments were cloned into the *Sma*I site of pBluescriptII/SK⁻ (Stratagene, La Jolla, CA) and sequenced using Sequenase Version 2.0 (United States Biochemicals, Cleveland, OH). Two clones were sequenced and a longer insert was found to contain 171 bp fragment that encoded a 57 amino acid polypeptide exhibiting strong sequence similarity to the pea FTase subunit at both nucleotide and amino acid levels.

Library screening and cDNA cloning

The 171 bp fragment, labeled with ³²P-dCTP using a random primer labeling system (Gibco BRL), was used to screen an *N. glutinosa* cDNA library (gift from Dr. B. Baker, University of California, Berkeley). Duplicate filters were hybridized in a solution containing 6 X SSPE (1 X SSPE is 0.15 M NaCl, 10 mM NaH₂PO₄, 10 mM EDTA), 5 X Denhardt's solution (1

```

1 -----GTSGRILE--DQWVSRVRELVNFVYS Tobacco
1 IEASITA-----AETPFDMSQRDQWLESDVFATVQLAN Pea
1 -----Arabidopsis
1 IASSSFTYYCPPSSSPVMSEPLVSLRP--EHARERLQDQVEVITSIEYAKAEKIQVVFSSVKF Rat
1 IQRVGRSI--ARAKFINTALLGRKRPVNERVYDIAHVDSKAIQPLHRELETDTEARQVYVLS Yeast

28 I-----SRBHETS TKHFD--KVTGALR-KGAS-SVVDANRMLTQWVILHSIALLG-SSVTRQ Tobacco
36 I-----PNAQ-----SIIAPWLCWVILHSIALLG-SSVTRQ Pea
1 -----Arabidopsis
65 N---HLVPRRMLQRKHAH--GKAGLR-QLTAYECDAARWLCWVILHSIELLQ-SPVQIV Rat
64 VLEIYDDEKNTSALTSERKHLQDYAFEDSIPQNTAIDASDQWLVWVANSKLVADRWLSDIT Yeast

86 ENQIDQLSQQMEGGVGGGQDTPHLATVYAAVNEIILGSPKALSSINHEKLVTFMLQIKIT Tobacco
68 ENITQDFLYGGDPNGGVAAGGQDTPHLATVYAAVNLITLGG-EVSLASINRVLVGFTRIKIT Pea
41 ENQIDQLSQQMEGGVGGGQDTPHLATVYAAVNALVILGG-DKALSSINREKASCELRTRIKIT Arabidopsis
123 ATQCCQLELGGSPGGFGGGQDTPHLATVYAAVNALVIGT-EEAVNMINREKLDVYVSLKIT Rat
130 KRRIWMLFTISPSGGPFGGGQDTPHLATVYAAVNALVIGT-EEAVNMINREKLDVYVSLKIT Yeast

151 NAGFRH-HDGEIDWRACYTATSWASVLIITDDELTKNAGD-ILSCOTYEGGCRGEP-GSEAHGGV Tobacco
133 NAGFRH-HDGEIDWRACYTATSWASVLIITDDELTKNAGD-ILSCOTYEGGCRGEP-GSEAHGGV Pea
106 NAGFRH-HDGEIDWRACYTATSWASVLIITDDELTKNAGD-ILSCOTYEGGCRGEP-GSEAHGGV Arabidopsis
188 DGEF-TIHYGGEVDWBSHYGHSWASVLIITDDELTKNAGD-ILSCOTYEGGCRGEP-GSEAHGGV Rat
196 NAGFRKTCLEVGEDVTRGIVCHLSDIITLNLITEELTEGVNLGKNCQWEGG-GSCPHWDEAHGGV Yeast

215 IFGGLAAILIIEWRLLDPRIDWVFRQGV-EGGFGRINRLEVDGCVSFWQAVVFLIQR-KS Tobacco
197 IFGGLAAILIIEWRLLDPRIDWVFRQGV-EGGFGRINRLEVDGCVSFWQAVVFLIQR-KS Pea
170 IFGGLAAILIIEWRLLDPRIDWVFRQGV-EGGFGRINRLEVDGCVSFWQAVVFLIQR-KS Arabidopsis
252 IFGGLAAILIIEWRLLDPRIDWVFRQGV-EGGFGRINRLEVDGCVSFWQAVVFLIQR-KS Rat
262 IFGGLAAILIIEWRLLDPRIDWVFRQGV-EGGFGRINRLEVDGCVSFWQAVVFLIQR-KS Yeast

279 IAHQVGLSNELSIESADDSSELSSEH-DIASSHWQRTCPVGGEG-NAADPTKLDIIVDQV Tobacco
261 IIEQDRAEASQVWVSD-----APEERECIDATSSHA--TSHIRHEGIMECSSD-WKNTLVNFM Pea
234 IVDQVHGGSHISEGNTNE--EHHAHVEDDSDSDDDDSD---DEDNDSDVNGHRUHHIS-TYI Arabidopsis
318 QGIPAAS-----RFGVY- Rat
323 -----RFGVY- Yeast

345 NATIANRANLDSFYDQWVLSQDQ-GEFADKAPGGRDQVHAWVGLSGLSITQVSWITNEAIPAP Tobacco
318 SEWRYSEPLHSIALDQWVLSQDQ-GEFADKAPGGRDQVHAWVGLSGLSITQVSWITNEAIPAP Pea
292 NATIADL--WDSGLDQWVLSQDQ-GEFADKAPGGRDQVHAWVGLSGLSITQVSWITNEAIPAP Arabidopsis
326 SHALF-----HQAQDEVLTICCPAG-GLDKPGKSDVYHTCVLGLSGLSITQVSWITNEAIPAP Rat
327 -----GQCENKHALRDVILVYSDKEKEQPLADKAPGGRDQVHAWVGLSGLSITQVSWITNEAIPAP Yeast

409 IADVDFP--GQNLLEQIHLVWMLDRVYHRSF-----SC Tobacco
383 IADVDFP--GQNLLEQIHLVWMLDRVYHRSF-----SQ Pea
355 IADVDFP--GQNLLEQIHLVWMLDRVYHRSF-----KAA Arabidopsis
381 IADVDFP--GQNLLEQIHLVWMLDRVYHRSF-----LQRPVGFEECEDAVTSDPATD Rat
387 HNRCTPDRLLIGSSKIDVWVGLPIENVYIIVY-----KSNLSSPS Yeast

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Figure III.3. Amino acid sequence comparison of FTase β subunits from *N. glutinosa*, pea, Arabidopsis, rat, and yeast.

The sequences are from GenBank. The accession numbers are *N. glutinosa*: U73203, pea: L08664, Arabidopsis: U46574, rat: A40037, and yeast: S07864. Consensus residues are indicated by black rectangles. Gaps introduced into alignments are indicated by dashes. Regions used for primers FT1 and FT2 are delineated with underlines. Boxed region represents the 50-amino acid insertion. MegAlign (DNA star Inc., Madison, WI) program was used for the sequences alignment.

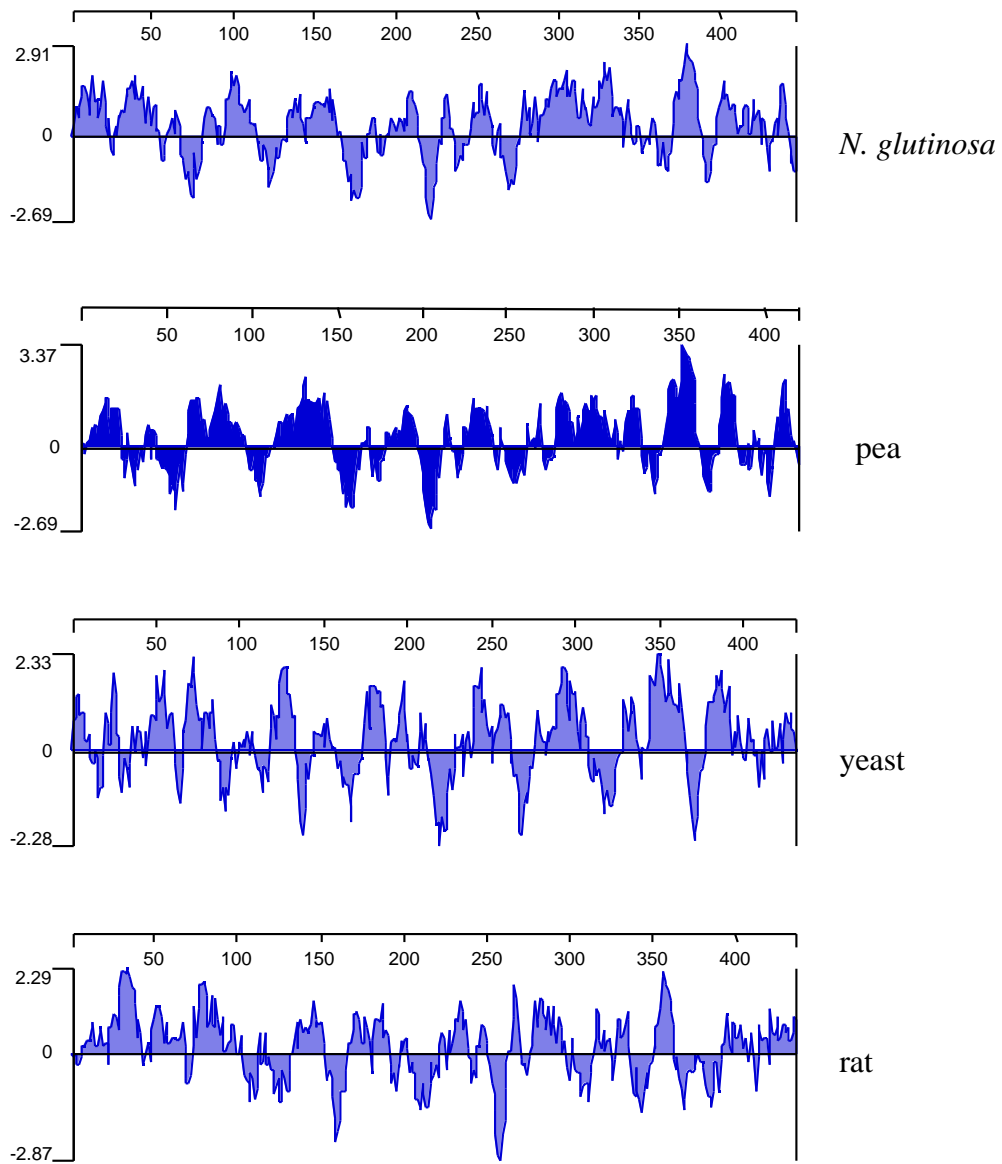


Figure III.4. Hydrophilicity plots of FTase β subunit from *N. glutinosa*, pea, yeast, and rat.

Protein analysis program of DNASTar was used for the application.

X Denhardt's solution is 0.4% [w/v] Ficoll, 0.4% [w/v] PVP, 0.4% [w/v] BSA), 50% formamide, and 100 mg/mL sonicated single-stranded salmon DNA at 42°C. Of 2×10^6 plaques screened, about 7 positive clones were identified. Two clones were subjected to sequence analysis following *in vivo* excision of the phagemid and/or subcloning of a 1.3 kb *XhoI* fragment that cross-hybridized with the 171 bp probe. Further digestion revealed two *PstI* sites in the 1.3 kb insert. To facilitate sequencing, the 1.3 kb fragment was excised and subcloned into pBluescriptII/SK⁻ at the *PstI* site. Sequence data from all the inserts were combined and resulted in a total of 1449 nucleotides encoding 446 amino acids. However, sequence comparison indicated that the 5' end of the cloned cDNA is truncated and about 30 nucleotides downstream of the start codon may be missing.

MegAlign program from DNASTar Inc. in Wisconsin was used in sequence analyses.

ACKNOWLEDGMENTS

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CHAPTER IV.

DEVELOPMENTAL AND ENVIRONMENTAL REGULATION OF TISSUE- AND CELL-SPECIFIC EXPRESSION FOR A PEA PROTEIN FARNESYLTRANSFERASE GENE IN TRANSGENIC PLANTS

This chapter is presented in the format of a paper submitted to the Plant Journal.

Dafeng Zhou, Daqi Qian, Carole L. Cramer, and Zhenbiao Yang. (1997) Developmental and environmental regulation of tissue- and cell-specific expression for a pea protein farnesyltransferase gene in transgenic plants. Submitted.

ABSTRACT

Farnesylation mediates membrane targeting and *in vivo* activities of several key regulatory proteins such as Ras and protein kinases in yeast and mammals, and is implicated in cell cycle control and abscisic acid (ABA) signaling in plants. In this study, we examined the developmental expression of a pea farnesyltransferase (FTase) gene, using transgenic expression of the -glucuronidase (GUS) gene fused to a 3.2 kb 5'-upstream sequence of the gene encoding the pea FTase subunit. Coordinate expression of the GUS transgene and endogenous tobacco FTase subunit gene in tobacco cell lines suggests that the cloned 3.2 kb region contains the key FTase promoter elements. In transgenic tobacco plants, GUS expression is most prominent in meristematic tissues such as root tips, lateral root primordia, and the shoot apex. This expression pattern is consistent with a role for FTase in the control of the cell cycle in plants. GUS activity was also detected in mature embryos and imbibed embryos, a pattern in accord with a role for FTase in ABA signaling that modulates seed dormancy and germination. In addition, GUS activity was detected in regions that border two organs, *i.e.*, flower receptacles, and junctions between stems and leaf petioles, cotyledons and hypocotyls, roots and hypocotyls, and primary and secondary roots. GUS is expressed in vascular bundles (primarily phloem) that are adjacent to actively growing tissues such as young leaves, roots of light-grown seedlings, and hypocotyls of dark-grown seedlings. Both light and sugar (*e.g.*, sucrose) treatments repressed GUS expression in the dark-grown seedlings. These expression patterns suggest a possible involvement of FTase in the regulation of nutrient transport or allocation into actively growing tissues.

INTRODUCTION

Protein isoprenylation is a post-translational modification required for membrane targeting and activation of many important signaling proteins, including the Ras superfamily of small GTP-binding proteins, the γ -subunit of heterotrimeric GTP-binding proteins, cGMP phosphodiesterase, fungal mating factors, nuclear lamins, and protein kinases and phosphatases (reviewed in Clarke, 1992; Cates *et al.*, 1996). Isoprenylation involves covalent attachment of an isoprenyl moiety (C-15 farnesyl or C-20 geranylgeranyl) to the cysteine residue of a CXXX motif located at the carboxyl terminus of target proteins. Three distinct enzymes catalyzing the isoprenylation reactions have been identified, each modifying proteins with a unique C-terminal motif (reviewed in Zhang and Casey, 1996). Protein farnesyltransferase (FTase) modifies proteins with CAAX (C, cysteine; A, aliphatic amino acids; X, any amino acids except for leucine). In contrast, protein geranylgeranyltransferase I (GGTase I) requires CAAL (L, leucine) at the C-terminus of substrate proteins. GGTase II recognizes proteins with C-terminal sequence CC, CCXX or CXC plus an unidentified internal sequence.

