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Isoprenylation of the plant molecular chaperone ANJ1 facilitates membrane association and function at high temperature

(DnaJ homolog/CaaX motifs/protein farnesylation)

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ABSTRACT **We demonstrate that ANJ1, a higher plant homolog of the bacterial molecular chaperone DnaJ, is a substrate *in vitro* for protein farnesyl- and geranylgeranyltransferase activities present in cell extracts of the plant *Atriplex nummularia* and yeast *Saccharomyces cerevisiae*. Isoprenylation did not occur when cysteine was replaced by serine in the CAQQ motif at the carboxyl terminus of ANJ1, indicating that this sequence functions as a CaaX consensus sequence for polyisoprenylation (where C is cysteine, a is an aliphatic residue, and X is any amino acid residue). Substitution of leucine for the terminal glutamine did not result in the expected geranylgeranylation as occurs with mammalian proteins containing a carboxyl-terminal leucine. Unlike the wild-type ANJ1, neither of the proteins containing these amino acid substitutions could functionally complement the yeast temperature-sensitive mutant *mas5*. Farnesylation enhanced the association of ANJ1 with *A. nummularia* microsomal membranes. Electrophoretic mobility of ANJ1 from the plant indicated that the protein is isoprenylated *in vivo*.**

ANJ1 is a homolog of the bacterial molecular chaperone DnaJ identified from the higher plant *Atriplex nummularia* (1). DnaJ is essential for phage DNA replication and bacterial growth at high temperatures (2, 3). As a molecular chaperone, DnaJ is involved in regulation of activity and stability of the heat shock transcription factor σ^{32} (4), the positive regulatory protein CRP (cAMP receptor protein) (5), and the chaperone function of DnaK (6). Recent studies in the yeast *Saccharomyces cerevisiae* indicate that eukaryotic DnaJ homologs function with ≈70-kDa heat shock proteins during protein folding and import into organelles (7, 8). Because ANJ1 can complement a mutation in the yeast cytoplasmic DnaJ homolog MAS5 (i.e., YDJ1), the *A. nummularia* protein presumably has a role in protein translocation into organelles of higher plant cells (1).

We have previously indicated (1) that the CAQQ sequence at the carboxyl terminus of ANJ1 may conform to the CaaX (C, cysteine; a, aliphatic amino acid residue; X, any amino acid) motif that functions in protein isoprenylation (9, 10). Protein isoprenylation involves a series of posttranslational processing that includes the transfer of a polyisoprene moiety (i.e., farnesyl or geranylgeranyl) to the cysteine through the formation of a thioether linkage, proteolytic cleavage of the aaX residues, and subsequent methylation of the α-carboxyl group (9). The terminal X has been determined to confer isoprenylation specificity (9, 11). Polypeptides containing a carboxyl-terminal serine, alanine, methionine, or glutamine residue are farnesylated, whereas those containing a leucine residue are geranylgeranylated. Proteins containing a carboxyl-terminal CC or CXC motif are also geranylgeranylated (12–14).

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Protein isoprenylation has been determined to function in cell cycle control, growth, and signaling reactions and, with the notable exception of plants, has been reported to occur in both lower and higher eukaryotes (9, 15). Proteins known to be isoprenylated include the Ras proteins and many of the other Ras-related small GTP-binding proteins (16), the γ subunit of the heterotrimeric GTP-binding proteins (G proteins) (17), nuclear lamins (18, 19), cGMP phosphodiesterase (20), fungal mating pheromones (21), rhodopsin kinase (22), and hepatitis δ virus large antigen (23). The prevailing belief is that the hydrophobic isoprene moiety targets the prenylated proteins to and facilitates association with cellular membranes (10, 24). Evidence indicates that the isoprenoid group interacts with hydrophobic portions of the membrane bilayer as well as with specific membrane proteins, perhaps even receptors (9).

