

## AtFACE-2, a functional Prenylated Protein Protease from *Arabidopsis thaliana* Related to Mammalian Ras-converting Enzymes\*

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**Eukaryotic proteins containing a CAAX (A is aliphatic amino acid) C-terminal tetrapeptide sequence generally undergo a lipid modification, the addition of a prenyl group. Proteins that are modified by prenylation, such as Ras GTPases, can be subsequently modified by a proteolytic event that removes a C-terminal tripeptide (AAX). Two distinct proteases have been identified that are involved in the CAAX proteolytic step, FACE-1/Ste24 and FACE-2/Rce1. These proteases have different enzymatic properties, substrate specificities, and biological functions. However, a proposal has been made that plants lack a FACE-2/Rce1-type protease. Here, we describe the isolation of a cDNA from *Arabidopsis thaliana* that encodes a 311-aa protein with characteristics that are similar to the FACE-2/Rce1 group of enzymes. Northern blot analysis demonstrates widespread expression of this gene in plant tissues. Heterologous expression of the *A. thaliana* cDNA in yeast restores CAAX proteolytic activity to yeast lacking native CAAX proteases. The recombinant protein produced in this system displays an *in vivo* substrate specificity profile distinct from AtSte24 and cleaves a farnesylated CAAX tetrapeptide *in vitro*. These results provide evidence for the existence of a previously unsuspected plant FACE-2/Rce1 ortholog and support the evolutionary conservation of dual CAAX proteolytic systems in eukaryotes.**

Maturation of eukaryotic proteins containing the C-terminal CAAX<sup>1</sup> domain (C, cysteine; A, aliphatic amino acid; X, one of

several amino acids) requires an ordered series of posttranslational modifications. This modification pathway starts with the addition of a prenyl group (farnesyl or geranylgeranyl) to the side chain of the cysteine residue. The -AAX tripeptide is proteolytically removed, and finally, the carboxyl group of the prenylated cysteine is methylated. These modifications occur to many eukaryotic proteins, including proteins of great biological and pathological relevance such as the small GTPases of the Ras superfamily, the heterotrimeric G-proteins, and certain nuclear lamins. These modifications may impart membrane binding, alter activity, and/or regulate protein-protein interactions (reviewed in Refs. 1–3). Because of the central role of oncogenic Ras proteins in human cancer and the fact that improperly modified Ras has significantly reduced activity, the enzymes responsible for each of the steps in CAAX maturation have attracted interest as potential targets of antitumoral therapies. For example, inhibitors of the farnesyltransferase that carries out the first step of this processing cascade are currently being evaluated in clinical trials for the treatment of cancer (4, 5). It has been proposed that the enzymes involved in the proteolytic and methylation steps could also provide feasible points of therapeutic intervention.

Two genes encoding distinct CAAX proteases have been identified in several organisms with the first set being identified in *Saccharomyces cerevisiae* (6). In yeast, Ste24p was identified as being required for the maturation of the prenylated mating pheromone **a-factor** (6–8). Yeast Ste24p is a metalloprotease that cleaves the farnesylated **a-factor** precursor after the farnesyl-cysteine residue and also at a second point toward the N terminus of the polypeptide (8). The completely unrelated Rce1p protein was also determined to cleave after the farnesyl-cysteine residue of **a-factor** (6). In addition, Rce1p seems to be responsible for removing the C-terminal tripeptide (AAX) from other prenylated proteins including proteins of the Ras superfamily (6). Thus, Ste24p and Rce1p appear to have partially redundant function in cleaving yeast prenylated proteins. Moreover, the yeast enzymes have been reported to have partially overlapping substrate specificity (9). Using the yeast sequences, we and others (7, 10–13) have identified the counterparts of the two CAAX proteases in other eukaryotic systems such as mouse, human, and nematode. Similar to the yeast enzymes, these orthologs contain several putative transmembrane domains and are likely to be localized at the cellular endomembrane system where the yeast enzymes have been localized (14).