Among the three enzymes, the structure and function of FTase has been most extensively studied. FTase consists of two non-identical subunits, α and β . Genes encoding both subunits have been cloned from various organisms including plants, yeast and mammals (Chen *et al.*, 1991a; 1991b; Goodman *et al.*, 1988; He *et al.*, 1991; Qian *et al.*, 1996; Yang *et al.*, 1993). The catalytic site for FTase appears to be composed of an interfacial cleft at the contact site between the two subunits (Ying *et al.*, 1994). Although the α subunit has a role in substrate binding and the transfer reaction, the β subunit determines substrate specificity (reviewed in Zhang and Casey, 1996). Moreover, the FTase β subunit is shared by GGTase I in yeast and mammals (Zhang and Casey, 1996), but it is unknown whether this also holds true in plants. In mammals and yeast, protein farnesylation is involved in the modulation of many important processes including signal transduction, cell cycle control, protein trafficking, and development, which reflects the role of specific farnesylated proteins (Cox *et al.*, 1992; Gibbs *et al.*, 1994; Hara and Han, 1995; Kauffmann *et al.*, 1995; Kisselev *et al.*, 1994; Marshall, 1993; Scheer and Gierschik, 1995). Farnesylated proteins in these organisms include Ras proteins, the β subunit of G-proteins, protein kinases and phosphatases, cGMP phosphodiesterase, nuclear lamins, and the chaperone DnaJ (Cates *et al.*, 1996; Clarke, 1992; Marshall *et al.*, 1993; Ong *et al.*, 1995; Zhu *et al.*, 1993).

Protein farnesylation also plays a key role in plant growth and development (Taylor, 1996). Using a seed germination assay, several *Arabidopsis thaliana* mutants showing enhanced response to abscisic acid (ABA) were identified (Cutler *et al.*, 1996). Surprisingly, one of the mutants was characterized as a T-DNA insertion mutation in the gene encoding an *Arabidopsis* FTase β subunit. This finding indicates a key role for FTase in ABA signaling or ABA-mediated growth regulation (Cutler *et al.*, 1996). There is also strong evidence that FTase is involved in the cell cycle control in plants (Qian *et al.*, 1996; Morehead *et al.*, 1995). The FTase-specific inhibitor manumycin inhibited mitosis but not cell expansion in suspension-cultured BY-2 cells. It also blocked the cell cycle when added at the S phase but not at the G₂ phase in synchronized BY-2 cells. Furthermore, FTase gene activation is associated with tissues having active cell division in pea plants, and FTase enzyme activity is correlated with mitotic activity in suspension-cultured BY-2 cells (Qian *et al.*, 1996). In tomato, high FTase activity was associated with but not limited to meristematic tissues, e.g., high levels of FTase activity were also found in stems (Schmitt *et al.*, 1996); this is consistent with the notion that FTase has other functions in addition to cell cycle control and ABA signaling.

The multiple functions for FTase in plants are further supported by the identification of farnesylated proteins such as homologs of the chaperone DnaJ and heavy metal-binding proteins (Biermann *et al.*, 1994; Biermann *et al.*, 1996; Randall *et al.*, 1996; Zhu *et al.*, 1993).

To further our understanding of the physiological role for FTase in higher plants, we analyzed the temporal and spatial expression of the pea FTase β subunit gene (*PsFTb*) using transgenic tobacco plants that contain the β -glucuronidase gene fused to the *PsFTb* promoter. Histological analyses of transgenic plants revealed a developmental regulation of *PsFTb* expression in a cell- and tissue-specific manner and an environmental regulation by light and exogenous sugars. *PsFTb* is expressed in mature and imbibed embryos and in meristematic tissues such as root tips, lateral root primordia, and shoot apices. These expression patterns further support previously proposed functions for FTase including in the control of cell division and ABA signaling. In addition, *PsFTb* expression is localized to the phloem and junctions between organs and is down-regulated by light and sucrose, raising an interesting possibility that FTase is involved in the regulation of nutrient transport and mobilization.

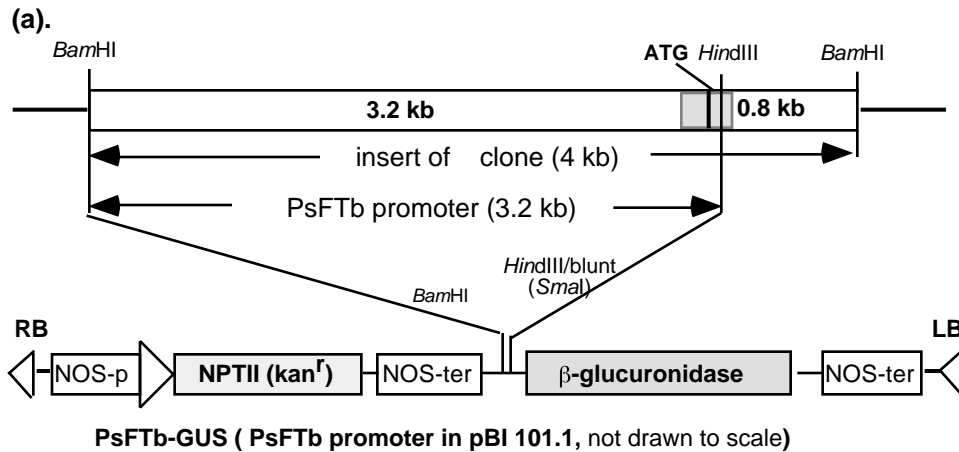
RESULTS

Isolation and analysis of the *PsFTb* promoter

In previous studies, we cloned a cDNA for the pea FTase β subunit (*PsFTb*) and showed that co-expression of *PsFTb* cDNA with a pea α subunit gene (*PsFTa*) in *E. coli* reconstituted FTase activity (Qian *et al.*, 1996; Yang *et al.*, 1993). To isolate the promoter sequences for the *PsFTb* gene, the *PsFTb*-coding sequence was used to screen a pea genomic library (Clontech) under high stringency conditions. Restriction and partial sequence analyses of a 4.0 kb genomic clone revealed that it contains a 5' portion of *PsFTb*-coding sequences and about 3.2 kb sequences upstream of *PsFTb* ATG codon. The 3.2 kb fragment was translationally fused to the β -glucuronidase gene (*uidA* gene from *Escherichia coli*) in pBI101.1 (Jefferson *et al.*, 1987) (Figure IV.1a). As shown in Figure IV.1b, sequencing confirmed the GUS gene was fused in frame with the *PsFTb* initiation codon ATG. Particle bombardment-mediated transient expression of the resulting fusion gene *PsFTb:GUS* in tobacco BY-2 cells (Nagata *et al.*, 1992) demonstrated the presence of promoter activity in the 3.2 kb fragment (data not shown).

PsFTb promoter-mediated GUS expression resembles the expression of a tobacco gene encoding the FTase β subunit in suspension-cultured BY-2 cells

To further assess the fidelity of the *PsFTb* promoter, the *PsFTb:GUS* fusion gene was stably introduced into BY-2 cells by *Agrobacterium tumefaciens*-mediated transformation (An *et al.*, 1988) and GUS expression patterns were compared to a tobacco gene encoding the endogenous FTase β subunit (Zhou *et al.*, 1996). Three cell lines containing the GUS fusion gene were selected and subsequently maintained in suspension cultures.



(b).

5'tatatcagtggggtgctactcaccacagctgtcgatataagatcgaaggctcatattcattacacgatcctgctat
acagtagaacatctgaatccttcgcttgtagcaaaaaaaaaaagcgactctggaagttcttttcaattcaacaac
cagttactcaaaccaccgaggagtaacacatg gaG GGT GGT CAG TCC CTT ATG 3'

Figure IV.1. Isolation and partial sequence of the *PsFTb* promoter.

(a). A 3.2 kb upstream fragment containing the *PsFTb* ATG initiation codon was inserted in front of the promoterless GUS gene in pBI101.1. Details of the plasmid construction are described in Experimental Procedures. (b). The junction region between *PsFTb* (lower case) and GUS (upper case) was sequenced to confirm that the fusion is in frame and that the promoter region contains a putative TATA box.

These cell lines were used to analyze growth kinetics and transcript levels for both GUS and a tobacco FTase subunit (Zhou *et al.*, 1996). All three cell lines showed similar growth kinetics and GUS expression patterns. Data from a representative line are shown in [Figure IV.2](#). Little cell growth occurred within the first two days; logarithmic growth began on day 3 and continued until day 7. Thus, the growth curve for the transgenic cell line is identical to that for untransformed BY-2 cells (Qian *et al.*, 1996). Low levels of tobacco FTb transcripts were detected within 6 hr and reached a first plateau by 24 hr. Following a slight decline at 36 hr, mRNA levels increased steadily, reached a second major peak between day 3 to 5, declined by day 6, and returned to basal levels by day 7. The kinetics of GUS mRNA expression resemble that of tobacco FTb mRNA, suggesting that the *PsFTb* promoter activity accurately reflects the expression of the *PsFTb* gene. The increase in FTb mRNA levels is correlated with the increase in FTase enzyme activity described previously (Qian *et al.*, 1996); however, the decline in FTb mRNA levels lag behind that of enzyme activity, suggesting that FTase is also subject to a posttranscriptional and/or posttranslational modification.

Temporal and spatial expression during seed germination and seedling development

The temporal and spatial expression pattern of the *PsFTb* gene was investigated using transgenic tobacco plants (*Nicotiana tabacum* cv. Xanthi) expressing the *PsFTb:GUS* gene. Eleven independently transformed plants (T_0) were analyzed by histochemical GUS staining (Gallagher, 1992) and all plants examined showed similar staining patterns although they varied in intensity. The first generation (T_1) seeds of selected transgenic plants that show relatively strong GUS activity and contain a single transgene, were used for subsequent analyses of GUS activity in seedlings and mature plants.

Histological analyses revealed dynamic changes in tissue-specific GUS expression during seed germination and seedling development under continuous light ([Figure IV.3](#)). No GUS activity was detected in immature seeds, although mature seeds showed GUS activity ([Figure IV.3a](#)). Shortly (within 1 hr) after imbibition, GUS activity was mainly concentrated at the tip of embryonic roots ([Figure IV.3b](#)) and the endosperm (data not shown). GUS activity remained in root tip within the first 24 hr ([Figure IV.3c](#)). One day after imbibition, GUS expression shifted to the central region of the embryo axis ([Figure IV.3d](#)). By day 3, GUS staining returned to root tips ([Figure IV.3e](#)). This reappearance of GUS expression in root tips is consistent with the activation of cell division in the root meristem. In 5 day-old seedlings, GUS activity was found not only in root tips but also in vascular bundles of all organs and the junction between hypocotyls and cotyledons ([Figure IV.3f](#)). In fully emerged 7-day old seedlings, GUS was expressed in the root tip, the vascular tissue in roots, and the junction between roots and hypocotyls ([Figure IV.3g](#)). In older seedlings (2 to 4 week-old), GUS activity was concentrated in root tips, shoot apices, root-shoot junctions, nodes (leaf and stem junctions), the vascular tissue of leaves ([Figure IV.3h](#)) and lateral root primordia ([Figure IV.3i](#)).

Expression of *PsFTb:GUS* fusion gene in mature transgenic plants

Histochemical analyses of mature transgenic plants showed a tissue-specific GUS expression pattern similar to that observed in seedlings. GUS activity was found in the shoot apex ([Figure IV. 4a](#)), junctions between the primary and secondary roots ([Figure IV. 4b](#)) and between leaves and stems ([Figure IV. 4c](#) and [4f](#)), regions from which auxiliary buds are being

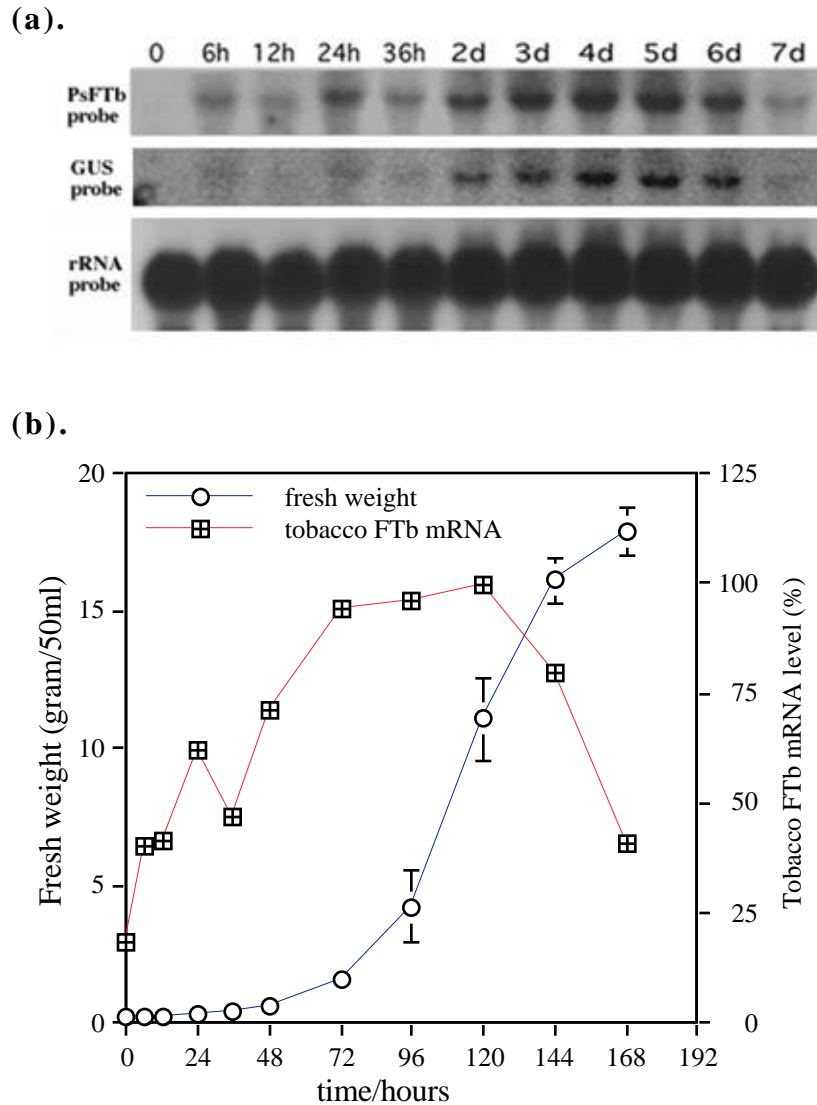


Figure IV.2. RNA gel blot hybridization analysis of GUS and FTb mRNA levels in the BY-2 cell line expressing the *PsFTb:GUS* fusion gene.

Suspension-cultured BY-2 cells were harvested at indicated times after subculture and used for the isolation of total RNAs. Thirty micrograms of total RNAs were separated on an agarose gel, transferred to membranes, and hybridized with the GUS-coding sequence and a tobacco FTb cDNA (Zhou *et al.*, 1996), respectively. The consistency of RNA loading was confirmed by rehybridizing the filter with a pea DNA fragment encoding a 26S ribosomal RNA (Yang *et al.*, 1993). The experiment was repeated twice and data from a representative experiment is shown (a). RNA signals were quantified using a densitometer. The kinetics of FTb mRNA accumulation was compared to cell fresh weights determined from a 50-ml suspension culture (b).