Here we provide direct evidence of the isoprenylation of a specific higher plant protein. The DnaJ homolog from *A. nummularia*, ANJ1, can be farnesylated and, to some degree, geranylgeranylated *in vitro* and *in vivo* by plant as well as yeast isoprenyltransferases. Isoprenylation of ANJ1 was dependent on the C in the CAQQ motif at the carboxyl terminus. Farnesylation substantially increased the association of ANJ1 with *A. nummularia* microsomes, indicating a function involving membrane attachment. ANJ1 isoprenylation was required for complementation of the temperature-sensitive growth phenotype of the yeast *mas5* mutant, indicating significance of the modification for chaperone function.

MATERIALS AND METHODS

ANJ1 Expression in *Escherichia coli*. The open reading frame of ANJ1 cDNA was amplified from clone pANJ1 (1) by polymerase chain reaction (PCR) using the sense primer 5′-ATGGCCGGATCCCATGTTTGGAAGAGCACCAA-3′ and the antisense primer 5′-TCCACGGGATCCAGATCACTGTTGAGCACA-3′. The amplified DNA was digested with *Nde* I and *Bam*HI and inserted into the analogous sites of the polylinker region in the bacterial expression vector pET9c (25) to generate pETANJ1. Constructs pETANJ1S and pETANJ1L were similarly generated by PCR using the same sense primer and the antisense primers 5′-TCCACGGGATCCAGATCACTGTTGAGCAGA-3′ and 5′-CCGGAATTCGGATCCAGATCACAGTTGAGCACA-3′, respectively. The plasmids were transformed into *E. coli* BL21(DE3) and protein expression was induced with isopropyl β-D-thiogalactopyranoside (IPTG) (26). The recombinant proteins produced by the three constructs were identical except for differences in the carboxyl-terminal four amino acids; CAQQ for ANJ1, SAQQ for ANJ1S, and CAQL for ANJ1L.

Production of Anti-ANJ1. *E. coli* extract containing ANJ1 (from construct pETANJ1) was subjected to preparative

Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

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SDS/PAGE and the ANJ1 protein was isolated by electroelution of gel slices. An emulsion of ANJ1 in Freund's adjuvant was injected subcutaneously into a hen; three injections of 300, 200, and 200 μg of ANJ1 were made at 2-week intervals (27). Polyclonal antibodies were purified from egg yolks by differential polyethylene glycol fractionation (28).

***A. nummularia* Extract Preparation.** Cell-free extracts that contained protein isoprenyltransferases were prepared by homogenizing linear-phase *A. nummularia* cells with a glass homogenizer in one-sixth volume of extraction buffer [50 mM Tris·HCl, pH 8.0/200 mM NaCl/0.1 mM EDTA/5% (vol/vol) glycerol] plus proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride; leupeptin, pepstatin, aprotinin, and chymostatin each at 1 μg/ml). The homogenate was centrifuged at 13,000 × *g* for 10 min at 4°C, and the supernatant was used in isoprenylation assays.

Fractions used to determine subcellular association of ANJ1 were prepared as follows: control (26°C) and heat-treated (37°C, 2.5 hr) cells were homogenized as described (1). The homogenate was filtered through eight layers of cheesecloth. The filtrate was centrifuged for 5 min at 700 × *g* to pellet cell debris. The supernatant was then centrifuged for 15 min at 13,000 × *g*, and the resultant supernatant was centrifuged again for 1 hr at 100,000 × *g*. Proteins were precipitated from the supernatant as described (1).