Although Rce1p and Ste24p have partially overlapping function, the relative importance of these enzymes to cellular physiology is quite distinct. Gene disruption has demonstrated the

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<sup>1</sup> The abbreviations used are: CAAX, C is cysteine, A is aliphatic amino acid, and X is one of several amino acids; FACE, farnesylated protein-converting enzyme; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone; At, *A. thaliana*; ORF, open reading frame; HA, hemagglutinin.

essential role of FACE-2/Rce1 in the processing and correct subcellular localization of Ras proteins (15). Not surprisingly, the loss of FACE-2/Rce1p is lethal in mice. FACE-2-deficient cells are also less susceptible to Ras-mediated transformation, supporting the inhibition of this enzyme as an anti-tumoral therapy (15, 16). By contrast, targeted disruption of FACE-1/Ste24 results in more subtle effects. Mice lacking FACE-1 are apparently normal at birth, but their growth slows after a few weeks when they begin to show accelerated aging, bone alterations, and symptoms characteristic of muscular dystrophies. These effects are apparently due to the incomplete processing of lamin A, a farnesylated component of the nuclear lamina, because the lamin A precursor accumulates in FACE-1-deficient cells and tissues (17, 18).

A FACE-1/Ste24 ortholog was the first CAAX protease to be identified in plants. AtSTE24 isolated from *Arabidopsis thaliana* has been functionally characterized and shown to have broad substrate specificity (19). This finding and the absence of an obvious plant Rce1p ortholog has led to the proposal that plants lack FACE-2/Rce1-like enzymes. According to this hypothesis, AtSTE24 is proposed to have evolved into a broader biological role as the only CAAX protease in these organisms (19). This hypothesis is consistent with the observation that *A. thaliana* lacks Ras GTPases (20), which have thus far been shown to be FACE-2/Rce1-specific targets in other organisms. Here, we describe the identification of a widely expressed *A. thaliana* gene encoding a FACE-2-type CAAX protease. This plant enzyme can substitute for the yeast enzyme *in vivo* and can cleave a farnesylated peptide *in vitro*. Together with the recent identification of two CAAX proteases belonging to FACE-1/Ste24 and FACE-2/Rce1 types from *Caenorhabditis elegans* (13), these findings support the evolutionary conservation of this proteolytic system in all eukaryotes.

#### EXPERIMENTAL PROCEDURES

**Materials**—Restriction endonucleases and other molecular cloning reagents were purchased from Roche Applies Science. [ $\alpha$ - $^{32}$ P]dCTP was from Amersham Biosciences. [ $^3$ H]Farnesyl pyrophosphate was from PerkinElmer Life Sciences. Oligonucleotides were purchased from Sigma-Genosys (The Woodlands, TX). Double-stranded DNA probes were radiolabeled using a commercial random-priming kit from Amersham Biosciences. *A. thaliana* (ecotype Columbia) were provided by D. García and E. Cervantes (University of Salamanca) and cultivated under standard conditions.

**RNA Isolation and Analysis**—Total RNA was extracted from *A. thaliana* seeds or tissues following the procedure described by Vicent and Delseny (21). Reverse transcriptase-PCR was performed with the SuperScript kit (Invitrogen) using oligo(dT) as primer in the reverse transcriptase reaction. PCR reactions were carried out in a GeneAmp 2400 PCR system from PerkinElmer Life Sciences/Cetus (Boston, MA) for 40 cycles of denaturation (94 °C, 15 s), annealing (64 °C, 20 s), and extension (72 °C, 2 min). The coding sequence of AtFACE-2 was amplified with primers F2At-forward (5'-CGGAGCTTCCTCAACGAT-TATGG-3') and F2At-reverse (5'-TTGGAGAAGTGTCATTCACAAACA-3'). Nylon filters containing 20  $\mu$ g of total RNA from *A. thaliana* seeds and tissues were prehybridized at 42 °C for 3 h in 50% formamide, 5 $\times$  saline/sodium phosphate/EDTA (1 time = 150 mM NaCl, 10 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM EDTA, pH 7.4), 10 $\times$  Denhardt's solution, 2% SDS, and 100  $\mu$ g/ml denatured herring sperm DNA and then hybridized with radiolabeled AtFACE-2 cDNAs for 20 h under the same conditions. Filters were washed with 0.1 $\times$  SSC, 0.1% SDS for 2 h at 50 °C and exposed to autoradiography.