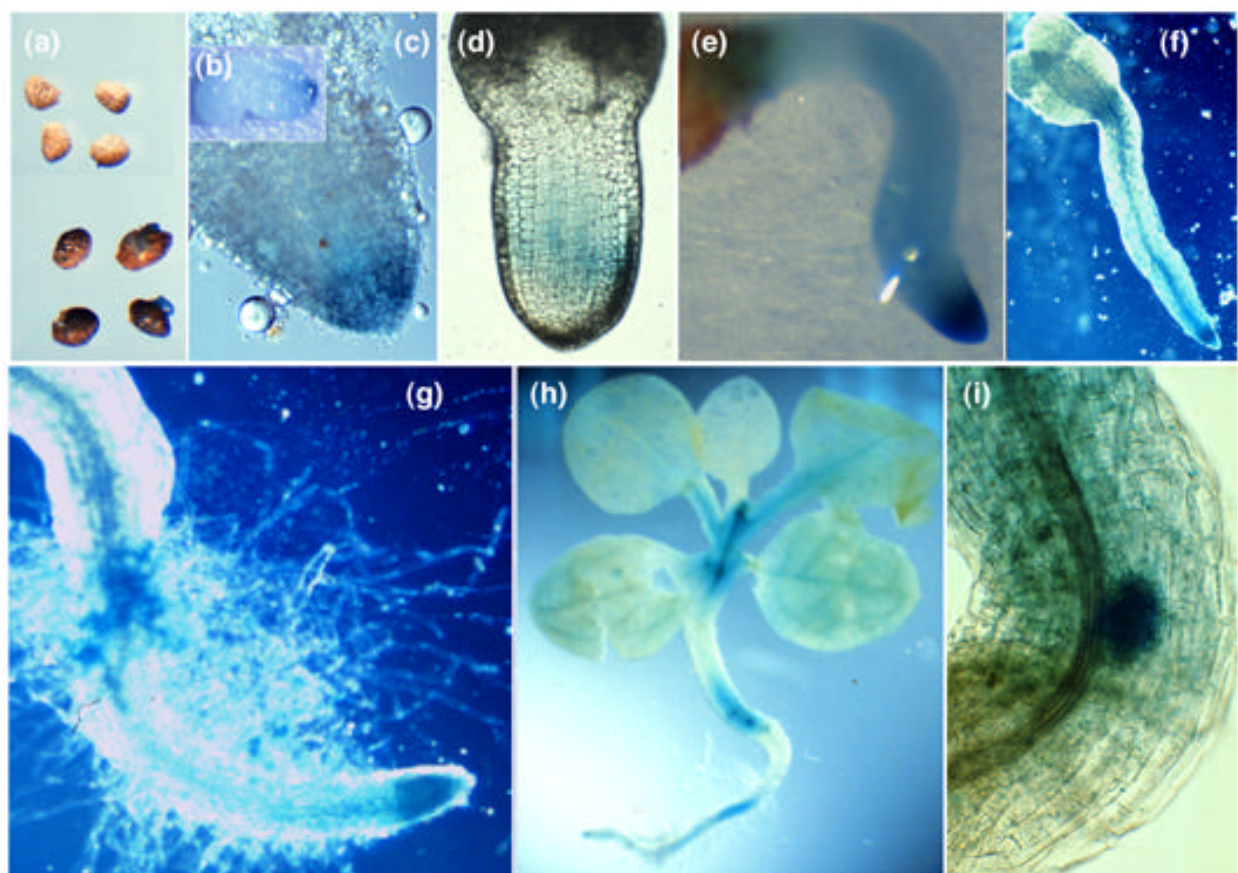


Figure IV.3. Histological GUS staining of transgenic tobacco seedlings expressing the *PsFTb:GUS* fusion gene.

Embryos and seedlings of different stages were stained with 5-bromo-4-chloro-3-indolyl -D-glucuronic acid as described in text. Whole mount seedlings or tissues were examined and photographed using a Zeiss stereoscope or a Zeiss microscope equipped with the Normaski optics.

- (a). Developing (top rows) and mature (bottom rows) seeds.
- (b). Embryonic roots from seeds imbibed for 1 hr.
- (c). Higher magnification of the similar root apex shown in (b).
- (d). Embryo from seeds imbibed for 24 hr.
- (e). A seedling three days after imbibition.
- (f). A 5-day old seedling.
- (g). Root and part of the hypocotyl of a 7-day old seedling.
- (h). A three-week-old seedling.
- (I). Lateral root primordium from a three-week old seedling.

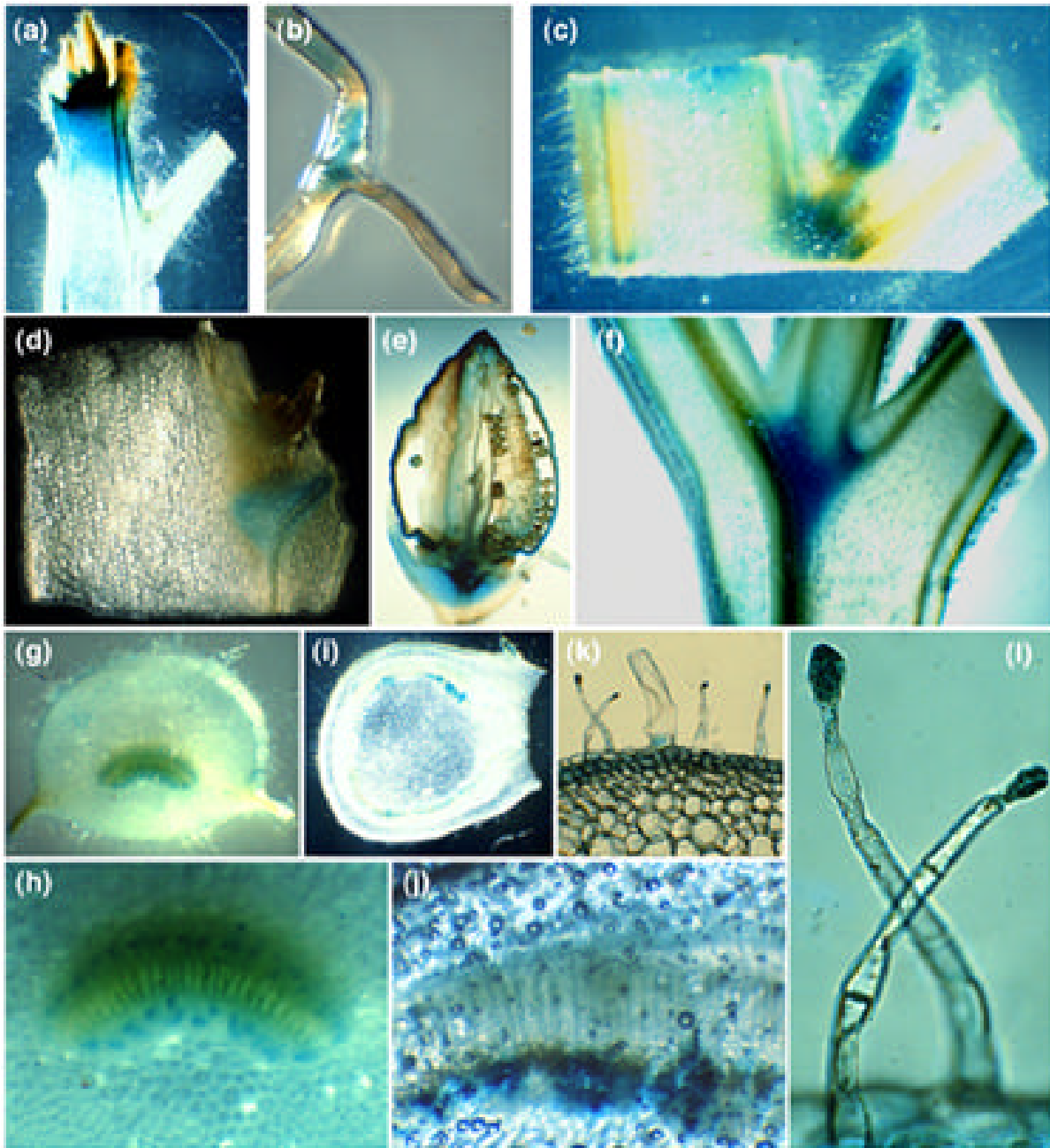


Figure IV.4. Histological GUS staining of transgenic tobacco plants expressing the *PsFTb:GUS* fusion gene.

Different parts of mature tobacco plants were prefixed and stained with 5-bromo-4-chloro-3-indolyl -D-glucuronic acid as described in text. Whole mount or hand-sectioned tissues were examined and photographed using a Zeiss stereoscope or a Zeiss microscope equipped with the Normaski optics.

- (a). Shoot apex.
- (b). Lateral root.
- (c). Junctions between stems and a region of stems from which a auxiliary bud is initiated.
- (d). Junctions between leaf petioles and stems.
- (e). Mature flower (petals, sepals, and stamens are removed).
- (f). Side branch of the shoot.
- (g). Cross section of leaf petioles.
- (h). Higher magnification the vascular bundle shown in (g).
- (i). Cross section of stems.
- (j). Higher magnification of the stem section (i) showing staining in inner phloems.
- (k). Cross section of a stem containing trichomes.
- (l). Higher magnification of stained trichomes shown in (k).

formed (Figure IV. 4d), flower receptacles (Figure IV. 4e), expanding young leaves and petioles (Figure IV. 4c), and the joint part of branch shoots (Figure IV. 4f). Cross-sections of leaf petioles showed that GUS was specifically expressed in the phloem (Figure IV. 4g and 4h). In stems, only inner phloem complexes were highly stained (Figure IV. 4i and 4j). GUS expression was also detected in the terminal cells of trichomes which were undergoing cell division (Figure IV. 4k and 4l). Little GUS activity was found in fully expanded leaves (data not shown).

Effects of light and exogenous sucrose on GUS expression in transgenic seedlings

Our previous studies indicated that the accumulation of *PsFTb* transcripts in leaves of pea seedlings was repressed by continuous white light (Yang *et al.*, 1993). To assess if the *PsFTb* promoter is responsible for the light-regulated expression, we examined the effect of light on the expression of the *PsFTb:GUS* fusion gene in transgenic tobacco seedlings. Surprisingly, striking differences in the spatial pattern of GUS expression between the dark-grown and light-grown seedlings were observed (Figure IV. 5). In 7-day-old light-grown seedlings, strong GUS activity was found in root tips and root-hypocotyl junctions; little GUS activity was detected in hypocotyls and the mature portion of roots (Figure IV. 5a). In contrast, no GUS activity was found in roots of 7-day-old dark-grown seedlings; GUS expression was primarily restricted to the vascular tissue of cotyledons, hypocotyls, and cotyledon-hypocotyl junctions (Figure IV. 5b). In 14-day-old light-grown seedlings, GUS expression was restricted to root tips, root-hypocotyl junctions, and the vascular tissue of leaves (Figure IV. 5c). Compared to 7-day seedlings, 14-day-old dark-grown seedlings contained little GUS activity in the cotyledon and lower portion of the hypocotyl, whereas strong GUS staining remained in most parts of hypocotyls (Figure IV. 5d). In the hypocotyl, GUS activity was primarily restricted to the phloem (Figure IV.5e).

To determine whether light has a quantitative effect on GUS expression, seedlings were grown in the dark for 10 days and then exposed to continuous light for various times. As shown in Figure IV.6, exposure to light for 1 to 3 days reduced total GUS activity by 2-3 fold. Dark-grown seedlings that were exposed to light for 3 days and followed by 7-day dark treatment showed similar GUS activity to seedlings grown in continuous darkness, suggesting that the light-mediated repression of GUS expression is reversible.

The expression of FTase in the phloem and inter-organ junctions together with light-mediated redistribution and repression of GUS activity in transgenic plants raises an interesting possibility that FTase expression is associated with the transport and/or mobilization of photosynthates. To test this idea, we determined the effect of various sugars on *PsFTb:GUS* expression. As shown in Figure IV.7a, 2% sucrose and glucose significantly repressed GUS expression in seedlings grown in the dark. Two-day treatments had a greater effect than one-day treatments, suggesting that sufficient levels of sugar must accumulate to suppress GUS expression. In contrast, 2% mannitol had no effect. One- and two-day treatments of 2% sucrose on 10-day old light-grown seedlings also reduced total GUS activity by 40% to 60% (data not shown).

Sucrose and glucose also repressed the expression of *PsFTb:GUS* in suspension-cultured BY-2 cells (Figure IV. 7b). Three-day-old cells were washed and resuspended in medium with or without sugars and GUS activity was determined at various times. In the medium without sucrose, GUS activity increased within 12 hr (data not shown) and remained high in the following days (Figure IV. 7b). In the presence of 3% sucrose, GUS activity remained unchanged. In contrast to seedlings, GUS expression was also repressed to some extent in the presence of 3%

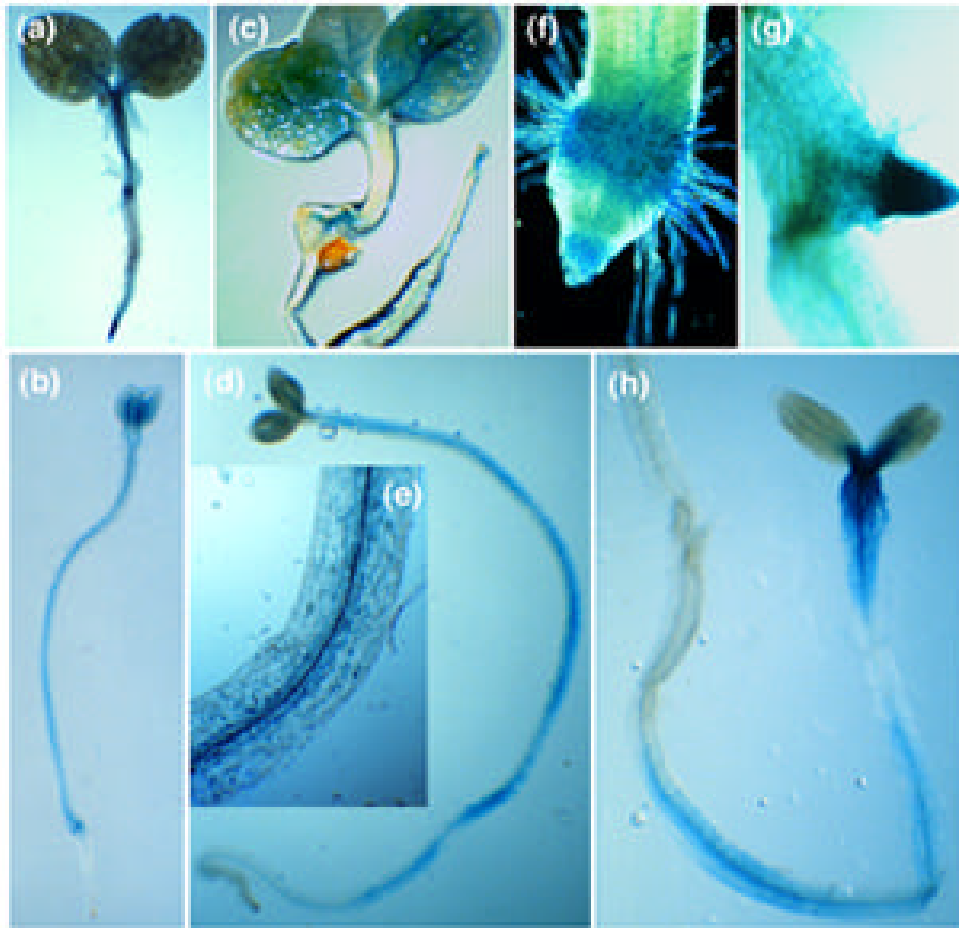


Figure IV.5. Effects of light and auxin treatments on the spatial pattern of *PsFTb:GUS* expression.