***In Vitro* Isoprenylation Assays.** Isoprenylation assay conditions were modified from those of Caplan *et al.* (26). Briefly, to an Eppendorf tube were added 10 μl of 5× assay buffer (250 mM Tris·HCl, pH 8.0/100 mM KCl/25 mM $MgCl_2$/50 μM $ZnCl_2$/50 mM dithiothreitol); 20 μg of *E. coli* extract containing recombinant ANJ1, ANJ1S, or ANJ1L; 70 μg of cell-free extract from *A. nummularia* or *S. cerevisiae*, and 0.1 nmol of [1-^{3}H]farnesyl pyrophosphate (triammonium salt; 60 Ci/mmol; American Radiolabeled Chemicals, St. Louis) or [1-^{3}H]geranylgeranyl pyrophosphate (triammonium salt; 12 Ci/mmol; American Radiolabeled Chemicals; 1 Ci = 37 GBq) with a final volume of 50 μl. The mixture was incubated at 37°C for 1 hr. The reaction was terminated by the addition of 50 μl of 2× SDS/PAGE sample buffer. Aliquots (20 μl) of the mixture were electrophoresed in an SDS/10% polyacrylamide gel. The gel was then fixed for 30 min in 50% isopropanol/10% acetic acid, immersed for 30 min in a solution of Amplify fluorographic reagent (Amersham), and dried onto filter paper before fluorography. Incorporation of ^{3}H into proteins was quantitated as described (26).

Metabolic Labeling of Recombinant ANJ1 and ANJ1S and Association with *A. nummularia* Microsomes. *E. coli* BL21(DE3) cells containing either pETANJ1 or pETANJ1S were incubated with [^{35}S]methionine as described (29). Aliquots (10^5 cpm) of nonfarnesylated metabolically labeled pETANJ1 extract or farnesylated (with nonradioactive farnesyl pyrophosphate) metabolically labeled pETANJ1 or pETANJ1S extracts were mixed and incubated with *A. nummularia* microsomal membranes (50 μg of 100,000 × *g* pellet from cells as described above) for 2 hr at room temperature in 50 mM Tris·HCl, pH 8.0/150 mM KCl/1% bovine serum albumin (final volume, 250 μl). The membrane suspension was pelleted for 30 min at 100,000 × *g* and resuspended in 4 ml of the same buffer. This process was repeated five times and the final pellets were each suspended in 50 μl of SDS sample buffer. Aliquots (20 μl) of the samples were subjected to SDS/PAGE and the labeled protein associated with microsomes was detected by autoradiography.

Manipulations in Yeast. PCR-amplified ANJ1, ANJ1S, and ANJ1L cDNAs were inserted between *Bam*HI and *Eco*RI sites of the yeast expression vector pYES downstream from the *GAL1* promoter to create pYANJ1, pYANJ1S, and pANJ1L, respectively. The constructs were transformed into the yeast strain MYY391, which is unable to grow at 37°C because of a *mas5* mutation (1).

Yeast extracts containing protein isoprenyltransferases were prepared by homogenizing MYY391 cells with a French pressure cell in extraction buffer plus proteinase inhibitors (described above). The homogenate was centrifuged for 10 min at 13,000 × *g* at 4°C and the supernatant was retained for isoprenylation assays.

The yeast strain MYY391 containing either pYES, pYANJ1, pYANJ1S, or pYANJ1L was also grown at 28°C to midlogarithmic phase in 5 ml of liquid YPD or YPG medium (30). The cultures were harvested, and the cells were disrupted in 150 μl of 20 mM Tris·HCl, pH 7.9/10 mM EDTA/5% glycerol/1 mM dithiothreitol plus proteinase inhibitors by vortex mixing with glass beads (0.45-mm diameter). One hundred fifty microliters of 2× SDS sample buffer was added and samples were analyzed by SDS/PAGE and immunoblotting (1) with anti-ANJ1.

RESULTS

Production of Recombinant ANJ1 Proteins in *E. coli*. Upon induction with IPTG, recombinant ANJ1, ANJ1S, and ANJ1L all accumulated to over 30% of total cell protein (Fig. 1). Although the predicted molecular mass of ANJ1 is 46.6 kDa based on the length of its open reading frame, the recombinant proteins all have apparent molecular masses of 51 kDa. ANJ1 from *A. nummularia* and ANJ1 expressed in yeast have similar molecular sizes of about 51 kDa (Fig. 2*C*; see Fig. 7). Consistent with ANJ1 being a homolog of the yeast protein YDJ1 (1), recombinant ANJ1 reacted with polyclonal anti-YDJ1.