**DNA Sequencing and Sequence Analysis**—PCR products were gel-purified, treated with Klenow fragment, kinased, and subcloned in the polylinker region of pBlueScript or pCDNA3. DNA sequencing was carried out using an ABI PRISM<sup>TM</sup> 310 genetic analyzer (Applied Biosystems, Foster City, CA). All of the nucleotide sequences were identified in both strands. Computer analysis of DNA and protein sequences was performed with the GCG software package of the University of Wisconsin Genetics Computer Group (22). Database similarity searches were performed with the BLAST algorithm (23). Multiple alignments were performed with the program ClustalX (24) and edited

TABLE I  
Yeast strains used in this study

Strain	Genotype	Reference
SM1058	<i>MATa trp1 leu2 ura3 his4 can1</i>	27
SM1068	<i>MAT<math>\alpha</math> lys1</i>	27
SM1086	<i>MAT<math>\alpha</math> rme his6 met1 can1 cyh2 sst2-1</i>	28
SM3041	pRS316 [ <i>CEN URA3</i> ] transformant of SM1058	7
SM3103	<i>MATa trp1 leu2 ura3 his4 can1 ste24::LEU2</i>	7
SM3613	<i>MATa trp1 leu2 ura3 his4 can1 rce1::TRP1</i>	8
SM3614	<i>MATa trp1 leu2 ura3 his4 can1 rce1::TRP1 ste24::LEU2</i>	8
SM3637	pRS316 [ <i>CEN URA3</i> ] transformant of SM3103	7
SM3643	pRS316 [ <i>CEN URA3</i> ] transformant of SM3613	8
SM3650 <sup>a</sup>	pRS316 [ <i>CEN URA3</i> ]	8
SM3689	<i>MATa trp1 leu2 ura3 his4 can1 mfa1-<math>\Delta</math>1 mfa2-<math>\Delta</math>1 ste24::LEU2</i>	8
yWS122 <sup>a</sup>	pSM1314 [ <i>CEN URA3 RCE1-HA</i> ]	14
yWS148 <sup>a</sup>	pWS399 [ <i>CEN URA3 AtFACE-2</i> ]	This study
yWS149 <sup>a</sup>	pWS400 [ <i>CEN URA3 AtFACE-2-HA</i> ]	This study
yWS163 <sup>a</sup>	pWS402 [ $2\mu$ <i>URA3 HA-AtFACE-2</i> ]	This study
yWS164	<i>trp1 leu2 ura3 his4 can1 mfa1-<math>\Delta</math>1 mfa2-<math>\Delta</math>1 rce1::TRP1 ste24::KAN<sup>R</sup></i>	This study
yWS167 <sup>a</sup>	pWS413 [ <i>CEN URA3 HA-AtSte24</i> ]	This study
yWS180 <sup>a</sup>	pWS448 [ $2\mu$ <i>URA3 AtFACE-2-HA</i> ]	This study
yWS182 <sup>a</sup>	pWS450 [ $2\mu$ <i>URA3 HA-AtSte24</i> ]	This study

<sup>a</sup> These strains are transformants of SM3614.

with GeneDoc (www.psc.edu/biomed/genedoc). Transmembrane regions were predicted with the help of the programs TopPred2 (25) at the Theoretical Chemistry Protein Prediction Server, University of Stockholm and TMpred at EMBnet (26).

**Yeast Strains and Plasmids**—The *Saccharomyces cerevisiae* strains used in this study are listed in Table I. Yeast were grown at 30 °C in complete (YPD) or synthetic media containing appropriate supplements (SC-) as described previously (27, 28). Plasmid-transformed strains were created according to published methods (29). The plasmids used in this study are listed in Table II. The plasmids encoding tagged and untagged yeast Rce1p and Ste24p have been described previously (7, 14). Low copy yeast expression vectors (*CEN*) encoding epitope-tagged and untagged CAAX proteases from other organisms were generated by replacing the appropriate yeast open reading frame (ORF) with the corresponding ortholog cDNAs by PCR-directed homologous recombination (30). Thus, the expression of Rce1p and Ste24p orthologs is under control of the native promoters for yeast *RCE1* and *STE24*, respectively. Multi-copy plasmids ( $2\mu$ ) encoding the CAAX protease ORFs were also created by recombinational cloning using linearized pRS426 as the recipient vector for the ortholog-containing fragments (31). In addition, the high expression vector encoding AtFACE-2 was generated by subcloning this cDNA into the polylinker of pSM703, a  $2\mu$  vector containing the constitutive phosphoglycerate kinase promoter (31, 32). A *STE24* disruption plasmid, pWS405, was created by replacing the entire *STE24* ORF of pSM1093 with the *KanMX* gene by homologous recombination (8). A *PvuII* and *NdeI* digestion fragment from the resultant plasmid containing the *KanMX* gene flanked by ~800 bp of sequence homologous to the *STE24* 5'- and 3'-untranslated regions was transformed into SM3613 to create yWS164. Transformed cells were selected on YEPD plates containing G418 (200  $\mu$ g/ml). Two plasmid-encoding CAAX variants (CTLM and CASQ) of the wild type *a*-factor gene (*CVIA*) were created by PCR-directed homologous recombination using the yeast *a*-factor gene encoded on pWS438. All of the plasmids constructed were analyzed by restriction digest and/or sequencing analysis to verify that the proper inserts were present.

