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Protein Isoprenylation in Plants

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CONTENTS

I. INTRODUCTION

Phytosterols are important components of plant cell membranes. Like all isoprenoids, they are derived from mevalonic acid. The synthesis, structure, and function of phytosterols, including campesterol, sitosterol, and stigmasterol, [1–5] as well as other plant isoprenoids, including cytokinins,[6] cyclic monoterpenes,[7] sesquiterpenoid phytoalexins,[8–12] ubiquinone,[4,5,13–15] plastoquinone,[4,5,15,16] dolichols,[13,14] steroid glycoalkaloids,[1,2,8,9,11,12,17] brassinosteroids,[18] gibberellins,[19,20] carotenoids,[3,21,22] chlorophylls,[3,23] and abscisic acid,[22] are discussed elsewhere (Figure 1). The accompanying chapters by Drs. Adler, Bach, Brosa, Chappell, Groenveld, Nes, and Norton also cover much of this material. Accordingly, this chapter is limited to recent advances in the study of plant protein isoprenylation (the isoprenoid modification of plant proteins), a relatively new area of plant isoprenoid research.

The biosynthesis of phytosterols is inevitably coupled to the synthesis of other plant isoprenoids (including isoprenylated proteins). This principle is best illustrated by the plant defense response to wounding and pathogen attack. Various studies suggest that elicitation of a hypersensitive response in wounded tissues alters the production of isoprenoids from the wound-associated steroid glycoalkaloids to the pathogen-associated sesquiterpenoid phytoalexins. This change is associated with changes in the activities of enzymes involved in isoprenoid biosynthesis, which shifts the flow of isoprenoid precursors from sterols and steroid glycoalkaloids to sesquiterpenoid phytoalexins.[17,24–28] For example, upon wounding of potato tubers, increases in the abundance of steroid glycoalkaloids and in the activities of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGR, both microsomal and organellar) and squalene synthetase have been observed (HMGR catalyzes the first committed step in isoprenoid biosynthesis, and squalene synthetase catalyzes the first committed step in sterol and steroid glycoalkaloid biosynthesis).[8,9,11,12] However, upon elicitor treatment of wounded tissues, increased sesquiterpenoid phytoalexin and decreased steroid glycoalkaloid accumulation have been observed, along with altered HMGR activity (increased microsomal HMGR activity), increased sesquiterpene cyclase activity, and decreased squalene synthetase activity (sesquiterpene cyclase catalyzes the first committed step in sesquiterpenoid biosynthesis).[12,17,24–29] Consistent with these findings, different HMGR genes have been shown to respond differently to wounding and pathogen attack.[30,31] These observations suggest that the biosynthesis of non-sterol isoprenoids influences phytosterol biosynthesis. That mevalonate biosynthesis appears to be rate-limiting for phytosterol biosynthesis further supports this suggestion (increased HMGR activity has been associated with increased sterol biosynthesis in a tobacco mutant and pharmacological inhibition of HMGR has been shown to decrease phytosterol biosynthesis in plant seedlings and cell cultures).[3,4,32–35]

II. PROTEIN ISOPRENYLATION IN MAMMALIAN AND YEAST CELLS

Unlike protein isoprenylation in plant cells, protein isoprenylation in mammalian and yeast cells has been extensively reviewed.[14,36–40] The discovery of protein isoprenylation followed an investigation into