ANJ1 Is Associated with Cellular Membranes in *A. nummularia* Cells. Polyclonal anti-ANJ1 reacted with a single band at 51 kDa in 13,000 and 100,000 × *g* pellets but detected no protein in the 100,000 × *g* supernatant from *A. nummularia* cells (Fig. 2). These results indicate that virtually all ANJ1 in *A. nummularia* cells is associated with cellular membranes. Moreover, the level of ANJ1 did not increase after 2 hr (data not shown) or 2.5 hr of heat treatment at 37°C. This is in contrast to ANJ1 message, which was induced substantially by heat treatment (1).

Isoprenylation of ANJ1 *in Vitro*. Isoprenylation of ANJ1, ANJ1S, and ANJ1L *in vitro* was determined by incubating recombinant protein (in *E. coli* extract) with *A. nummularia* cell-free extract in the presence of either [^{3}H]farnesyl pyrophosphate or [^{3}H]geranylgeranyl pyrophosphate. ANJ1

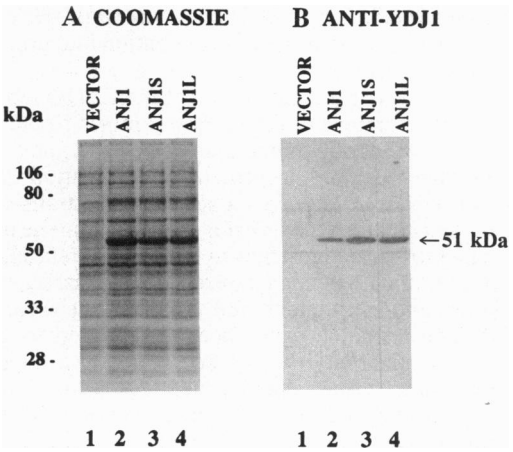

FIG. 1. Production of recombinant ANJ1 proteins in *E. coli*. (*A*) Coomassie blue-stained gel of total protein (IPTG-induced) from *E. coli* BL21(DE3) containing either pET9c (vector, lane 1), pETANJ1 (ANJ1, lane 2), pETANJ1S (ANJ1S, lane 3), or pETANJ1L (ANJ1L, lane 4). (*B*) Immunoblot probed with rabbit polyclonal serum (1:1000 dilution) against the yeast homolog YDJ1 (26).

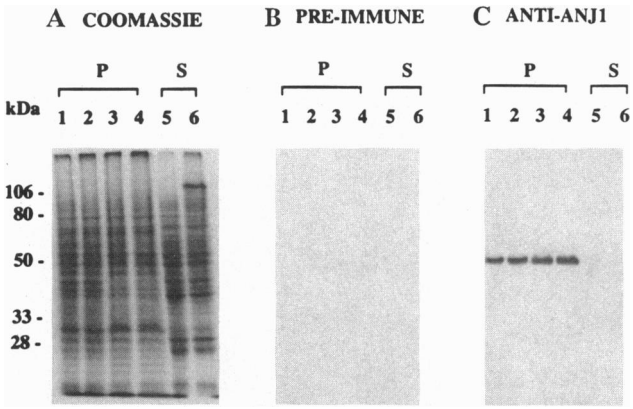

FIG. 2. Subcellular association of ANJ1 in *A. nummularia* cells. Lanes 1 (26°C) and 2 (37°C) are 13,000 × *g* pellets; lanes 3 (26°C) and 4 (37°C) are 100,000 × *g* pellets; lanes 5 (26°C) and 6 (37°C) are soluble fractions (100,000 × *g* supernatants). Pellet (P) and soluble (S) fractions from control (26°C) and 2.5-hr heat (37°C)-treated *A. nummularia* cells (30 μg of protein per lane) were subjected to SDS/PAGE and stained with Coomassie blue (*A*) or blotted to nitrocellulose and probed with chicken preimmune serum (2 hr, 1:1000 dilution) (*B*) or chicken immune serum against ANJ1 (2 hr, 1:2000 dilution) (*C*).