***a*-Factor Production Assays**—To test the ability of the putative CAAX proteases to promote *a*-factor production in yeast, halo and mating tests were carried out using established biological assays (7, 27). For both tests, cells grown in selective medium were replica-plated onto a lawn of SM1086 (halo test) or SM1068 (mating test) that was previously spread onto a rich (YPD) or minimal (SD) agar plate, respectively. Halo formation and diploid production were recorded after 24 and 48 h, respectively, of incubation at 30 °C. For the specificity study, cells were grown to saturation in 100- $\mu$ l cultures in microtiter plates, washed twice with H<sub>2</sub>O, and resuspended into 10  $\mu$ l of sterile H<sub>2</sub>O. For each

TABLE II  
Yeast expression plasmids used in this study

Plasmid	Genotype	Reference
pRS316	CEN URA3	31
pSM703	2 $\mu$ URA3	32
pSM1093	CEN URA3 STE24	7
pSM1097	CEN LEU2 HA-STE24	7
pSM1275	CEN URA3 RCE1	14
pSM1314	CEN URA3 RCE1-HA	14
pWS399	CEN URA3 AtFACE-2	This study
pWS400	CEN URA3 AtFACE-2-HA	This study
pWS402	2 $\mu$ URA3 HA-AtFACE-2	This study
pWS413	CEN URA3 HA-AtSte24	This study
pWS438	2 $\mu$ LEU2 MFA1	This study
pWS440	2 $\mu$ LEU2 MFA1-CASQ	This study
pWS441	2 $\mu$ LEU2 MFA1-CTLM	This study
pWS448	2 $\mu$ URA3 AtFACE-2-HA	This study
pWS450	2 $\mu$ URA3 HA-AtSte24	This study

strain examined, a portion of the resuspended cells (2  $\mu$ l) was spotted onto the corresponding lawn.

**CAAX Proteolytic Assay**—CAAX proteolytic assays were performed essentially as described previously (33). Farnesyltransferase that was partially purified from calf brains by ammonium sulfate precipitation (30–50%), hydrophobic interaction chromatography, and cation exchange was used to farnesylate the tetrapeptide Ac-CVIS using [<sup>3</sup>H]farnesyl pyrophosphate as donor of the prenyl group. The farnesylated peptide was purified by organic extraction, lyophilized, and dissolved in Me<sub>2</sub>SO at a final concentration of 10  $\mu$ M (~7250 Ci/mmol). Membrane fractions were prepared as described previously (34) with the following modifications: cells were resuspended in 5 mM MgCl<sub>2</sub>, 100 mM NaCl, 200 mM Hepes, pH 7.4 (FACE buffer), and the membranes were recovered by centrifugation at 16,000  $\times$  g. CAAX proteolytic assays were performed in FACE buffer in a final volume of 100  $\mu$ l for 1 h at 37 °C and stopped by the addition of 0.8 ml of chloroform:methanol (1:1). After the addition of 0.8 ml of 1 M citric acid, the organic phase containing the farnesylated peptides was collected, vacuum-dried, and analyzed by thin layer chromatography in 20  $\times$  20-cm cellulose plates using chloroform:acetone:methanol:acetic acid (60:20:10:5) as mobile phase. After chromatography, the TLC plates were dried and exposed to Biomax x-ray film (Eastman Kodak Co.) at –80 °C for 1 week.

**Western Blot**—Yeast membrane extracts (40  $\mu$ g) were resolved by SDS-PAGE gels and transferred to nitrocellulose membranes following standard procedures. The 12CA5 anti-hemagglutinin monoclonal antibody was purchased from Roche Applied Science. Equal loading of the different samples was assessed by reversible staining of the membranes with Ponceau S. Detection of the immunoreactive bands was carried out with horseradish peroxidase-coupled secondary antibodies and the PicoSignal chemiluminescent reagent from Pierce.