Seeds were germinated under complete darkness or continuous white light for 1 or 2 weeks (a-e). Light- or dark-grown seedlings were treated with auxins (f-h). Seedlings were stained and examined as described in Figure IV.3.

- (a). Seven-day-old light-grown seedlings.
- (b). Seven-day-old dark grown seedlings.
- (c). Fourteen-day-old light-grown seedlings.
- (d). Fourteen-day-old dark-grown seedlings.
- (e). Higher magnification of the hypocotyl in (d).
- (f). Hypocotyl and root of a 7-day-old light-grown seedling germinated on MS medium containing 2 μ M 2,4-D.
- (g). Lateral root of a 12-day-old light-grown seedling germinated on MS medium for 10 days and transferred to MS medium containing 2 μ M IAA for 2 days.
- (h). Fourteen-day old dark-grown seedlings germinated on MS medium for 12 days and transferred to MS medium containing 2 μ M 2,4-D for 2 days.

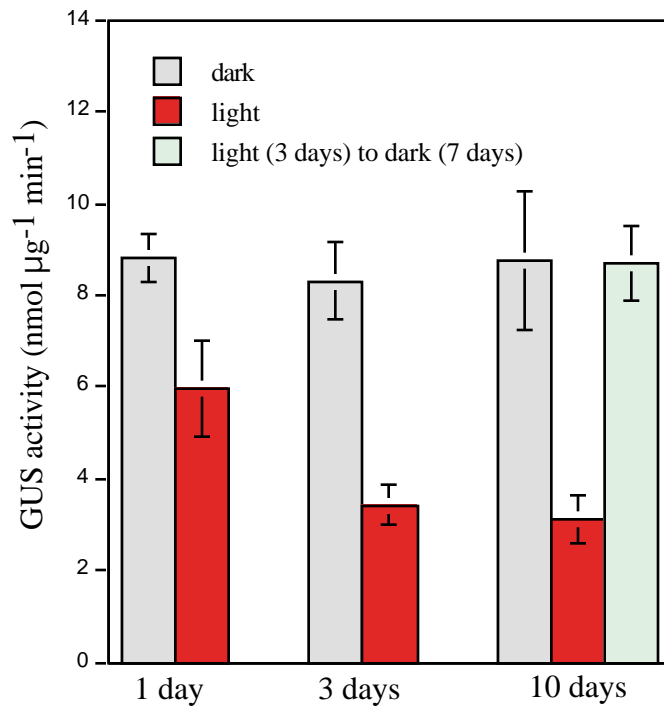


Figure IV.6. Effects of light treatments on the level of *PsFTb:GUS* expression in tobacco seedlings.

Seedlings were germinated in complete darkness for 10 days and exposed to light for various days as indicated. The control seedlings were kept in the dark until light treated seedlings were harvested. Proteins were extracted from whole seedlings and used for quantitative GUS assays as described in text.

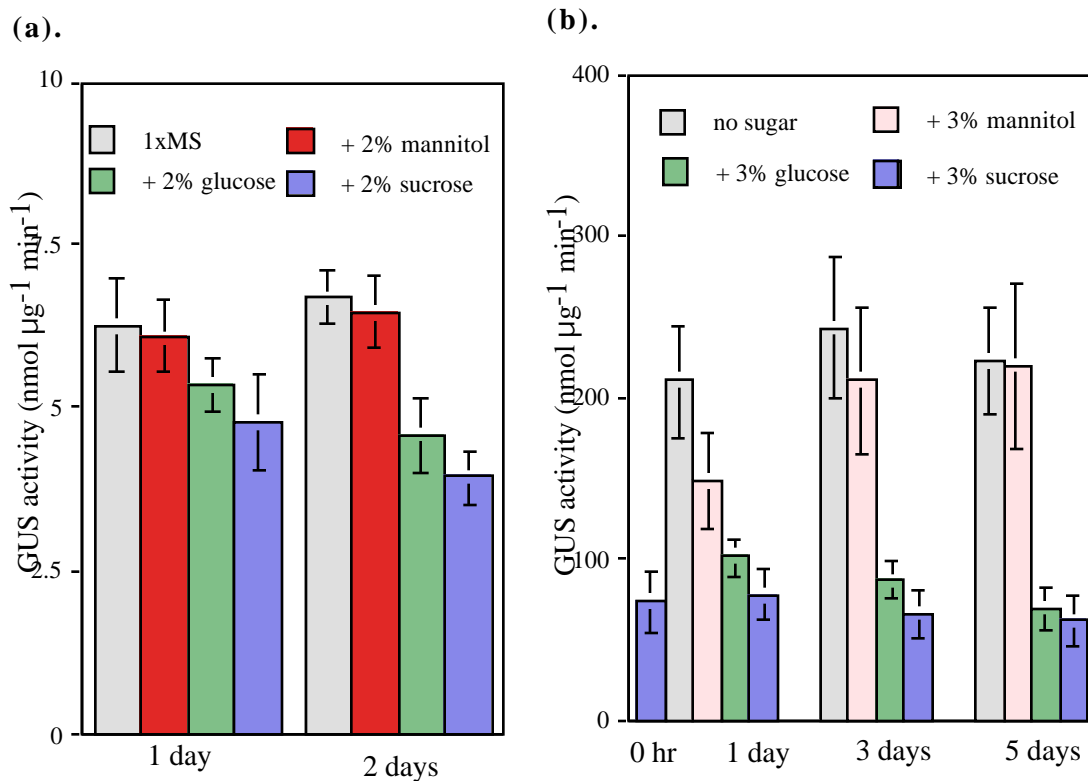


Figure IV.7. Effects of sugar treatments on the expression of the *PsFTb:GUS* gene in tobacco seedlings and in suspension-cultured BY-2 cells.

(a). Ten-day-old dark-grown seedlings (germinated on MS medium without sucrose) were incubated in MS medium containing no sugar, 2% mannitol, 2% glucose, or 2% sucrose for additional 1 or 2 days. GUS assays were performed as described in Figure IV.6.

(b). Three-day-old suspension-cultured BY-2 cells (time 0 hr) were washed with MS medium without sucrose and resuspended in media containing no sugar, 3% mannitol, 3% glucose, or 3% sucrose. Cells were harvested on indicated days after resuspension and used for GUS assays.

mannitol at early times (especially 1 day). This effect may be due to the presence of other sugars in the mannitol preparation.

Effects of phytohormones on *PsFTb*:*GUS* expression

To assess whether growth hormones contribute to the developmental regulation of the expression of *PsFTb*:*GUS* fusion gene, light-grown transgenic seedlings germinated on MS medium were treated with 2,4-D, kinetin, or ABA. Compared to seedlings germinated in MS medium alone, treatments with 2,4-D and kinetin enhanced GUS expression in three locations: root tips, root-shoot junctions and the basal portion of cotyledon (data not shown). In addition, 2,4-D treatment induced novel GUS expression in root hairs and in the region of roots where hairs are formed (Figure IV. 5f). In light-grown seedlings treated with auxin, strong GUS staining was evident at the site of root hair (Figure IV. 5f) and lateral root (Figure IV. 5g) initiation. In etiolated seedlings, 2,4-D treatment resulted in a pronounced increase in GUS activity at the top of the hypocotyl and the bottom of cotyledons (compare Figure IV. 5h to Figure IV. 5d). Treatments with ABA or kinetin alone did not have significant effect on *PsFTb*:*GUS* expression (data not shown).

DISCUSSION

The current data indicate that *PsFTb* expression exhibits unique tissue- and cell-specific expression that is regulated both by developmental cues and a number of growth-regulating factors. The *PsFTb*:*GUS* fusion gene is expressed in mature embryos, germinating embryos, meristematic tissues, phloem complexes, junctions between organs, and the terminal cells of trichomes. In addition, *PsFTb* gene expression is down-regulated by light and sucrose, but is promoted by auxins. These expression patterns and the existence of multiple farnesylated proteins in plants (Biermann *et al.*, 1996; Qian *et al.*, 1996; Zhu *et al.*, 1993) are consistent with the notion that the plant FTase has multiple specific functions, such as in the regulation of cell division and ABA-mediated seed germination (Cutler *et al.*, 1996; Qian *et al.*, 1996).

The 3.2 kb upstream fragment contains *cis*-elements required for the regulation of *PsFTb* gene

The temporal and spatial expression of the *PsFTb* gene during development was analyzed using transgenic expression of the GUS reporter gene fused to a 3.2 kb fragment upstream of the *PsFTb* coding sequence. Several lines of evidence indicate that this promoter truly reflects the activity of the *PsFTb* gene. First, the kinetics of GUS mRNA accumulation is similar to that for a tobacco FTase subunit gene in suspension-cultured tobacco cells containing the fusion gene. Moreover, a similar kinetics for FTase enzyme activity has been previously shown in BY-2 suspension cultures (Qian *et al.*, 1996). Second, GUS expression patterns in transgenic tobacco plants agreed with the accumulation of *PsFTb* transcripts in various organs of pea plants, *i.e.*, transcript levels were relatively high in roots and very low in stems, leaves and flowers of light-grown plants (Qian *et al.*, 1996). Finally, both the accumulation of *PsFTb* mRNA in pea seedlings (Yang *et al.*, 1993) and the transgenic GUS expression were repressed by light. Thus, we conclude that the 3.2 fragment contains all key *cis*-elements for the regulation of *PsFTb* mRNA

accumulation and that the GUS expression pattern observed in transgenic tobacco largely reflects the *PsFTb* expression pattern in pea plants.

Transgenic FTase gene expression in meristematic tissues supports a role for FTase in the control of cell division

Our recent studies demonstrated that a FTase-specific inhibitor blocks the cell cycle and that FTase activity is correlated with mitotic activity in suspension-cultured BY-2 cells, suggesting a critical role for FTase in the control of the cell cycle in plants (Qian *et al.*, 1996). This role is further supported by the expression pattern for the *PsFTb:GUS* fusion gene in tobacco suspension cultures and plants described in this study. In the transgenic suspension cultures, there is an increase in GUS mRNA levels at the log phase of cell growth, which is correlated with an increase in FTase activity and precedes the activation of cell division (Qian *et al.*, 1996). In transgenic plants, strong accumulation of GUS activity in root tips begins three days after seed imbibition, coincident with the activation of cell division in root apical meristems. GUS expression in root tips is associated with the activity of root growth; it was detected in seedlings grown under light (which stimulates root growth) but not in the dark (which inhibits root growth). Strong GUS expression was also found in lateral root primordia and the tip of lateral roots; auxin, which promotes the formation of lateral roots, enhanced GUS expression in these tissues. The high level of *PsFTb:GUS* expression in tissues associated with rapid cell division is consistent with a recent study in tomato that indicates that tissues such as calli, apical buds, and young fruits exhibit high levels of FTase activity (Schmitt *et al.*, 1996).

Elucidation of the specific role of FTase in mediating cell cycle control must await the identification of farnesylated protein(s) that regulates or is required for this process. In mammals and yeast, farnesylation is known to modulate the membrane association and the activity of the mitotic signaling proteins, Ras small GTPases (Itho *et al.*, 1993; Kato *et al.*, 1992; Zhang and Casey, 1996). A mammalian protein phosphatase that promotes cell proliferation was also found to be farnesylated (Cates *et al.*, 1996). Further, farnesylation is required for targeting of nuclear lamins in *Xenopus*, which are thought to be involved in the regulation of DNA replication as well as nuclear envelope assembly (Moir *et al.*, 1995). In accord with a role for the farnesylation of these proteins in cell cycle control, evidence indicates that Ras proteins and lamins are preferentially isoprenylated during the G₁ and S phases in human cells, respectively, and that FTase mRNA and enzyme activity levels are higher in Ras-induced human skin carcinomas than in normal skin cells (Khan *et al.*, 1996; Sepp-Lorenzino *et al.*, 1991). In suspension-cultured BY-2 cells, radioactively-labeled FTase substrate (farnesyl pyrophosphate) is incorporated into proteins with molecular weights similar to Ras and lamins (Qian *et al.*, 1996), and the fluctuation in FTase activity is correlated with the isoprenylation of these proteins (Morehead *et al.*, 1995). These observations raise the possibility that conserved farnesylated proteins such as Ras and lamins might also be involved in the control of cell division in plants.

If FTase plays an essential role in plant cell cycle control as suggested by inhibitor studies (Qian *et al.*, 1996), flower primordia would be expected to have high FTase activity. Indeed mRNA for the pea FTase subunit is relatively abundant in floral buds (Qian *et al.*, 1996). In *Arabidopsis*, both FTase activity and the expression of a FTase subunit gene were also found to be highest in floral buds among different tissues (Cutler *et al.*, 1996). Surprisingly, both RNA gel blot analysis (Qian *et al.*, 1996) and transgenic analysis of the GUS gene described in this study indicate that *PsFTb* is not actively expressed in floral buds. Genomic DNA hybridization analyses

under moderate stringency suggest that the plant FTase subunit is likely to be encoded by a small gene family (Qian and Yang, unpublished data). These genes might be differentially expressed, and a distinct isogene may be responsible for FTase activity in flower primordia. Additional support for this notion comes from the study by Schmitt and colleagues (1996), which suggests that the protein level for a tomato FTase subunit is correlated with FTase activity in some tissues but not in other tissues. In yeast and mammals, only one gene for the FTase subunit has been identified to date (Zhang and Casey, 1996). Identification of additional FTase subunit genes from plants will facilitate testing this hypothesis as well as revealing any functions unique to the plant FTase.