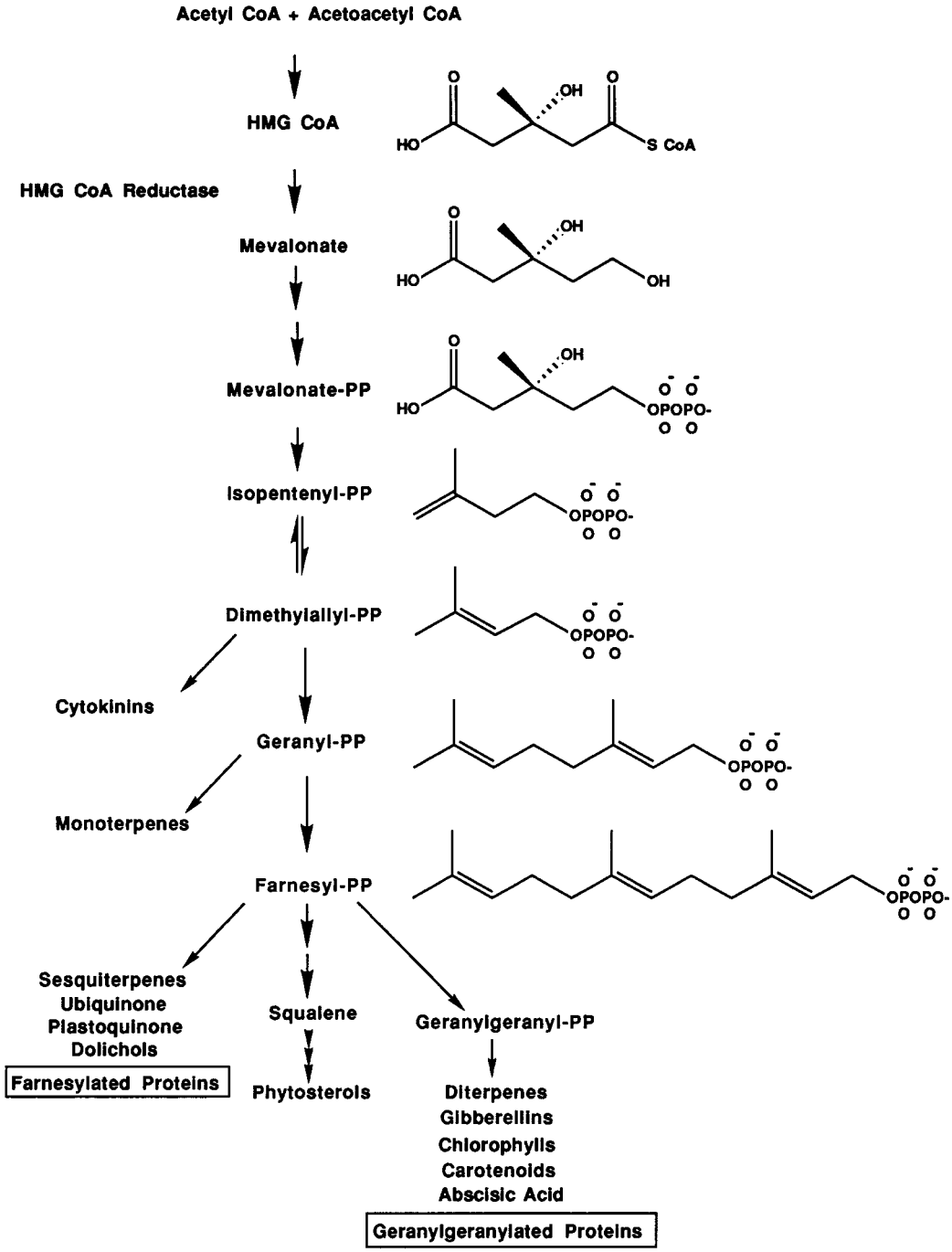

Figure 1 Isoprenoid biosynthesis in plant cells. CoA, coenzyme A; HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; -PP, pyrophosphate.

the growth requirement of mammalian cells for mevalonate-derived materials. This work led to the observation that exogenous cholesterol, dolichol, and ubiquinone would not restore growth to mammalian cells treated with lovastatin (a potent inhibitor of HMGR, also called mevinolin) suggesting that other isoprenoid product(s) of mevalonate were also required for growth.[13,14,36,41,42] Consistent with this hypothesis, it was shown by Schmidt et al. that radiolabeling of mammalian cells with [^{3}H]mevalonate in the presence of lovastatin resulted in significant incorporation of radioactivity into protein.[43]

Isoprenylated proteins are modified at the carboxy-terminus by cysteinyl thioether linkage to either a 15- carbon farnesyl or a 20-carbon geranylgeranyl moiety (most are geranylgeranylated). The prenylated cysteine residue is generally carboxymethylated and fatty acylated (i.e., palmitoylated) cysteine residues are often found within a few residues of the prenylated carboxy-terminus.[36,38,44–49] The steps involved in the carboxy-terminal processing of prenylated proteins are well understood for Ras, a protein involved in the regulation of cell growth and division.[50,51] Ras farnesylation occurs at Cys-186, after which amino acids 187-189 are removed by proteolysis. The farnesyl-cysteine residue at the carboxy-terminus is then methylated and, for H-Ras and N-Ras, upstream cysteine residues are palmitoylated.[44,47,52–55] Multiple activities (both soluble and membrane-associated) appear to catalyze the proteolysis of prenylated substrates *in vitro* and a membrane-associated carboxy-terminal methyltransferase (the product of the *STE14* gene in *Saccharomyces cerevisiae*) catalyzes the methylation of prenylated carboxy-termini (both farnesylated and geranylgeranylated).[56–64]