readily incorporated the [^{3}H]farnesyl moiety (Fig. 3*C*, lane 2), however, substitution of serine for cysteine in the protein (ANJ1S) resulted in no incorporation of the [^{3}H]farnesyl group (lane 3). A low level of incorporation of [^{3}H]geranylgeranyl moiety into ANJ1 was detected, but again no label was associated with ANJ1S. These results indicate that (*i*) *A. nummularia* cells contain both protein farnesyltransferase and protein geranylgeranyltransferase activities, (*ii*) ANJ1 is a very good substrate for protein farnesyltransferase and a poor substrate for protein geranylgeranyltransferase, and (*iii*) cysteine-414 is essential for ANJ1 isoprenylation.

Studies with Ras proteins have indicated that the last amino acid in the CaaX motif determines isoprenylation specificity (9). Specifically, if the carboxyl-terminal amino acid is leucine then the geranylgeranyl instead of the farnesyl moiety is conjugated to the protein. Surprisingly, ANJ1L (carboxyl-terminal CAQL) did not incorporate any [^{3}H]geranylgeranyl in *in vitro* assays (Fig. 3*D*, lane 4). ANJ1L was farnesylated, but to a much lower level than the wild-type ANJ1 (Fig. 3*C*, lane 4). Quantitative measurements illustrated that farnesyl incorporation into ANJ1 was 5-fold greater than geranylgeranyl incorporation (Fig. 4). ANJ1 can be similarly modified by protein isoprenyltransferases present in the yeast cell extract (Fig. 4).

Farnesylation Increases the Capacity of ANJ1 to Associate with Cellular Membranes *in Vitro*. Farnesylated ANJ1 (Fig. 5, lane 4) bound to microsomal membranes more than either nonfarnesylated ANJ1 (lane 3) or "farnesylated" ANJ1S (lane 5). This indicates that ANJ1 association with plant membranes (Figs. 2 and 5) requires that the protein be isoprenylated.

Isoprenylation of ANJ1 Is Essential for Function at High Temperature in Yeast. In the yeast *S. cerevisiae*, the cytoplasmic DnaJ homolog YDJ1 is required for growth at elevated temperatures (7, 8, 26). The yeast strain MYY391 is unable to grow at 37°C because it carries a mutant allele of the gene encoding YDJ1, *mas5*. We previously reported (1) that expression of ANJ1 in this yeast strain was able to complement its temperature-sensitive phenotype. Fig. 6 shows that MYY391 carrying pYANJ1 (CAQQ) but not pYANJ1S (SAQQ) can grow at 37°C when induced by galactose. Cell extracts from MYY391 can farnesylate ANJ1 but not ANJ1S *in vitro* (Fig. 4). These results indicate that farnesylation is essential for ANJ1 function at elevated temperatures. Consistent with this observation, ANJ1L, which is a very poor substrate for farnesylation, did not complement the temperature-sensitive phenotype of MYY391, either (Fig. 6).

Electrophoretic Mobility Shift Reveals That ANJ1 Is Isoprenylated *in Vivo*. Isoprenylation increases protein mobility in SDS/polyacrylamide gels, and this characteristic has been used to monitor protein isoprenylation *in vivo* (16, 19, 31, 32). The electrophoretic mobilities of ANJ1 from *A. nummularia* and yeast were similar, and these proteins migrated slightly faster in the gel (<1-kDa difference in apparent molecular mass) than ANJ1S and ANJ1L from yeast and recombinant ANJ1 from *E. coli* (Fig. 7). When the gel was overloaded, a very small amount (<5%) of ANJ1L with the same mobility as the wild-type ANJ1 could be detected (data not shown). These results indicate that ANJ1 is isoprenylated *in vivo* in plant cells and are consistent with the *in vitro* isoprenylation data.