FTase gene expression during embryo development and seed germination

Recently, several *Arabidopsis era* mutants (enhanced response to ABA) were isolated based on the hypersensitivity of seed germination to ABA, and one *era* locus, *eral*, encodes an FTase subunit (Cutler *et al.*, 1996). Because *eral* mutants display enhanced dormancy, which is known to be mediated by ABA, it was suggested that farnesylation may negatively regulate the ABA-signaling pathway that controls seed dormancy (Cutler *et al.*, 1996). In agreement with the phenotypes of the *eral* mutant, our data suggest that the *PsFTb* gene is not expressed in developing embryos but is activated in mature embryos as well as in imbibed seeds. During seed maturation, the concentration of ABA rises, leading to seed dormancy (reviewed in McCarty, 1995). The expression of *PsFTb* in the mature embryo supports the notion that FTase is involved in the ABA signal transduction by desensitizing ABA responses (Cutler *et al.*, 1996). In animals, isoprenylation is involved in the deactivation of G-protein-coupled receptors by two different mechanisms. In one case, farnesylation and subsequent membrane localization of rhodopsin kinase is required for the desensitization of rhodopsin (Inglese *et al.*, 1992). In another case, the β -adrenergic receptor kinase must interact with the membrane-associated β subunits of G-proteins to deactivate the receptor; the membrane association of the G protein β subunit is dependent upon its isoprenylation (Inglese *et al.*, 1992). Interestingly, there is some evidence that G-proteins are involved in the regulation of ABA-mediated K^+ channels in guard cells (see reviews in Assmann, 1996; Yang, 1996). Identification of the farnesylated protein involved in the control of ABA-mediated seed dormancy will facilitate our understanding of the cellular significance of protein farnesylation in this process.

The expression of *PsFTb* in imbibed embryos is dynamically regulated in a temporal and spatial manner. GUS staining first appears in the root apex of embryo axis within hours of imbibition and then shifts to the central region of the axis one day after imbibition, before returning to the tip of the radicle by day 3. In general, little cell division occurs in the embryo before day 3, suggesting that early *PsFTb* gene expression in imbibed embryos is unlikely to be related to cell cycle control. Immediately after imbibition, nutrients stored in the endosperm and cotyledons are mobilized and transported into the embryo; this process is promoted by GA and inhibited by ABA. FTase could be involved in the negative regulation of nutrient mobilization by ABA. Alternatively, FTase might be a positive regulator in other signaling events such as in the regulation of cell growth or of nutrient transport into cells undergoing rapid expansion. This latter is consistent with the shift in the spatial pattern of GUS expression in imbibed embryos. Comparison of temporal and spatial expression patterns between *PsFTb* and *eral* will facilitate assessing whether *PsFTb* and *eral* have overlapping expression patterns and functions in mature embryos and early stages of

seed germination, but have differential expression patterns and functions in other developmental stages in pea and Arabidopsis as discussed above.

Possible involvement of FTase in the control of nutrient transport and partitioning

Specific expression of *PsFTb* in the phloem and junctions between two organs suggests an additional role for FTase in plants. One reasonable explanation for this expression pattern is that FTase is involved in the regulatory mechanism that modulate nutrient mobilization and transport toward the sink or tissues undergoing active growth. Several observations are consistent with this notion. First, *PsFTb* is expressed in the vascular tissue of young leaves and petioles but not in mature leaves. Second, *PsFTb* is expressed in the vascular tissue of roots and hypocotyls under the conditions that promote elongation (i.e., root elongation in the light and hypocotyl elongation in the dark), but not under the conditions that inhibit growth. Third, *PsFTb* expression was observed only in certain phloem complexes of stems and petioles, such as inner phloem. Finally, levels of *PsFTb* expression in seedlings are similarly suppressed by both continuous light and sugar treatments. Conceivably, sugar accumulation in sinks might negatively feedback-regulate FTase expression to down-regulate transport activity, analogous to the repression of photosynthetic genes by photosynthates (Jang and Sheen, 1994). Effect of light on *PsFTb* expression could be the result of its regulation of sucrose accumulation. Thus, light and sucrose treatments induce changes both in the spatial pattern and the level of *PsFTb* expression that are consistent with a role for FTase in the modulation of nutrient transport.

Nutrient transport and allocation is highly regulated and both sinks and sources can act as signals to regulate these processes (Stitt, 1996). However, the molecular mechanism for this regulation is essentially unknown. Whatever the mechanism might be, phloem loading and unloading clearly plays a key role in the regulation of nutrient transport and allocation (Stitt, 1996). The expression of *PsFTb* in the phloem adjacent to actively growing tissues raises the possibility that FTase might be a regulator of unloading. Like loading, unloading involves active transport, which is primarily driven by proton motive force generated by the plasma membrane H⁺-ATPase pump (Serrano, 1989). There is evidence that protein phosphorylation plays a role in the regulation of the ATPase (Xing *et al.*, 1996). It would be interesting to determine whether farnesylation may also be part of the mechanism controlling the ATPase activity and nutrient transport. Expression of antisense-RNA for *PsFTb* using a phloem specific promoter (DeWitt *et al.*, 1991) may facilitate testing the potential role of FTase in the control of nutrient transport and allocation.

METHODS

Isolation of *PsFTb* genomic clones and construction of *PsFTb* promoter:*GUS* fusion gene

To isolate *PsFTb* upstream sequences, we screened a pea genomic library (Clontech) using a near full-length *PsFTb* cDNA clone (Yang *et al.*, 1993). Several positive clones were obtained and were digested with *Bam*HI. Digested DNA was analyzed by Southern blot hybridization with the 230 bp *Hind*III/*Bam*HI fragment located at the 5'-end of the *PsFTb* cDNA clone (Yang *et al.*, 1993). A 4 kb *Bam*HI fragment that hybridized was subcloned into the pBlueScript II/KS- vector

(Stratagene). Sequence analysis showed that this fragment contained 0.8 kb *PsFTb*-coding region and 3.2 kb sequences upstream of the *PsFTb* translation initiation codon (Figure IV. 1a).

The 4 kb clone was digested with *Hind*III (located just downstream of *PsFTb* ATG start codon), blunt-ended by Mung Bean exonuclease digestion, and then digested with *Bam*HI. The resulting 3.2 kb fragment was fused in frame with the GUS gene into *Bam*HI/*Sma*I-digested pBI101.1 (Clontech). This resulting plasmid was designated p*PsFTb*:GUS (Figure IV.1).

Plant transformation

p*PsFTb*:GUS was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (An *et al.*, 1988). The chimeric gene was introduced into tobacco (*Nicotiana tabacum* cv. Xanthi) using leaf disc transformation (Horsch *et al.*, 1985). Primary transgenic plantlets were selected on Murashige and Skoog medium (MS, Sigma) containing 300 µg/ml kanamycin. Eleven independent transgenic plants were analyzed by histochemical GUS staining (Jefferson *et al.*, 1987) and showed similar GUS expression patterns. Two primary lines that showed 3:1 co-segregation for kanamycin resistance and GUS staining in T₁ seedlings were chosen for further analyses of GUS expression in T₁ generations.

Seed germination, seedlings growth, and treatments

Seeds were surface sterilized and germinated on Whatman 3MM paper wetted with 1 X MS salts (pH 5.7) or on 0.4% agar plates containing 1 X MS salts (Sigma). Seedlings were grown under constant white light (150 µmol photons m⁻² sec⁻¹) or in complete darkness at 25 °C. For time course analyses of GUS expression, seeds or seedlings were harvested at various times after being planted MS medium. For sugar treatments, seeds were germinated on MS medium for 10 days in dark and then transferred to fresh MS medium containing 2% sucrose, 2% glucose, or 2% mannitol, respectively. For hormone treatments, seeds were germinated directly on MS medium containing phytohormones [2 µM 2, 4-dichlorophenoxyacetic acid (2,4-D) and/or 2 µM kinetin (Sigma)], or 12-day-old seedlings germinated on MS medium were transferred to MS medium containing the phytohormones for 2 days.

Histochemical and quantitative GUS assays

Histochemical assays for GUS activity in transgenic tobacco plants were performed as described by Jefferson *et al.* (1987) with minor modifications. Parts of transgenic plants or entire seedlings were prefixed in 0.2% glutaraldehyde and 4 mM paraformaldehyde for 15 min and washed 3 times with 1 X PBS buffer (pH 7.4). Fixed tissues were submerged in GUS staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl -D-glucuronic acid (X-gluc, Research Products International Co., IL), 100 mM sodium phosphate (pH 7.5), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, and 0.1% Triton X-100. Following vacuum infiltration (3 min), tissues were incubated at 37 °C for 24 hr. Chlorophyll in green tissues was cleared by washing in 70% ethanol. To examine cell-specific GUS staining, tissues were hand-sectioned and photographed under a Zeiss stereoscope or a Zeiss microscope equipped with the Normaski optics.

Quantitative GUS assays were performed according to Gallagher (1992). Briefly, 100 mg of frozen tobacco BY2 cells or seedlings were ground in 200 μ l of extraction buffer (50 mM NaPO₄, pH7, 10 mM EDTA, 10 mM β -mercaptoethanol, 0.1% Triton X-100 and 0.1% sarcosyl) in a microcentrifuge tube using a tooth-polisher (Magnabrite™, 1992 Dental Concepts Inc., NY). Cell debris was removed from the homogenates by centrifugation at 12,000 rpm for 15 min. Protein concentrations were determined by the Bradford method (BioRad). Ten to 100 μ g of protein extracts in 100 μ l were mixed with 400 μ l GUS assay buffer [2 mM 4-methylumbelliferyl -D-glucuronide (4-MUG) in extraction buffer] and incubated at 37 °C. Aliquots of 100 μ l were taken at 0, 30 and 60 min, and reactions were stopped by adding 1.9 ml stop buffer (0.2 M Na₂CO₃). Fluorescent products were quantified using a fluorometer (Sequoia-Turner, model 450).

Culture and transformation of tobacco BY-2 cells

Tobacco BY-2 cell suspension cultures were routinely maintained in liquid BY-2 medium as described (Qian *et al.*, 1996). For stable transformation of BY-2 cells, 5 ml of 3-day old suspension-cultured cells were incubated with 0.1 ml of log-phase *Agrobacterium tumefaciens* (O.D600 = 1.0) bearing p*PsFTb:GUS* at room temperature for two days. Bacterial cells were removed by washing BY-2 cells several times with liquid MS medium. BY-2 cells were plated on solid BY-2 medium containing 300 μ g ml⁻¹ kanamycin and 500 μ g ml⁻¹ carbenicillin. Transformed calli were visible after 2-3 weeks at 25 °C under continuous light. GUS expression in transformed calli were confirmed by GUS staining. GUS-positive calli were transferred to liquid BY-2 medium to generate suspension cultures.

RNA gel blot hybridization analysis

Suspension-cultured cells were collected on Miracloth (Calbiochem) at indicated times after subculture, and fresh weights were measured as described previously Qian *et al.* (1996). Total RNA was isolated according to Thompson *et al.* (1983). Thirty micrograms of total RNA were denatured and separated on 1.2% agarose-formaldehyde gel as described (Sambrook *et al.*, 1992). RNAs were visualized by staining with 0.5 μ g/ml ethidium bromide in 0.1 M ammonium acetate and photographed before being transferred to the Maximum-Strength Nytran filter (Schleicher & Schuell). Filters were hybridized with the 1.3 kb cDNA encoding a *N. glutinosa* FTase subunit (Zhou *et al.*, 1996). The filter was stripped and rehybridized with the GUS gene (a 1.87 kb *Xba*I-*Sst*I GUS fragment from pBI221, Clontech). Hybridization was conducted at 42 °C in 50% formamide, 6 X SSPE, 5 X Denhardt's solution, 0.5% SDS, and 100 μ g ml⁻¹ denatured salmon teste DNA (Sigma). Filters were washed in 0.2 X SSC, 0.5% SDS at 65 °C for 15 min. To confirm RNA loading, filters were rehybridized with a pea DNA fragment encoding a 26S ribosomal RNA (Yang *et al.*, 1993). The hybridization signals were scanned using a Personal Densitometer (Molecular Dynamic Inc.) for quantification. Relative mRNA levels were presented as percentage of maximum. Data presented are the scanning average of 2 independent RNA analysis results.

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CHAPTER V.

**THE EXPRESSION OF FTASE α PROMOTER GENE
FUSIONS IN TRANSGENIC PLANTS**

ABSTRACT

Previous work has identified cDNA clones encoding both (*PsFTa*) and (*PsFTb*) subunits of FTase from pea (*Pisum sativum*). The study of expression of *PsFTb* revealed a tissue- and cell-specific pattern for the FTase subunit in pea and transgenic plants. Preliminary results also suggested that the expression of *PsFTa* and *PsFTb* are somewhat different in plants. To address the correlation between the expression of these two genes, a 2 kb fragment containing the 5' upstream sequence of *PsFTa* was isolated from a pea genomic library and fused with reporter genes encoding β -glucuronidase (GUS) and green fluorescent protein (GFP). Both chimeric constructs were introduced into tobacco (*Nicotiana tabacum* cv. Xanthi). In addition, the *PsFTa:GFP* construct was used to transform *Arabidopsis* (ecotype Columbia). Transient expression of *PsFTa:GUS* was observed in tobacco suspension cells following vector introduction by particle bombardment. However, neither GUS nor GFP was detected histochemically or at the transcript level in stably-transformed plants. The possible explanations for this result are discussed.

INTRODUCTION

Posttranslational farnesylation of many membrane-associated proteins is critical for the subcellular localization and biological function of these proteins (Omer and Gibbs, 1994). Protein farnesyltransferase (FTase), which catalyzes the attachment of farnesyl moieties to target proteins (discussed in Chapter I), is a heterodimer composed of non-identical α and β subunits (Reiss *et al.*, 1990; Kohl *et al.*, 1991). In both yeast and mammals, the FTase α subunit is shared by another prenyltransferase--type I geranylgeranyltransferase (GGTase I), whereas the β subunit is unique for FTase (Seabra *et al.*, 1991). cDNA clones encoding both FTase subunits have been isolated from yeast and mammals (Chen *et al.*, 1991a; 1991b; He *et al.*, 1991; Omer *et al.*, 1993). Mutation analysis demonstrated that both subunits are required for the enzyme catalytic activity (Goodman *et al.*, 1990; Chen *et al.*, 1991a; 1991b).

Evidence from yeast and mammalian systems suggests that the function of each subunit of the FTase is different. While the yeast mutant with a deletion of *RAM1* (encoding the α subunit gene of yeast FTase) is viable, a deletion of *RAM2* (encoding the β subunit gene of yeast FTase) is lethal (Goodman *et al.*, 1990; He *et al.*, 1991). The α subunit of FTase is found to bind target proteins specifically (Reiss *et al.*, 1991). The role of the β subunit is not clear, however, indirect evidence suggests it might be involved in binding of isoprenyl pyrophosphate (Reiss *et al.*, 1991).

Because of the evolutionary conservation of farnesylation, the fundamental function of FTase in mediating cell cycle control and development is likely to exist in plants as well. In tobacco suspension culture, changes in FTase mRNA levels and enzyme activity are correlated with cell division (Morehead *et al.*, 1995; Qian *et al.*, 1996). Furthermore, specific inhibitors of FTase can severely impair cell growth and appear to block specific stages in the cell cycle (Qian *et al.*, 1996).

Although progress has been made in molecular cloning of FTase genes in plants (Yang *et al.*, 1993; Cutler *et al.*, 1996; Schmitt *et al.*, 1996; previous chapters in this dissertation), the biochemistry and function of plant FTase are only poorly understood. Moreover, the interaction between the two subunits of plant FTase and the regulation of gene expression remain to be answered. The cloned *PsFTa* and *PsFTb* provide powerful tools to explore the correlation between these two subunits at the molecular level.