Three distinct prenyl:protein transferases, a farnesyl:protein transferase and two geranylgeranyl:protein transferases (types I and II) have been described.[47,54,65–68] The farnesyl:protein transferase prenylates protein substrates containing a CaaX carboxy-terminal sequence, where C is the target cysteine, a is usually, but not always, an aliphatic amino acid, and X is serine, methionine, alanine, cysteine, or glutamine.[47,67] The type I geranylgeranyl:protein transferase prenylates protein substrates containing a similar CaaX carboxy-terminal sequence, where X is usually leucine.[65,67] The type II geranylgeranyl:protein transferase, on the other hand, prenylates protein substrates containing a XXCC, CCXX, or CXC carboxy-terminal sequence (additional internal sequences are also required).[67,68] Both farnesyl:protein transferase and geranylgeranyl:protein transferase type I are $\alpha\beta$ heterodimers consisting of identical α subunits (the product of the *RAM2* gene in yeast) and similar β subunits (the products of the *RAM1* and *CDC43* genes in yeast, respectively).[47,66,69–71] In contrast, the type II geranylgeranyl:protein transferase (i.e., Rab geranylgeranyl:protein transferase) consists of α and β subunits (the products of the *BET4* and *BET2* genes in yeast, respectively) that are related to the α and β subunits of the other prenyl:protein transferases, as well as a third component.[68,72–75] This third component, once called component A and now called the Rab escort protein (REP), appears to bind Rab proteins and present them to the type II geranylgeranyl:protein transferase (originally called component B). Mutations in the gene that encodes REP are associated with choroideremia, an X-linked form of retinal degeneration in humans.[76]

Isoprenylated proteins are involved in regulation of cell growth, signal transduction, organization of the nuclear matrix and cytoskeleton, intracellular vesicle transport, and other cellular functions. Many of the known isoprenylated proteins include fungal lipopeptide mating pheromones,[51,77,78] small GTP-binding proteins of the Ras superfamily,[50,55,79–91] heterotrimeric G-protein γ subunits,[92–96] nuclear lamins,[97–102] protein kinases,[103–106] cyclic nucleotide phosphodiesterases,[107,108] molecular chaperones,[109,110] and others.[111–114]

Several isoprenylated proteins participate in regulation of cell growth and division. For example, the yeast **a**-factor causes cell cycle arrest in haploid cells of the opposite mating type by activating a signal transduction mechanism that ultimately leads to the direct inhibition of the CLN2-CDC28 complex (a G1 cyclin-cdk complex).[115,116] This inhibition is at least partially dependent on **a**-factor isoprenylation.[38,60] In mammalian cells, farnesylation of Ras (and perhaps other proteins) is required for regulation of cell proliferation and differentiation.[81,117–119] Consequently, inhibitors of farnesyl:protein transferase are being vigorously sought as possible cancer chemotherapeutic agents.[120–126] Other small GTP-binding proteins also participate in cell growth and division. For example, isoprenylation of Rho1p and Cdc42p is indispensable for growth of *Saccharomyces cerevisiae*.[127] In addition, the isoprenylated small GTP-binding proteins Rho (from *Aplysia californica*) and RC21/R-Ras2 have been shown to cause cellular transformation when overexpressed in NIH3T3 cells.[128,129] The isoprenylation of heterotrimeric G-protein γ subunits further implicates protein isoprenylation in regulation of cell growth and division.[92–96]

Many isoprenylated proteins participate in signal transduction. For example, various small GTP-binding proteins, including Ras,[118,119] participate in the transduction of extracellular signals. In addition, the isoprenylated γ subunits of heterotrimeric G-proteins, which were once thought to serve an accessory role in the transduction of extracellular signals, are now believed to directly transduce specific transmembrane signals.[130–134] Protein isoprenylation has also been shown to influence the activity of several signal-transducing protein kinases,[103–106] including receptor kinases,[104,105] and cyclic nucleotide phosphodiesterases.[107,108] Thus, isoprenylated proteins are, in some cases, necessary for the proper regulation of receptor function and cyclic nucleotide second messenger levels.