DISCUSSION

This report provides direct evidence that higher plant cells contain both protein farnesyltransferase and protein gera-

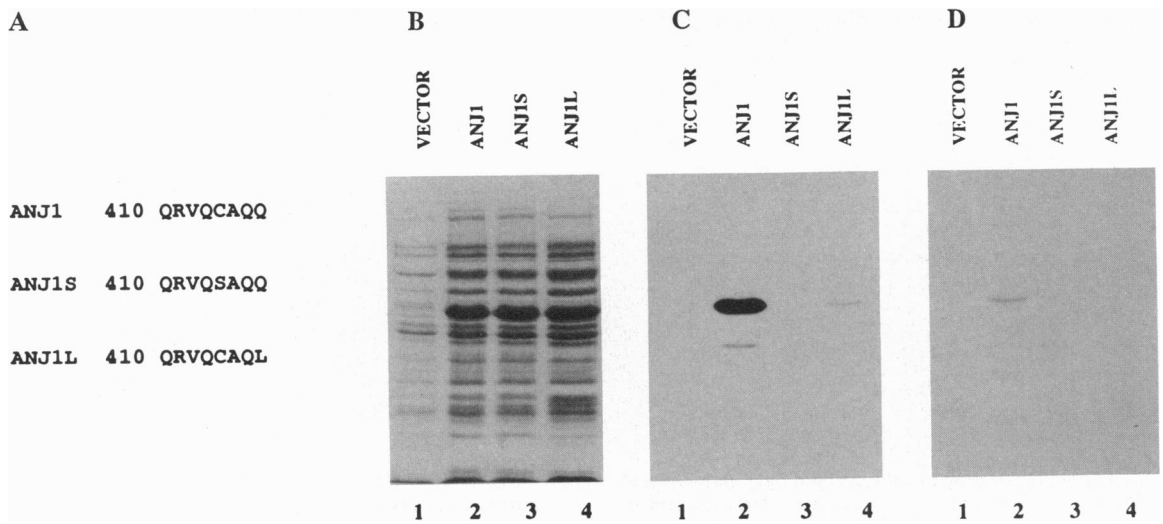

FIG. 3. Isoprenylation of ANJ1 *in vitro* with *A. nummularia* cell-free extracts. (*A*) Carboxyl-terminal amino acid sequences of ANJ1, ANJ1S, and ANJ1L. (*B*) Coomassie blue-stained gel of soluble extracts from IPTG-induced *E. coli* containing pET9c (vector, lane 1), pETANJ1 (ANJ1, lane 2), pETANJ1S (ANJ1S, lane 3), or pETANJ1L (ANJ1L, lane 4). (*C*) Fluorogram (1-week exposure) of farnesylation reaction products. (*D*) Fluorogram (1-week exposure) of geranylgeranylation reaction products.

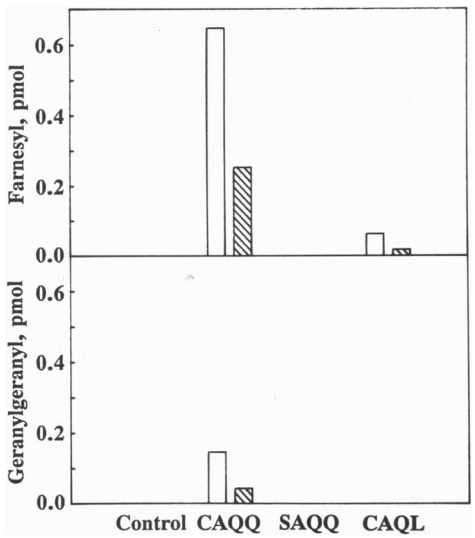

FIG. 4. Quantitation of [^{3}H]farnesyl (*Upper*) and [^{3}H]geranylgeranyl (*Lower*) incorporated into proteins. Open and hatched bars represent isoprenylation with *A. nummularia* and yeast cell-free extracts, respectively, in a 1-hr reaction.