The expression patterns of *PsFTa* and *PsFTb* suggest that both genes are developmentally regulated. Differences in transcript levels in some tissues (*e.g.*, in floral buds) between the two subunits further indicate that these genes are differentially regulated in certain organs of plants. Our previous study of the FTase α subunit gene in tobacco showed that its expression was most prominent in meristematic tissues such as root tip and shoot apex. In pea, the transcript level of *PsFTb* was the highest in root nodules in which plant cells are presumably actively dividing. Initial RNA analysis in pea revealed that the expression pattern of *PsFTa* was similar to that of *PsFTb* in most parts of the pea plants. However, the transcript level of *PsFTb* was barely detectable in floral buds, whereas *PsFTa* was relatively high (Qian *et al.*, 1996). Also, different expression patterns were observed for these two subunit genes in preliminary experiments with tobacco suspension cultures (Zhou *et al.*, unpublished data).

In the present work, we proposed to investigate the expression of *PsFTa* in detail. A 2 kb fragment upstream of the *PsFTb* coding region was cloned from a pea genomic library. This presumptive promoter sequence was inserted upstream of either GUS or GFP reporter genes in individual expression vectors. These two constructs were introduced into tobacco and Arabidopsis. The sensitivities of GUS and GFP detection methods were expected to help in elucidating the subtle expression of FTase subunit gene in plant. Analyses of transgenic plants for tissue- and cell-specific expression should address regulation of the subunit gene by developmental and environmental factors. By comparing expression patterns of both FTase subunit genes, we hoped to gain insight into the following questions: 1) are these two subunit genes coordinately or differentially expressed, and 2) are there cells where the FTase subunit is expressed in the absence of subunit that would form the first evidence that the plant subunit may be shared by GGTase I?

RESULTS

Isolation of *PsFTa* promoter

Isolation of cDNA clones encoding PsFTa, the pea FTase subunit, was described previously (Qian *et al.*, 1996). To isolate the promoter sequences for *PsFTa*, a 318 bp fragment (see [Figure V.1](#)) from the 5' coding region of the *PsFTb* cDNA was initially used as a probe to screen a pea genomic library (Clontech) under high stringency conditions.

A 2.3 kb *EcoRI* fragment from a positive plaque was found to specifically cross-hybridize with the 318 bp probe. This 2.3 kb fragment was subcloned by insertion into the *EcoRI* site of pBluescriptII/SK. Partial sequence analyses showed that this 2.3 kb fragment contains a portion of *PsFTa*-coding sequence and about 2 kb of sequences upstream of the *PsFTa* ATG start codon. Based on the *PsFTa* sequence, a 24-nucleotide primer (antisense-FTa) was designed containing the *PsFTa* initiation codon ATG and its flanking sequences. To facilitate subcloning, nucleotide substitutions were designed in the primer to provide an *XbaI* and a *BamHI* restriction enzyme site ([Figure V.2](#)). Both antisense-FTa and a modified T₃ primer were used to amplify the 2 kb sequence via polymerase chain reaction (PCR).

Generation of plants expressing the α subunit promoter:reporter gene fusions

The 2 kb PCR product, containing the presumptive promoter for the *PsFTa*, was translationally fused to the GUS gene (*uidA* gene from *Escherichia coli*) in pBI101.1 (Jefferson *et al.*, 1987) via a three-step cloning strategy ([Figure V.3](#)). Particle bombardment-mediated transient expression of the resulting fusion gene, *PsFTa:GUS*, in tobacco BY-2 cells (Nagata *et al.*, 1992) demonstrated the presence of promoter activity in the 2 kb fragment (data not shown).

In addition, the 2 kb PCR product was inserted upstream of the modified GFP reporter gene (Prasher *et al.*, 1992). The hygromycin resistant gene was used as a marker to facilitate subsequent selection of transgenic plants which have both *PsFTb:GUS* (Kan^r) and *PsFTa:mGFP4*



Figure V.1. The position of the 318 bp fragment used in screening pea genomic library relative to the *PsFTa* cDNA clone.

A.

Antisense-FTa primer

5'-CCTCTAGACATGTTCTGGATCCTTCTTC-3'
XbaI BamHI

B.

pea FTa genomic sequence (partial):

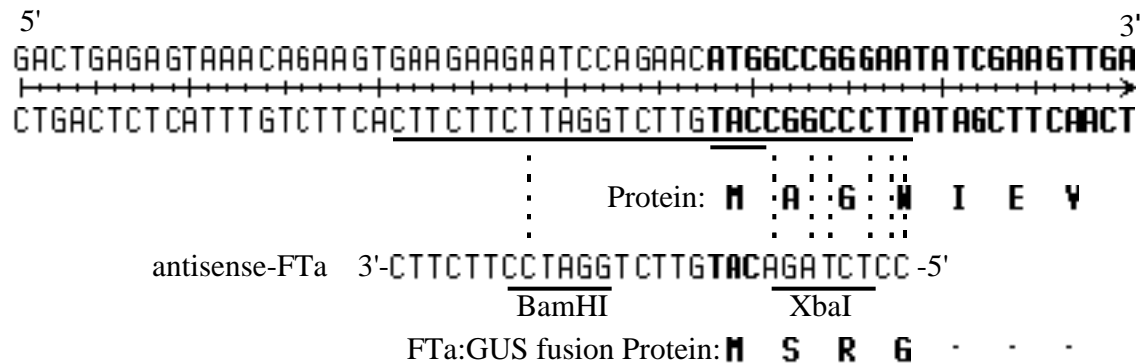


Figure V.2. Design of the antisense-FTase primer used in cloning of the putative promoter region of pea FTase α subunit gene.

- A. The primer sequence. The underlined restriction enzyme sites, *XbaI* and *BamHI* were used to generate expression constructs *PsFTa:GUS* and *PsFTa:mGFP4*, respectively.
- B. The location of the primer in the *PsFTa* genomic sequence. The *PsFTa* coding sequence is in bold and its start codon is double underlined. The genomic sequence corresponding to the primer is underlined. The substitution nucleotide sites are dashed. The substitution is to create an *XbaI* and a *BamHI* site in the primer to facilitate subcloning (see Figure V.3 & V.4).

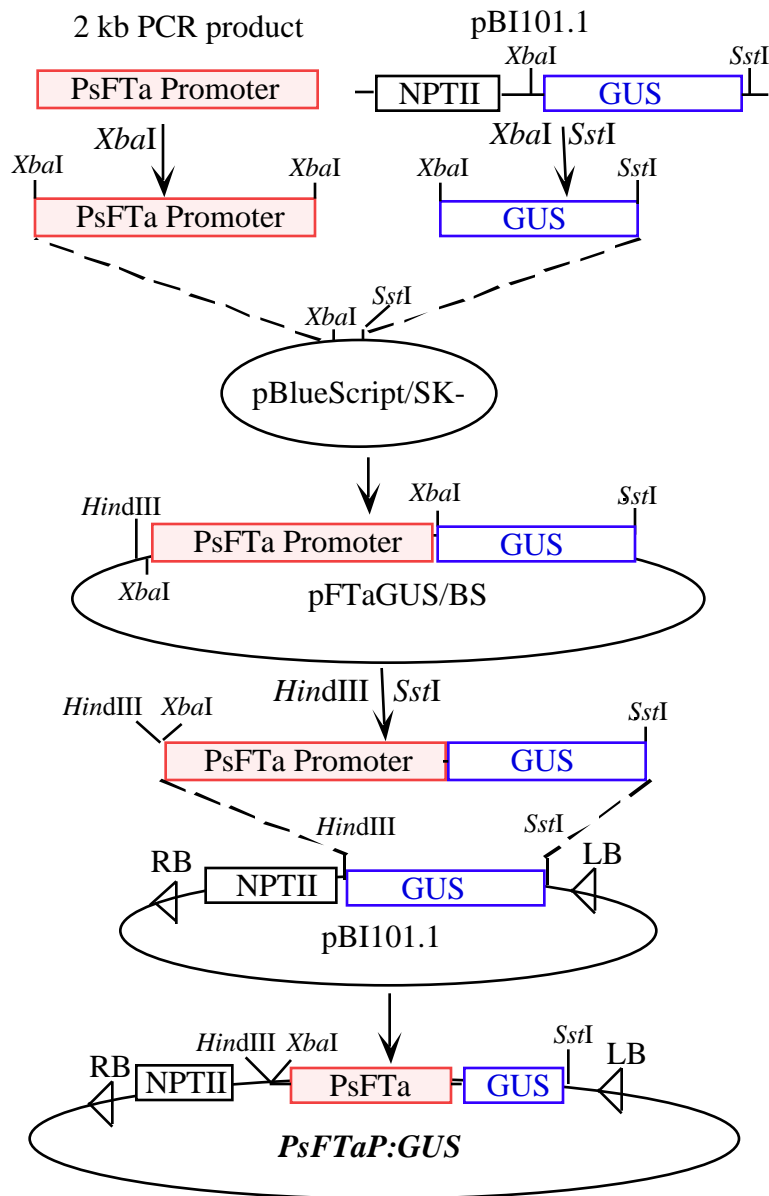


Figure V.3. Construction of pea FTase α subunit promoter:GUS (*PsFTa:GUS*) expression vector.

Three subcloning steps were involved (see methods for detail).

1. GUS fragment from pBI101.1 was inserted into pBlueScript vector and yielded pGUS/BS;
2. The 2 kb putative *PsFTa* promoter was inserted into pGUS/BS to produce pFTaGUS/BS;
3. The *FTa:GUS* cassette from pFTaGUS/BS was used to replace the GUS fragment in pBI101.1 to produce a final expression vector, *PsFTa:GUS*. NPTII, neomycin phosphotransferase conferring kanamycin resistance; LB and RB, left and right border sequences delineating DNA transferred to plant by *Agrobacterium tumefaciens*.

(Hyg^r) constructs stably transformed. The subcloning strategy for the construct is shown in [Figure V.4](#).

Both *PsFTa:GUS* and *PsFTa:mGFP4* were introduced into tobacco (*Nicotiana tabacum* cv. Xanthi) plants by *Agrobacterium tumefaciens*-mediated transformation (Horsch *et al.*, 1985). After kanamycin or hygromycin selection, about 20 individual primary transformants were selected for each construct. In addition, 18 transformed Arabidopsis (ecotype Columbia) lines were generated that contained the *PsFTa:mGFP4* construct.

Analyses of transgene integration and expression

Histochemical staining was used to examine GUS activity in 23 tobacco plants independently transformed with *PsFTa:GUS*. Analyses were focused on tissues known to express the subunit (see Chapter IV), *e.g.*, those plant regions involved in active cell division and growth. To determine if *PsFTa* has similar expression pattern, meristematic tissues such as root tip, shoot apex, and very young leaves were excised from the transformed tobacco for GUS staining. Among all *PsFTa:GUS* initial transformants, no detectable GUS activity was observed. In Northern analyses (see Chapter II), we observed relatively high levels of subunit transcript in floral buds but no subunit transcript. However, the floral buds from *PsFTa:GUS* transformed tobacco also did not contain any GUS activity.

Since the *PsFTa:GUS* is a translational fusion construct, one possible explanation for the lack of GUS activity is that the subcloning may have introduced a reading frame shift in the construct. However, sequence analysis confirmed that the GUS gene was fused in-frame with the *PsFTa* initiation codon ([Figure V.5](#)). This is further supported by the fact that GUS activity was observed in particle bombardment-mediated transient expression assays in BY-2 cells (data not shown).

In addition, tobacco plants transformed with the *PsFTa:mGFP4*, which is a transcriptional fusion construct, showed no detectable GFP. Since light down-regulated the expression of FTase subunit gene in tobacco (Chapter IV), we also used dark-grown tobacco seedlings to monitor the GFP activity by fluorescent microscopy. However, detection of very low levels of GFP may have been hindered by high levels of red fluorescence of the tobacco tissues.

Because of the autofluorescent characteristics of tobacco, the *PsFTa:mGFP4* construct was also introduced into Arabidopsis. The T₂ seedlings of 18 independently-derived transformants were germinated on medium containing hygromycin. Two lines were examined by fluorescence microscopy after three weeks germination on selective medium. Neither line showed any GFP fluorescence in these seedlings (data not shown). Seedlings of analogous developmental stage showed easily detectable levels of *PsFTb promoter:GUS* expression, especially in root/shoot junctions, root tips, and shoot apices (see Chapter IV).

To examine the possibility of false positive selection or unstable transformation, Southern blot analyses were performed on total genomic DNA isolated from transformed plants. At least one copy of the reporter gene was detected in all tested plants. Some plants contain multiple copies of the reporter gene integrated into the genome (data not shown).

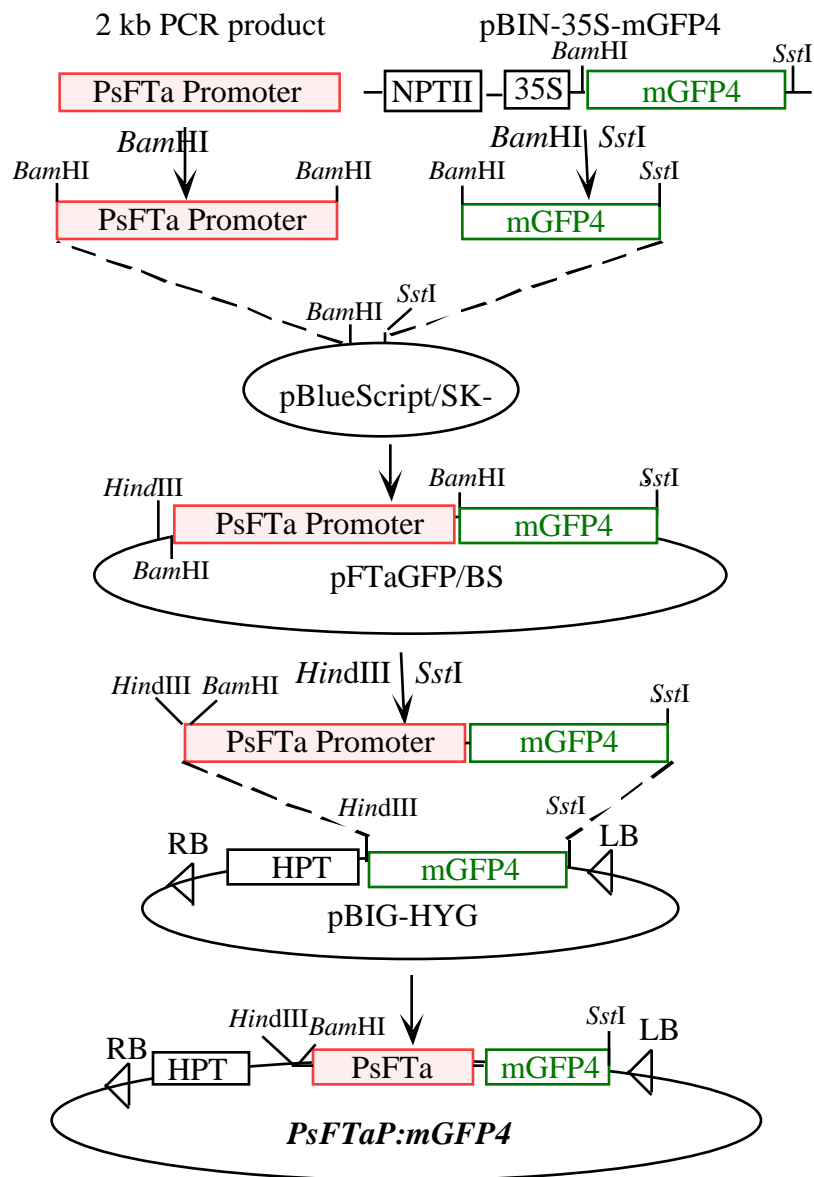


Figure V.4. Construction of pea FTase α subunit promoter:GFP (*PsFTa:mGFP4*) expression vector.