Certain isoprenylated proteins are involved in the organization of the nuclear lamina and cytoskeleton. The nuclear lamina, a proteinaceous matrix lining the inner surface of the nuclear envelope, is composed

of nuclear intermediate filament proteins called lamins. These proteins, which mediate interactions between chromatin and the nuclear envelope, also regulate the disassembly and reassembly of the nuclear envelope during mitosis. Interestingly, a subset of these nuclear lamin proteins are also isoprenylated.[97–102] In addition, the isoprenylated small GTP-binding proteins Rac and Rho influence the organization of the actin cytoskeleton.[135–137]

Other isoprenylated proteins are involved in the sorting and proper assembly of intracellular proteins. Members of the Rab family of isoprenylated small GTP-binding proteins, for example, have been shown to mediate many of the interactions necessary for proper transport of intracellular vesicles.[68,86,90,138–143] In addition, homologues of the *Escherichia coli DnaJ* protein have been shown to regulate the molecular chaperone Hsp70, which participates in the proper folding of proteins and assembly of protein complexes in eukaryotic cells.[109,110]

The role of the isoprenoid moiety in the function of isoprenylated proteins is currently under investigation. For many proteins, such as Ras, the isoprenoid moiety facilitates membrane association,[44,81,144] although additional modifications (i.e., fatty acylation) are often required for efficient membrane association.[44] In addition, a growing body of evidence suggests that protein isoprenylation is required for protein-protein interactions,[145] including the interaction between Ras2 and adenylate cyclase in yeast,[146] the interaction between Rab6 and RabGDI (Rab GDP-dissociation inhibitor protein),[147] the interaction between a G-protein γ subunit and rhodopsin,[148] the interaction between Ras and the guanine nucleotide exchange protein hSOS1,[149] and the interaction between Hepatitis large delta antigen and hepatitis B virus surface antigen.[150] In other cases, protein isoprenylation appears to regulate the assembly of protein complexes. Isoprenylation of prelamin A, for example, is required for its assembly into the nuclear lamina, but mature lamin A contains no isoprenoid moiety due to proteolytic removal of the isoprenylated carboxy-terminus.[99–102]

III. PROTEIN ISOPRENYLATION IN PLANTS

Protein isoprenylation in plants was first suggested by Bach, who stated that "...when intact etiolated radish seedlings or cell-free homogenates were incubated in the dark with [^{3}H]MVA, radioactivity was found associated with proteins passing a Sephadex column (Bach and Gupta, unpublished)."[5] The first data on protein isoprenylation in plants appeared in the literature in 1991, when Epstein et al. described a selective cleavage reaction for the quantitation of prenylcysteines.[49] In this report, the thioether bonds unique to prenylated proteins were shown to be cleaved by 2-naphthol under alkaline conditions to generate substituted naphthopyrans, which were resolved by high performance liquid chromatography (HPLC). Using this method, prenylcysteine residues from various organisms were analyzed. For example, in mammalian tissues, prenylcysteines were found at concentrations ranging between 0.36 and 1.4 nmol/mg of protein, with geranylgeranylcysteine representing 80–90% and farnesylcysteine representing 10–20% of the total prenylcysteine content. In contrast, prenylcysteines were found in spinach (*Spinacia oleracea*) at 0.06 nmol/mg protein, with geranylgeranylcysteine representing the majority. Prenylcysteines were also detected in a yeast (*Saccharomyces cerevisiae*), a fungus (*Phycomyces*), a brown alga (*Peletia fastigiata*), an insect (*Manduca sexta*), and an archaebacterium (*Methanobacterium thermoautotrophicum*), but not in *Escherichia coli*.[49]

The existence of prenylated proteins in plants was further suggested by the molecular cloning of a pea (*Pisum sativum* L. cv Alaska) homolog of the farnesyl:protein transferase β subunit gene.[151] This was accomplished by PCR (polymerase chain reaction) amplification of a cDNA sequence using total RNA from apical buds of pea seedlings and degenerate oligonucleotide primers corresponding to conserved regions within the farnesyl:protein transferase β subunit. The PCR-amplifed product was then used to isolate a full-length pea cDNA, the deduced amino acid sequence of which exhibited 48% and 40% identity to rat and yeast farnesyl:protein transferase β subunits, respectively. The pea farnesyl:protein transferase β subunit appears to be encoded by a single-copy gene and its expression, which is high in etiolated seedlings, declines by a factor of 5 during light-regulated leaf development.[151]