nylgeranyltransferase activities, since incorporation of [^{3}H]farnesyl and [^{3}H]geranylgeranyl into ANJ1 was observed. Two recent reports on the incorporation of mevalonate derivatives into proteins in tobacco and spinach cells indicate the presence of isoprenylated proteins in plants (33, 34). The isolation of a pea cDNA that encodes a polypeptide homologous to the β subunit of the rat farnesyltransferase (35) also indicates the occurrence of the transferases in plants.

Our results indicated that isoprenylation of ANJ1 was dependent on the CAQQ motif at the carboxyl terminus. The carboxyl-terminal sequence of ANJ1 does not conform strictly to the CaaX motif for signaling isoprenylation (36), because the penultimate amino acid, glutamine, is not an aliphatic residue. However, since ANJ1 proved to be a good substrate for protein isoprenyltransferases, the substrate recognition of the plant enzymes may be different from that of the animal and yeast forms.

Although ANJ1 appeared to be a poor substrate for geranylgeranyltransferase *in vitro*, it is not possible to conclude that the protein is farnesylated or geranylgeranylated in *A. nummularia* cells *in vivo*. The low level of [^{3}H]geranylgeranyl

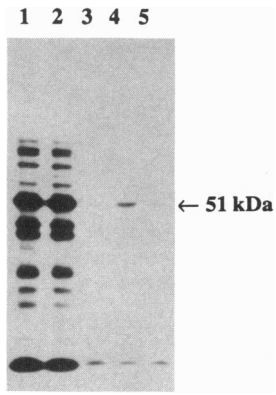

FIG. 5. Radiogram illustrating membrane binding of farnesylated [^{35}S]methionine-labeled ANJ1. Lanes 1 and 2, bacterial lysates of IPTG-induced pETANJ1 and pETANJ1S cultures, respectively, which were labeled metabolically with [^{35}S]methionine; lanes 3–5, *A. nummularia* microsomal membrane pellets (100,000 × *g*) incubated with nonisoprenylated ANJ1, farnesylated ANJ1, and "farnesylated" ANJ1S, respectively.

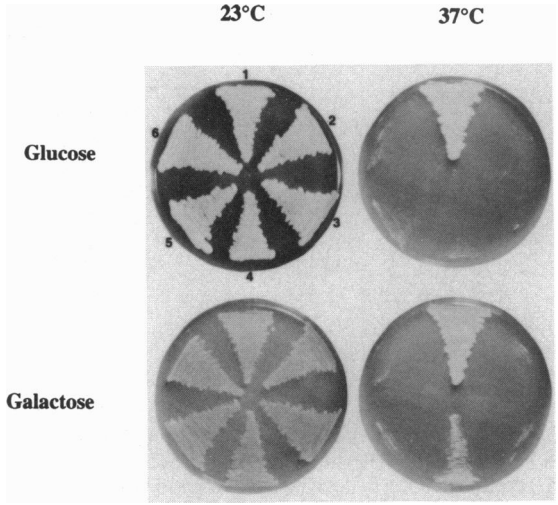

FIG. 6. Complementation of the yeast *mas5* mutation by the wild-type ANJ1 but not the mutated proteins ANJ1S or ANJ1L. Sectors: 1, wild-type yeast strain MYY290; 2, *mas5* mutant strain MYY391; 3, MYY391 transformed with pYES (vector); 4, MYY391 transformed with pYANJ1; 5, MYY391 transformed with pYANJ1S; 6, MYY391 transformed with pYANJ1L. Expression of ANJ1, ANJ1S, and ANJ1L is repressed in yeast growing on YPD (glucose) medium and is induced in yeast growing on YPG (galactose) medium.