Three subcloning steps were involved (see methods for detail).

1. The modified green fluorescent protein- encoding fragment, mGFP4, from pBIN-35S-mGFP4 was inserted into pBluscript vector and yielded pGFP/BS;
2. The 2 kb putative *PsFTa* promoter was inserted into pGFP/BS to produce pFTaGFP/BS;
3. The *FTa:GFP* cassette from pFTaGFP/BS was used to replace the GUS fragment in pBIG-HYG to produce a final expression vector, *PsFTa:mGFP4*. HPT, hygromycin B phosphotransferase conferring hygromycin resistance; LB and RB, left and right border sequences delineating DNA transferred to plant by *Agrobacterium tumefaciens*.

```

GATCGATACCCCTTCTATCACTATATAGTAATTGACTTTTTGTATATTGAC 50
TATTGGACTAAATACATTGACAATCAAATATATCAACAAGAAAAAAATC 100
AAATTTATTTATAAATGTGACGGGGAGGGTGTATTAGAAATATGGGACTCC 150
ACGTGTAAAGGATTAATAGGGGGTGCCCGGTTGAATCCCCAACACCTACC 200
TAGTTCTTCTAGTTCTGGTTCTAGGACTGAGAGTAAACAGAAGTGAAGAA 250
GGATCCAGAACATGTCTAGAGGATCCCCGGGTGGTCAGTCCCTTATGTTA 300
      M S R G S P G G Q S L M L
CGTCCTGTAGAAACCCCAACCCGTGAAATCAAAAAACTC 339
R P V E T P T R E I K K L

```

Figure V.5. Sequence analysis of the junction region of the expression vector *PsFTa:GUS*.

The first ATG (bold) start codon is from the *PsFTa* promoter and the second one (bold and underlined) is from the *GUS* gene. The encoded amino acid sequences are shown using single letter code.

To determine if the transgenes were active at the transcriptional level, RNA was isolated from the transformed hygromycin-resistant BY-2 cell lines for Northern hybridization analyses. No GFP expression was detected in any of the eight BY-2 cell lines that were stably transformed with *PsFTa:mGFP4*. However, the hygromycin gene was strongly expressed in seven of these lines (Figure V.6).

DISCUSSION

The cloning of *PsFTa* 5'-upstream sequence was successful. The fusion of cloned putative promoter sequence with two different reporter genes was completed as designed. Both expression constructs were introduced into plants and the analyses of transgenic plants were initiated.

Disappointingly, both reporter genes driven by the putative *PsFTa* promoter sequence were not expressed in the transgenic plants, or showed expression at an extremely low level that could not be detected. The latter explanation is less likely because GUS protein is quite stable in plant cells and very sensitive in detection.

Partial sequencing confirmed that the cloned 2.3 kb fragment indeed contains 309 bp of the *PsFTa* coding region and about 2 kb of the 5'-upstream sequence (Figure V.7). The partial coding region and its upstream sequences are over 99% identical with the corresponding region in the published *PsFTa* cDNA sequences and the genomic clone sequences (Figure V.7). Sequence analyses of the construct junction region plus the transient expression result exclude the possibility of reading frame shift in the final expression cassette. Genomic Southern analyses, antibiotic resistance selection, and the HPT (responsible for the hygromycin resistance) mRNA detection confirmed stable introduction of the transgene. In fact, we found 11 out of 18 individual transformed plants had GUS activity in the previous study of *PsFTb:GUS* expression in transgenic plants. Two plants that had high levels of GUS activity were used in subsequent analyses (see Chapter IV). Here, although more transgenic plants (23 lines of *PsFTa:GUS* transformed tobacco, 18 lines and 15 lines of *PsFTa:mGFP4* transformed tobacco and Arabidopsis, respectively) have been examined, none showed evidence of reporter gene expression. Positional effects are unlikely to account for this unusual result. The possibility that gene promoters of pea origin may not function in tobacco is unlikely. Numerous gene promoters of legume origin have been successfully studied in tobacco system (Li *et al.*, 1994; Prandl *et al.*, 1995; Ahn *et al.*, 1996). The promoter of pea FTase subunit is also well-regulated in transgenic tobacco (Chapter IV). All this information leads us to suspect that the 2 kb upstream region of the pea FTase subunit gene is insufficient to provide promoter function.

For many plant genes that have been examined, 1 to 1.5 kb of 5' sequence upstream of a coding region is sufficient to confer the proper expression pattern in transgenic plants (Fu *et al.*, 1995a). However, exceptions are reported. The most prominent sequence element that affects normal gene expression is the leader intron (Callis *et al.*, 1987; Fu *et al.*, 1995a; 1995b; Vain *et al.*, 1996). Studies of the leader intron of *Shrunken1* gene of maize showed that it can increase expression of a heterologous promoter up to 100-fold in transient assays (Maas *et al.*, 1991; Clancy *et al.*, 1994). Removal of the long first intron of *Sus4* gene from potato caused a significant reduction in expression in potato tubers and also changed its expression pattern in transgenic potatoes (Fu *et al.*, 1995a). The insertion of maize leader intron of *ubi1* (ubiquitin) between CaMV 35S promoter and its downstream GUS gene increased the GUS expression in transgenic maize about 71 fold (Vain *et al.*, 1996). There are two proposed mechanisms for the

A. GFP probe



B. HPT probe

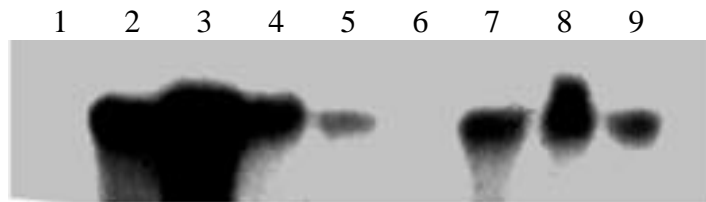


Figure V.6. RNA gel blot analyses of BY-2 cell lines transformed with *PsFTa:GFP* expression vector.

A. Blot hybridized with ^{32}P -labeled GFP coding region (about 700 bp). A total of 8 independent lines were tested. Lane 1 represents a *35S:mGFP4* transformed BY-2 cell line.

B. Blot hybridized with ^{32}P -labeled HPT gene (about 1.8 kb).



Figure V.7. Partial sequence comparison of the *Taq* polymerase synthesized pea FTase α subunit promoter with the *PsFT α* genomic and cDNA clones.

Within the available sequence information in the promoter region, no vital PCR error was observed. Consensus residues are indicated by black rectangles. Start codons are boxed. MegAlign program from DNASTar Inc. in Wisconsin was used in sequence analyses.

involvement of the leader intron in regulation of gene expression (Fu *et al.*, 1995a). One is that there are *cis* elements present in the intron sequence. The other is that the intron functions to facilitate RNA splicing.

In addition, it is believed that some *cis*-elements may exist in 3'-untranslated sequences. The 3' non-coding sequences are essential for the proper expression of the potato proteinase inhibitor II gene (Thornburg *et al.*, 1987) and the potato *Sus3* and *Sus4* genes (Fu *et al.*, 1995a; 1995b), oilseed rape *AX92* (Dietrich *et al.*, 1992), and Arabidopsis *GLABROUS1* (Larkin *et al.*, 1993). For example, replacing the *Sus4* 3'-sequence with the terminator from the nopline synthetase gene can cause an eight fold decrease in *Sus4* gene expression in potato tubers (Fu *et al.*, 1995b).

The further upstream sequence of 5' non-coding region may also have an effect on the normal function of pea FTase subunit gene. During analyses of the pea FTase subunit gene, we had made two different GUS fusion constructs. One contained a 3.2 kb sequence upstream of the PsFTb coding region and was functionally expressed in transgenic tobacco (Chapter IV). The other construct, containing only 1 kb upstream of PsFTb coding region, did not show any detectable GUS activity in more than 30 transgenic tobacco plants that were analyzed (Zhou, D., Cramer, C.L., Yang, Z., unpublished data). The result indicates that important elements for regulation of *PsFTb* expression are located further upstream than 1 kb. As in the case of the promoter for pea FTase subunit, the cloned 2 kb fragment may not be sufficient for functional expression.

A possible error due to the PCR amplification using *Taq* DNA polymerase could have been a cause of a lack of expression. Although the *Taq* polymerase is used widely for all the PCR applications, the relatively low fidelity of *Taq* is a possible consideration (Lundberg *et al.*, 1991). Sequence comparison of the PCR-cloned 2 kb with the original genomic clone was attempted to address this question. As shown in [Figure V.7](#), there was no error was introduced in the sequences surrounding the TATA box region by PCR amplification.

Based on the results presented here, we must conclude that the cloned 2 kb 5' flanking region is not sufficient for the gene expression. The first intron, the 3' untranslated sequence, or additional 5' non coding region may be required for the full function of PsFTa expression in plants. To answer this question, further promoter analysis and characterization the PsFTa genomic gene structure are needed.

METHODS

Isolation of *PsFTa* genomic clone

An 318 bp *EcoRI* fragment containing the 5' end of the PsFTa cDNA clone ([Figure V.1](#)) was used to screen a pea genomic library (Clontech, Palo Alto, CA). After several rounds of screening and purification, 10 positive plaques were randomly selected (originally from 4 independent clones) and amplified. DNA was extracted (Sambrook *et al.*, 1989) and digested. Southern blot analyses showed that a 2.3 kb *EcoRI* fragment hybridized with the 318 bp fragment probe (data not shown). The 2.3 kb fragment was cloned into the pBlueScriptII/KS⁻ vector

(Stratagene, La Jolla, CA). Partial sequencing showed that this 2.3 kb contained 309 bp of *PsFTa* coding region and 2 kb of upstream sequence. The modified T3 primer (5'-GCGCATTAACCCTCACTAAAGGG) and antisense-FTa oligonucleotide (5'-ACCTTCTAGATTCCCGGCCATGTTC) were used as primers in a polymerase chain reaction (PCR) to amplify the 2 kb fragment. The antisense-FTa primer was designed to span the ATG start codon and generate a flanking *Xba*I site (underlined) to facilitate translational fusion to the GUS reporter gene. PCR was performed in a 100 µl reaction mixture containing 20 ng of plasmid DNA, 400 pmol of each primer, and 5 units of *Taq* DNA polymerase (Promega, Madison, WI). Thirty five amplification cycles were conducted at 94° C for 1 min (denaturation), 57° C for 30 sec (annealing), and 72° C for 2min 30 sec (elongation). PCR was finished with a further 10 min incubation at 72° C. The PCR products were purified from gel using a gel purification kit (Qiagen, Chatsworth, CA).

Construction of *PsFTa:GUS* and *PsFTa:mGFP4* fusion genes

The 3-step constructions of *PsFTa:GUS* and *PsFTa:mGFP4* are illustrated in [Figure V.3](#) and [Figure V.4](#), respectively. Briefly, GUS and mGFP4 gene fragments were excised from of pBI101.1 (Clontech) and pBIN 35S-mGFP4 (gift from Dr. Jim Haseloff in Cambridge, UK; Prasher *et al.*, 1992), respectively. Each of the two gene fragments was inserted into a pBlueScriptII/KS vector, producing pGUS/BS and pGFP/BS, respectively. The *Xba*I-digested PCR-product was inserted into pGUS/BS digested with *Xba*I, yielding pFTaPGUS/BS. The *Bam*HI-treated 2 kb PCR-product and pGFP/BS were ligated to produce pFTaPGFP/BS. The FTaP:GUS and FTaP:GFP cassettes were derived from pFTaPGUS/BS and pFTaGFP/BS digested with *Hind*III and *Sst*I. The FTa:GUS was inserted into *Hind*III- and *Sst*I-treated pBI101.1 vector. This produced the *PsFTa:GUS* expression construct. The *FTaP:GFP* was insert into the *Hind*III/*Sst*I-digested pBIB-HYG vector to produce the *PsFTa:mGFP4* construct.

Plant transformation

The expression vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404 (for tobacco) or GA3101 (for Arabidopsis) by the freeze-thaw method (An *et al.*, 1988). Tobacco transformation is as described in CHAPTER IV. The protocol of vacuum infiltration of Arabidopsis was adapted from Bechtold *et al.*, (1994).

Seeds collected from *Agrobacterium tumefaciens*-treated Arabidopsis (ecotype Columbia) were sown on selective medium containing 30 µg/ml hygromycin. After three weeks, the young hygromycin-resistant seedlings were transferred to soil and propagated in a growth chamber. The seeds from these plants were collected and used for further analysis.

Histochemical GUS staining and RNA gel blot hybridization analysis

These methods were described previously in CHAPTER IV.

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CHAPTER VI.

CONCLUSIONS AND FUTURE DIRECTIONS

Farnesyltransferase (FTase), a heterodimeric enzyme, is responsible for the posttranslational farnesylation that is critical for the functional regulation and intracellular targeting of a broad range of proteins. The structure of FTase, modes of action, and preference of substrate have been extensively studied in yeast and mammalian systems (reviewed in Casey and Seabra, 1996). Results from studies of farnesylated proteins (*e.g.*, Ras and lamins) clearly demonstrate a role FTase in cell cycle control.

In plants, this conserved posttranslational modification exists as well. FTase activity has been detected in many plant cells, including tobacco (*Nicotiana tabacum*) tomato (*Lycopersicon esculentum*), pea (*Pisum sativum*), soybean (*Glycine max*), and Arabidopsis (*Arabidopsis thaliana*). Putative protein substrates for farnesylation have been described from several plant systems. Genes encoding FTase have also been identified in several plants. However, the nature of FTase and its role in plants are still poorly understood.

As an extension of our initial cloning of a gene encoding a pea FTase subunit (*PsFTb*), a cDNA encoding the subunit of pea FTase (*PsFTa*) and a cDNA clone encoding a tobacco homolog of FTase subunit (*NgFTb*) were isolated and characterized. The cloned *NgFTb*, although lacking about 30 bp in the 5' end, proved very useful in studying FTase gene expression in tobacco. The deduced protein has overall 61%, 61.6%, 21.8%, and 38.8% identity with the pea, Arabidopsis, yeast, and rat FTase subunits, respectively.