The existence of isoprenylated plant proteins was demonstrated in tobacco cultures (line BY-2, derived from *Nicotiana tabacum* cv Bright Yellow-2 callus).[152] Suspension-cultured tobacco cells were shown to cease growth in the presence of lovastatin and resume normal growth when supplemented with mevalonic acid,[152,153] suggesting that mevalonate-derived materials are essential for their growth in culture. Furthermore, when pretreated with lovastatin, these cells incorporated radioactivity from [^{14}C]mevalonic acid into proteins (high-molecular-weight, TCA-precipitable, acetone-insoluble, RNase-resistant, protease-sensitive macromolecules). Most of these proteins were membrane-associated, and

many were similar in mass to mammalian Ras-like GTP binding proteins (21–31 kDa) and nuclear lamins (55–66 kDa, see Figure 2). Extracts of cultured tobacco cells were also shown to contain both farnesyl:protein transferase and type I geranylgeranyl:protein transferase activity (farnesyl:protein transferase activity exceeded type I geranylgeranyl:protein transferase activity by 10-fold) using artificial yeast Ras1 protein substrates containing either an SVLS, CVLS, CAIM, or CAIL carboxy-terminus. These studies demonstrated that protein substrates containing a CAIM carboxy-terminus are preferentially farnesylated by tobacco extracts, and protein substrates containing a CAIL carboxy-terminus are preferentially geranylgeranylated by tobacco extracts. Protein substrates containing an SVLS carboxy-terminus were not detectably prenylated *in vitro*.[152] A more detailed substrate specificity analysis is shown in Figure 3.

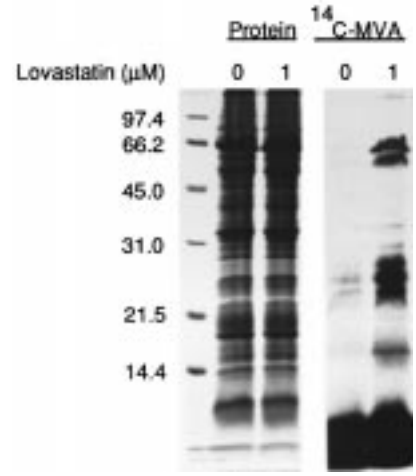

Figure 2 Cultured tobacco cells were pretreated for 16 h with or without 1 μ*M* lovastatin in Murashige-Skoog medium containing 0.9 μ*M* 2,4-dichlorophenoxyacetic acid at 26°C and then incubated with 7 μCi/ml (50 mCi/mmol) ^{14}C-mevalonic acid for 4 h in the same medium. Cells were washed twice and lysed directly by incubation at 90°C in SDS-sample buffer for 4 minutes. Following 14% polyacrylamide gel electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250, soaked in Amplify (Amersham) fluorographic reagent, dried, and fluorography was performed at –80°C for 2 weeks. The Coomassie-stained gel and its corresponding fluorogram are shown. ^{14}C-MVA, ^{14}C-mevalonic acid.

Mevalonate labeling of plant proteins in the 23-kDa range was also demonstrated by incubation of spinach leaves in the presence of [^{3}H]mevalonate.[154] Alkaline hydrolysis of the labeled protein fraction liberated phytol, polyprenyl phosphates 11-15 as well as polyprenols 11-15, whereas complete liberation of farnesol and geranylgeraniol from the protein fraction required iodomethane cleavage of thioether linkages. These results suggested that a subset of isoprenylated plant proteins may contain isoprenoid modifications other than thioether-linked farnesyl or geranylgeranyl moieties.