incorporation into ANJ1 may have been the consequence of insufficient protein geranylgeranyltransferase enzyme in *A. nummularia* extracts and/or unsuitability of the *in vitro* assay conditions for geranylgeranylation of plant proteins. The yeast homolog of ANJ1, YDJ1, has a similar *in vitro* isoprenylation pattern in normal yeast—i.e., substantially less geranylgeranylation (26). Only a small amount of YDJ1 is isoprenylated in the *ram1* (farnesyltransferase-deficient) strain, indicating that this protein is primarily farnesylated *in vivo* (26). Feeding plant cells with labeled mevalonic acid, followed by HPLC analysis of the chain length of the labeled isoprenyl group that is incorporated into ANJ1, will establish which isoprenoid is conjugated to the protein *in vivo*.

Consistent with numerous isoprenylation studies on mammalian and yeast proteins (9, 10), the cysteine-414 of the CAQQ box is absolutely required for *in vitro* incorporation of both [^{3}H]farnesyl and [^{3}H]geranylgeranyl into ANJ1. Substitution of serine for cysteine-414 also abolished isoprenylation

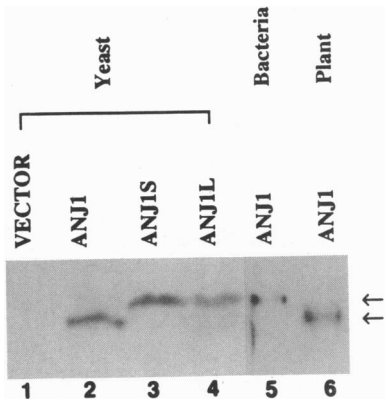

FIG. 7. Electrophoretic mobilities of ANJ1, ANJ1S, and ANJ1L expressed in yeast, bacterial, or plant cells. Cell extracts from yeast containing pYES (vector, lane 1), pYANJ1 (lane 2), pYANJ1S (lane 3), or pYANJ1L (lane 4), IPTG-induced *E. coli* BL21(DE3) containing pETANJ1 (lane 5), or *A. nummularia* cells (lane 6) were subjected to SDS/PAGE, electroblotted onto nitrocellulose, and probed with anti-ANJ1. Arrows indicate the two forms of ANJ1.

of ANJ1 *in vivo* as judged from the electrophoretic mobility differences between yeast-expressed ANJ1S and the wild-type ANJ1.

It was unexpected that substitution of leucine for the terminal glutamine in the CAQQ motif did not result in increased incorporation of [^{3}H]geranylgeranyl. Rather, this change abolished any geranylgeranylation of ANJ1. Interestingly, a low level of farnesylation of this mutant protein did occur. These results indicate that the earlier conclusion that specificity of isoprenylation (i.e., C_{15} versus C_{20}) is determined by the last amino acid in the CaaX box (11) may need to be reexamined.

The precise role of isoprenylation of ANJ1 in its functional activity at high temperatures in yeast is yet to be determined. In yeast, high temperature results in increased membrane association of the ANJ1 homolog, YDJ1, which may be linked to the function of the protein under these conditions (26). *In vitro* experiments established that farnesylation enhances the association of ANJ1 with microsomal membranes. It seems that virtually all ANJ1 in *A. nummularia* cells is isoprenylated and associated with membranes, and no difference is detected after high-temperature treatment. Association of ANJ1 with specific membranes has not been determined; however, targeting of Ras and nuclear lamin proteins to specific membranes occurs and requires other posttranslational modifications in addition to isoprenylation (16, 37). Although association of YDJ1 with membranes is apparently mediated by more than isoprenylation (26), membrane attachment of ANJ1 seems to be at least one function of isoprenyl modification. Another might be to interact with a specific membrane receptor so that ANJ1 can be positioned for more efficient interaction with a membrane translocating peptide and/or 70-kDa heat shock protein.

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