Our data on gene expression and regulation of FTase activity in tobacco cell culture systems support the hypothesis that farnesylation is involved in regulating plant cell cycle progression. In tobacco suspension culture, changes in the transcript level of the FTase subunit gene correlate with cell division and growth cycle. The mRNA of the FTase subunit gene increased rapidly following transfer to fresh medium at the early log phase of cell growth and declined dramatically before cells reached the stationary phase. The kinetics of FTase enzyme activity is similar to that observed at the mRNA level. Further studies using manumycin, a specific FTase inhibitor, showed that inhibition of FTase activity blocked cell cycle progression. Consistent with the report using other prenylation inhibitors (Morehead *et al.*, 1995), the inhibition of cell growth was effective only when the inhibitor was added before any increase in cell fresh weight (volume) was detectable. The data on synchronized tobacco cells further indicates that the involvement of FTase in cell cycle is in the G₀/G₁ to S phase transition.

Detailed study of *PsFTb:GUS* expression patterns in transgenic tobacco plants has provided additional evidence for the function of FTase in plant cell division and development. GUS activity was detected in the meristematic tissues including root tip, sites of lateral roots initiation, and shoot apices. Also, GUS was expressed in young leaves but not mature leaves.

In addition, changing patterns of GUS activity during the seed maturation and germination is consistent with the notion that FTase also functions in one of the plant signal transduction pathways—abscisic acid (ABA) signaling. In previous studies of Arabidopsis *Era* mutants (Culter *et al.*, 1996), it was found that defects in the FTase subunit resulted in an increased sensitivity to ABA. ABA is known for mediating plant physiological processes, such as seed dormancy and leaf senescence. During seed maturation, the ABA concentration rises and thus keeps seeds in dormancy. When gibberellic acid (GA) level rises upon stimulation by environmental factors, the ABA level begins to drop and seeds start to germinate (reviewed in McCarty, 1995). Coincident with the changes of ABA levels in seeds during maturation, GUS activity was found in mature and

imbibed embryos of the tobacco plants, whereas no detectable GUS activity was observed in premature embryos. This data provides further support for the possibility that FTase may negatively control the ABA signaling.

Besides substantiating previous studies suggesting FTase function in plants (Morehead *et al.*, 1995; Cutler *et al.*, 1996), our data on the temporal and spatial expression patterns of *PsFTb:GUS* suggest a potential role of FTase in another signal transduction pathway. In transgenic plants, the correlation between the expression of FTase genes and actively dividing tissue (*e.g.*, meristematic tissues) is not absolute. The *PsFTb:GUS* was expressed in certain vascular bundles (*e.g.*, primary phloem) that are adjacent to actively growing tissues, such as young leaves, roots of light-grown seedlings, and hypocotyls of dark-grown seedlings. GUS activity was also detected in connection regions of similar or different organs such as flower receptacles, and junctions between stems and leaf petioles, primary and secondary roots, cotyledons and hypocotyls, and roots and hypocotyls. Although different tissues and cell types are involved, all of these regions are extremely active in nutrients and metabolites transportation. A similar unexpected enzyme activity pattern was also reported in a study of tomato FTase (Schmitt *et al.*, 1996). Therefore, one reasonable speculation is that FTase may have a broader role in mediating nutrient transport and partitioning.

The effects of environmental factors on the expression of *PsFTb:GUS* are consistent with the notion that FTase may be involved in the regulation of nutrient mobilization and allocation of nutrients toward the sink or tissues undergoing active growth. In general, the expression of *PsFTb:GUS* in the young seedlings of transgenic tobacco is suppressed by continuous light. Sugar (*e.g.*, sucrose) also causes a reduction of GUS expression in the seedlings. Since light promotes photosynthesis leading to accumulation of sucrose in plant, it is feasible that inhibition of FTase subunit gene expression by both light and sucrose results from negative feedback-regulation by the *in vivo* accumulation of sucrose or its hydrolyzed forms, glucose and fructose, in plant cells (sink). In fact, a similar mechanism of regulation was proposed in the control of photosynthetic gene expression (Jang and Sheen, 1994; Jang *et al.*, 1997).

Results generated in a collaborative research project on the parasite effects on the expression of *PsFTb:GUS* in transgenic plants further strengthens our sink-source theory (Jim Westwood, Dafeng Zhou, Carole L. Cramer, unpublished results). *Orobanche aegyptiana*, a parasitic weed, was used to infect young tobacco seedlings containing the *PsFTb:GUS* fusion construct. Upon infection (Westwood *et al.*, 1996), GUS activity was observed specifically in the vascular tissue where the parasite attached to the tobacco root (Figure VI.1). The parasitic angiosperm is known for capture of nutrient resources by penetration into the vascular tissue of the host plant (reviewed in Stewart, 1990). Presumably, the vascular tissues around the infection site of *Orobanche* are most actively involved in the transportation of various nutrients.

Although our data indicates that FTase has multiple functions in plant differentiation and development, it remains to be determined how FTase mediates plant processes as well as how its activity is precisely regulated. For down regulation of FTase activity by both light and sucrose, the higher influx of sucrose and/or glucose may trigger the repression of FTase subunit genes through downstream signal transduction pathways (Figure VI.2). For functions of FTase in plant signal transduction pathways, it is possible that specific proteins that participate in regulation of the either ABA signaling or nutrient allocation may require farnesylation to function. In fact, the molecular mechanisms of ABA signaling and the nutrient allocation in plants are not yet known. Further understanding of these complex signal transduction pathways in plants will benefit from the study of FTase function.

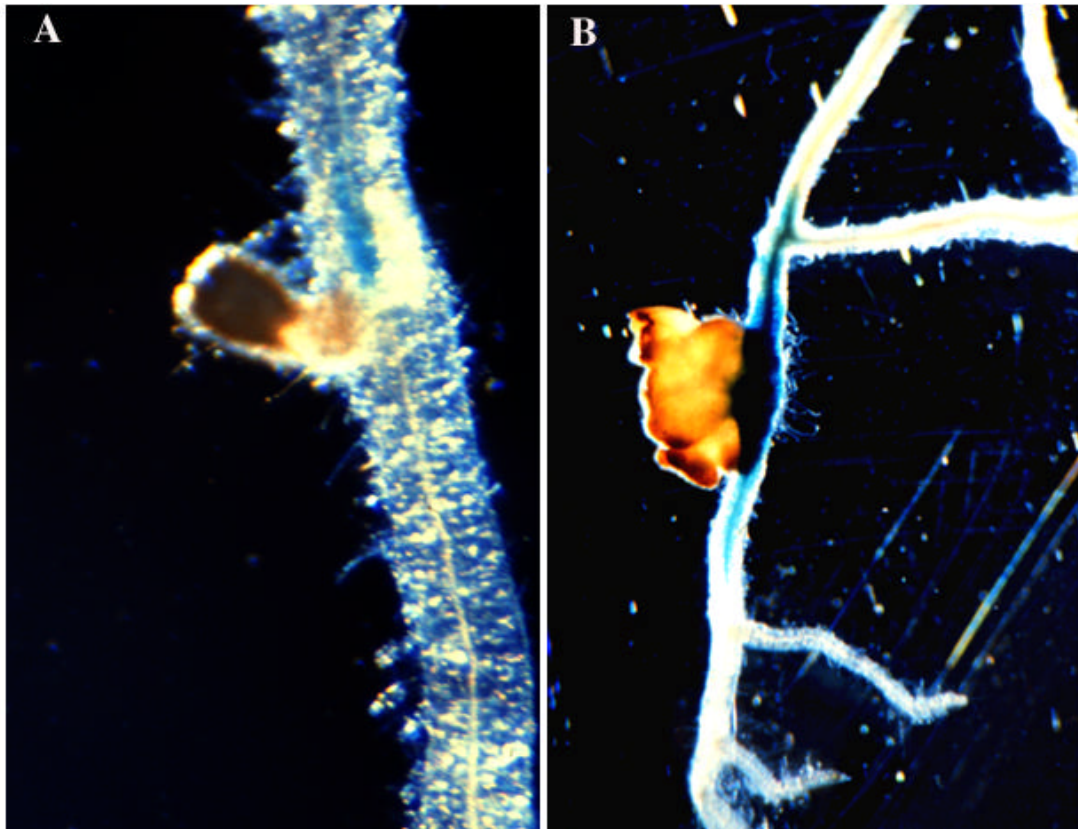


Figure VI.1. Histological GUS staining of transgenic tobacco seedlings expression the PsFTb:GUS fusion gene after inoculation with *Orobanche aegyptiaca*.

- A. 24 hours after inoculation. *Orobanche* seed (brown) has germinated and penetrated the tobacco root. GUS activity is localized to the vascular tissue proximal to attachment (blue).
- B. 10 days after inoculation. Strong GUS staining is evident in all areas of contact between the developing tubercle and the host root.

The fact that the subunit of FTase is shared with another prenyltransferase--geranylgeranyltransferase, although not yet proved in plants, could mean another regulatory mechanism for controlling FTase gene expression and enzyme activity. It was revealed during our research that pea FTase protein is relatively stable in *E. coli* only in heterodimer form; either single subunit of FTase is unstable. Thus, the interaction between the two subunits may also play a role in regulation of FTase activity in plants.

In the future, the study of FTase and farnesylation in plants should focus on the following:

1) to further identify FTase genes and farnesylated proteins in plants. Like the enzyme HMGR in plants, it is possible that, in order to deal with the more complicated isoprenoid pathway and plant developmental programs, FTase in pea or tobacco or even Arabidopsis is a multi-gene family (Cutler et al., 1996; Yang, Z., Zhou, D., Qian, D., Cramer, C.L., unpublished data). The identification of other FTase genes and additional farnesylated proteins in plant systems will provide a greater understanding of the function of FTase in plant development.

2) to identify and characterize a fully functional promoter of pea FTase subunit. Our attempt to study the expression of pea FTase subunit gene in transgenic plants was not successful. The promoter region (~2 kb) appears insufficient for the expression of pea FTase subunit. Further study of the leader intron, the 3'-non coding region, and further 5' upstream sequences of the *PsFTa* gene is necessary. Once a fully functional promoter of *PsFTa* gene is acquired, the *PsFTa* gene expression pattern and its regulation can be studied. Comparison of expression pattern and regulation of the two subunit genes of pea FTase will provide a basic understanding of the correlation between the two subunits at the transcript level.

3) to analyze FTase function at both the gene and protein levels. Using molecular approaches, such as mutation, overexpression and antisense repression, the function of FTase in plant development could be further elucidated.

4) to characterize the target proteins for farnesylation in plants. Since our study on pea FTase subunit showed a cell- and tissue-specific gene expression pattern, FTase genes may be actively mediating biological processes in these tissues (cells). Therefore, mRNA from these tissues could be used to generate expression libraries for identification of specific farnesylated proteins using the *in situ* farnesylation screening procedure described by Biermann *et al.* (1994). Strategies such as the two-hybrid method and/or epitope tagging, could then be performed to identify associated proteins in the relevant signal transduction pathways. The more we know about the FTase substrate proteins, the more insight we can gain into the involvement of FTase in plant cell cycle control and specific signal transduction pathways mediating plant development.

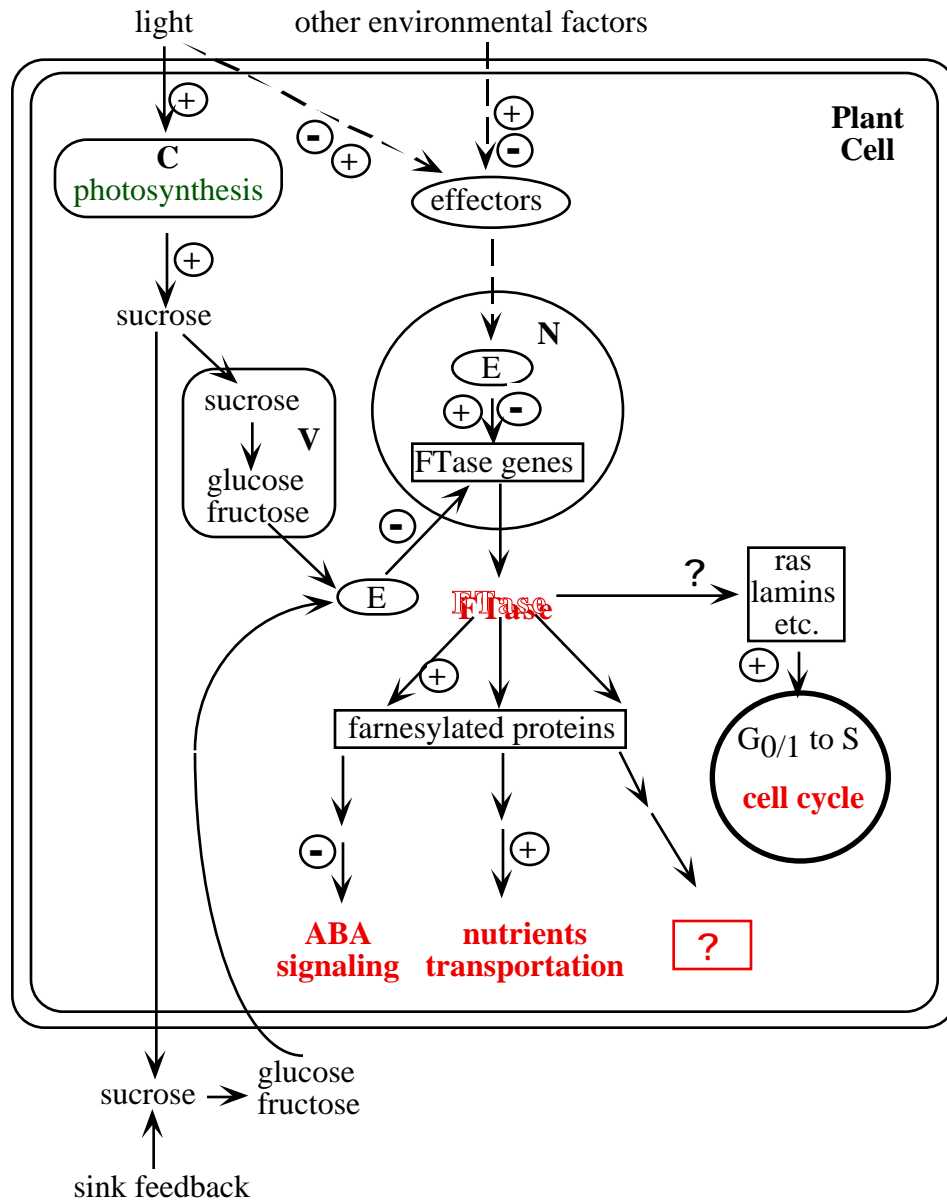


Figure VI.2. Model for regulation of FTase gene expression and FTase functions in plant cell.

C, chloroplast; E, effector; G_{0/1}, gap between mitosis and DNA synthesis; N, nucleus; S, DNA synthesis; V, vacuole; +, positive regulation; -, negative regulation.

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