The only plant protein to be definitively identified as an isoprenylated protein is the plant molecular chaperone ANJ1.[155,156] The *Atriplex nummularia* ANJ1 protein is a 47-kDa polypeptide with 35% amino acid sequence identity to the *Escherichia coli* DnaJ protein.[155] ANJ1 and other DnaJ-like proteins are thought to regulate the chaperone activity of Hsp70 proteins, which bind and stabilize polypeptides in non-native conformations at the expense of ATP. Thus, DnaJ-like proteins, in association with members of the Hsp70 family, are critically involved in the folding of proteins, the assembly of protein complexes, and the cellular response to heat shock.[110] Interestingly, the ANJ1 protein of *A. nummularia* complemented a temperature-sensitive yeast *mas*5 mutant (YDJ1/MAS5 is a yeast DnaJ-like protein) that has been shown to be defective in mitochondrial protein import as well as protein translocation across the endoplasmic reticulum.[157,158] ANJ1 was a substrate for *in vitro* isoprenylation (farnesylation and, to some extent, geranylgeranylation) when extracts from *Atriplex nummularia* or *Saccharomyces cerevisiae* were used as a source of prenyl:protein transferase activity.[156] Furthermore, isoprenylation of ANJ1, which was dependent on its carboxy-terminal CAQQ sequence, facilitated its association with *A. nummularia* microsomal membranes *in vitro* and was necessary for its function *in vivo* (i.e., its ability to complement the yeast *mas*5 mutation). The electrophoretic mobility of ANJ1 isolated from *A. nummularia* cells suggested that it was isoprenylated *in vivo*.[156] Heat shock has been shown to cause the accumulation of ANJ1 and Hsp70 transcripts in *A. nummularia* cells[155] and to increase the amount of prenylated DnaJ protein associated with glyoxosomal membranes in cucumber seedlings,[159] consistent with a role for DnaJ-like proteins in the heat shock response of plant cells.

Evidence for the existence of heterotrimeric G-proteins and small GTP-binding proteins in plants has accumulated in recent years.[160–176] In addition, proteins related to mammalian nuclear lamins have been described in plants.[177–182] While it is presumed that these proteins are isoprenylated, none have been directly tested for the covalent attachment of an isoprenoid moiety *in vivo* or *in vitro*. However, the

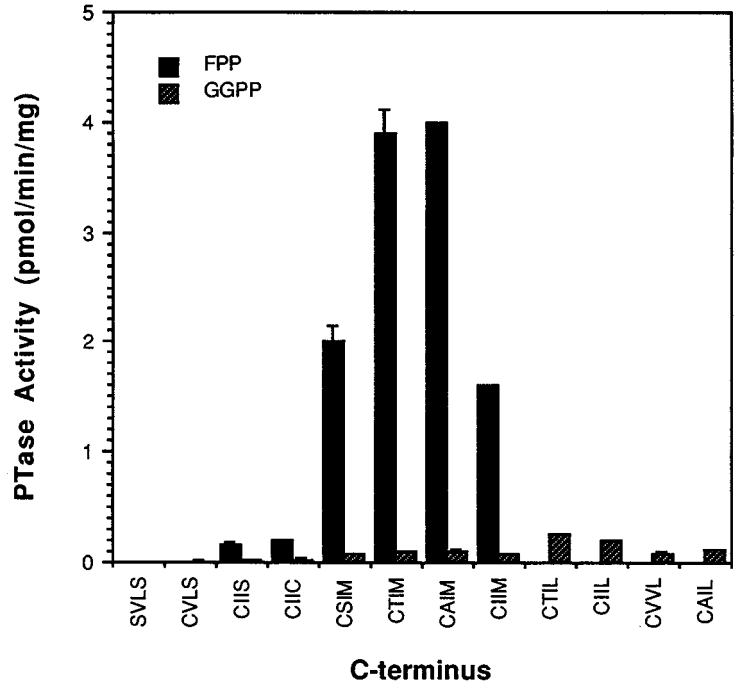

Figure 3 Tobacco prenyl:protein transferase activity (picomole of product formed per minute per milligram of tobacco cell protein) is shown using various yeast Ras1 substrate proteins (the carboxy-terminal sequences of the various substrate proteins are indicated along the horizontal axis). Prenyl:protein transferase activity was measured in the presence of 20 μCi/ml ^{3}H-farnesyl pyrophosphate or ^{3}H-geranylgeranyl pyrophosphate (15 Ci/mmol) using an extract of cultured tobacco cells as a source of prenyl:protein transferase activity. Assays were performed under conditions where product formation was linear with time and tobacco cell protein.[152] Substrate proteins with CaaX sequences ending in methionine were efficiently prenylated in the presence of ^{3}H-farnesyl pyrophosphate and only weakly prenylated in the presence of ^{3}H-geranylgeranyl pyrophosphate. In contrast, substrate proteins with CaaX sequences ending in leucine were prenylated in the presence of ^{3}H-geranylgeranyl pyrophosphate but not detectably prenylated in the presence of ^{3}H-farnesyl pyrophosphate. Ras-SVLS lacks a carboxy-terminal cysteine residue and was not detectably prenylated. PTase, prenyl:protein transferase; FPP, ^{3}H-farnesyl pyrophosphate; GGPP, ^{3}H-geranylgeranyl pyrophosphate.

amino acid sequences and membrane localization of several plant small GTP-binding proteins support the hypothesis that they are isoprenylated *in vivo*.[162,164,166,168–174,176]

Little is currently known about the identities and functions of isoprenylated plant proteins. As a step toward the identification of these proteins (homologues of mammalian and yeast isoprenylated proteins as well as novel isoprenylated proteins), a method of identifying cDNAs that encode protein substrates of the plant farnesyl:protein transferase was recently developed.[183] Colony protein blots of a plasmid cDNA expression library from soybean were prepared on replica nylon filters by lysing *E. coli* colonies *in situ* in the presence of sodium hydroxide and sodium dodecylsulfate. The blots were then isoprenylated *in vitro* by incubation in the presence of [^{3}H]-farnesyl pyrophosphate and a tobacco extract containing farnesyl:protein transferase activity (4.5 pmol/min/mg). Unincorporated radioactivity was removed by washing the filters in ethanol and *in vitro*-prenylated proteins were detected by fluorography. This procedure led to the identification of cDNAs encoding soybean homologues of ANJ1 (GMFP1, *Glycine max* farnesylated protein 1) as well as several novel proteins. All of the proteins identified by this method contained consensus carboxy-terminal CaaX motifs and one abundant class of proteins, represented by GMFP2 and GMFP3, contained a repeat motif upstream of the CaaX motif rich in lysine, glutamate, proline, and glycine.[183,184] This method is being used to screen for cDNAs encoding geranylgeranylated plant proteins and, conceivably, could be adapted to identify any cDNA encoding a protein that is post-translationally modified, either covalently or by the binding of a ligand (provided the protein determinant for modification is conferred by a small, denaturation-resistant domain).

IV. FUTURE DIRECTIONS

The steps involved in the processing of isoprenylated plant proteins remain to be characterized. In particular, it is not currently known whether isoprenylated plant proteins, particularly those modified at CaaX by either the addition of a farnesyl or a geranylgeranyl moiety, are subsequently proteolyzed and carboxymethylated. The possibility of further modifications of these proteins (e.g., fatty acylation of upstream cysteine residues) also has not been investigated.

Much work remains to be done on the identification of isoprenylated plant proteins and their role in physiological processes. The work currently being done on plant GTP-binding proteins and nuclear lamins will undoubtedly contribute much to our understanding of the functions of isoprenylated plant proteins. In addition, the expression library screen described above will lead to the identification of novel isoprenylated plant proteins and their functions. Other experiments using HMGR or farnesyl:protein transferase inhibitors suggest that protein farnesylation is essential for the growth of cultured tobacco cells, but only within the first 2 days of culture, before any increase in culture volume is detectable. This "window" of sensitivity to inhibitors of protein isoprenylation correlates temporally with a peak of farnesyl:protein transferase activity and an unusually complex pattern of protein labeling upon incubation of lovastatin-treated cells with [^{14}C]mevalonic acid. Interestingly, multiple cycles of DNA replication occur during this early time.[185] Thus, it may be possible to relate protein isoprenylation to critical events in the plant cell division cycle.

ACKNOWLEDGMENTS

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NOTE ADDED IN PROOF

While this article was in press, a number of important studies on protein isoprenylation in plants were published. These include studies on protein isoprenylation during the growth of suspension-cultured tobacco cells,[185] characterization of farnesyl:protein transferase from spinach,[186] identification of geranylgeranyl:protein transferase type II from plants,[187–189] localization and isoprenylation of a plant Rho GTPase,[190] and characterization of a plant Rac GTPase.[191] A detailed description of 'prenyl-screening' was also published.[192]

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