## RESULTS

**Identification and Characterization of the *A. thaliana* AtFACE-2 Gene**—Both FACE-1/Ste24 and FACE-2/Rce1 CAAX proteases have been described in yeasts, mammals, and nematodes (6, 7, 10–13). However, it has been recently proposed that the plant FACE-1-type enzyme might have evolved to acquire broader substrate specificity and function as the only CAAX protease (19). To test this hypothesis, we performed a TBLASTN search of the *A. thaliana* genomic databases using the human FACE-2 amino acid sequence as query. This approach revealed the presence of a putative gene in *A. thaliana* chromosome 2 with significant similarity to the human sequence. Furthermore, two expressed sequence tags corresponding to this genomic region were identified (AV557607 and AU231387), which encoded partial amino acid sequences similar to the C-terminal portion of FACE-2-type proteases. For one of these expressed sequence tags (AU331387), the nucleotide sequence corresponding to the 5' end of the cDNA clone from which it had been derived was also available in the data base (GenBank<sup>TM</sup> accession AU240018), allowing us to design oligonucleotides corresponding to both ends of the cDNA. These oligonucleotides were used in a reverse transcriptase-PCR experiment using total RNA from *A. thaliana* seeds as template,

after which we obtained a 966-bp cDNA fragment containing the coding sequence for a 311-amino acid polypeptide with a calculated molecular mass of 34,548 Da. This protein, which we have tentatively called AtFACE-2, displays a significant degree of sequence similarity to previously described enzymes of the FACE-2/Rce1 family (Fig. 1). The closest homolog of AtFACE-2 is the insect enzyme (45% similarity and 27% identity), whereas a hypothetical CAAX protease from *Candida albicans* is the most distantly related ortholog (33% similarity and 14% identity). As shown in Fig. 1, 21 amino acid residues are conserved across all of the FACE-2/Rce1 sequences. These invariably conserved positions include several residues previously reported to be required for the enzymatic activity of *S. cerevisiae* Rce1p (Glu-164, His-198, His-251, and Cys-254 in the *Arabidopsis* sequence) (35). 18 of the 21 invariably conserved residues are located in a region corresponding roughly to the third quarter of the polypeptide chain (residues 155–260 in AtFACE-2), suggesting that this part of the protein could be the most directly involved in its function.

Computer analysis of the amino acid sequence predicted several putative transmembrane helices (Fig. 2), suggesting that the plant enzyme is localized to membranes as has been determined for CAAX proteases in other systems (11, 14). However, it cannot be ruled out that some of the hydrophobic regions present in these polypeptides play alternative roles in the processing of prenylated proteins in the vicinity of the cellular membranes without being completely embedded in the lipid bilayer. The fact that some of the most conserved residues such as the QXXYXXXFG motif (residues 223–231 in AtFACE-2) are located in highly hydrophobic environments would be in agreement with this possibility. Taken together, the characteristics of this polypeptide indicate that the isolated cDNA encodes the first plant member of the FACE-2/Rce1 family of proteases.

**Expression of AtFACE-2**—Previous studies have shown that FACE-2/Rce1 enzymes from human and mice have widespread expression in adult tissues and are present throughout development (10, 12). To investigate the expression pattern of the gene encoding the putative *A. thaliana* CAAX protease described in this work, we carried out a Northern blot analysis of total RNA from *A. thaliana* seeds, stems, leaves, flowers, and fruits using the AtFACE-2 full-length cDNA as a probe. As shown in Fig. 3, bands corresponding to transcripts of ~1.7 and 1.3 kb were detected with different intensities in all of the samples. These transcripts were large enough to contain the complete AtFACE-2 open reading frame, and the different sizes probably reflect alternative splicing or polyadenylation events. The fact that AtFACE-2, like its mammalian counterparts, shows a widespread pattern of expression suggests that this enzyme has a housekeeping role both in plants and animals.

**In Vivo Activity of AtFACE-2**—To obtain experimental evidence on the ability of the isolated cDNA to encode a functional protein, we constructed a high expression *S. cerevisiae* vector containing the AtFACE-2 ORF fused to a HA epitope. Western blot of membrane-enriched extracts from yeast transformed with this plasmid using an anti-HA monoclonal antibody demonstrated the presence of an immunoreactive band corresponding to a protein of ~35 kDa, which corresponds to the expected molecular mass of AtFACE-2 in the absence of post-translational modifications (34.5 kDa) (Fig. 3B).

Because the cDNA isolated in this work encodes a protein with structural features characteristic of CAAX proteases of the FACE-2/Rce1 family, we tested its ability to complement the deficiency of CAAX proteolysis in yeast cells lacking both Ste24p and Rce1p. This approach allowed us recently to demonstrate the functionality of the *C. elegans* CAAX proteases

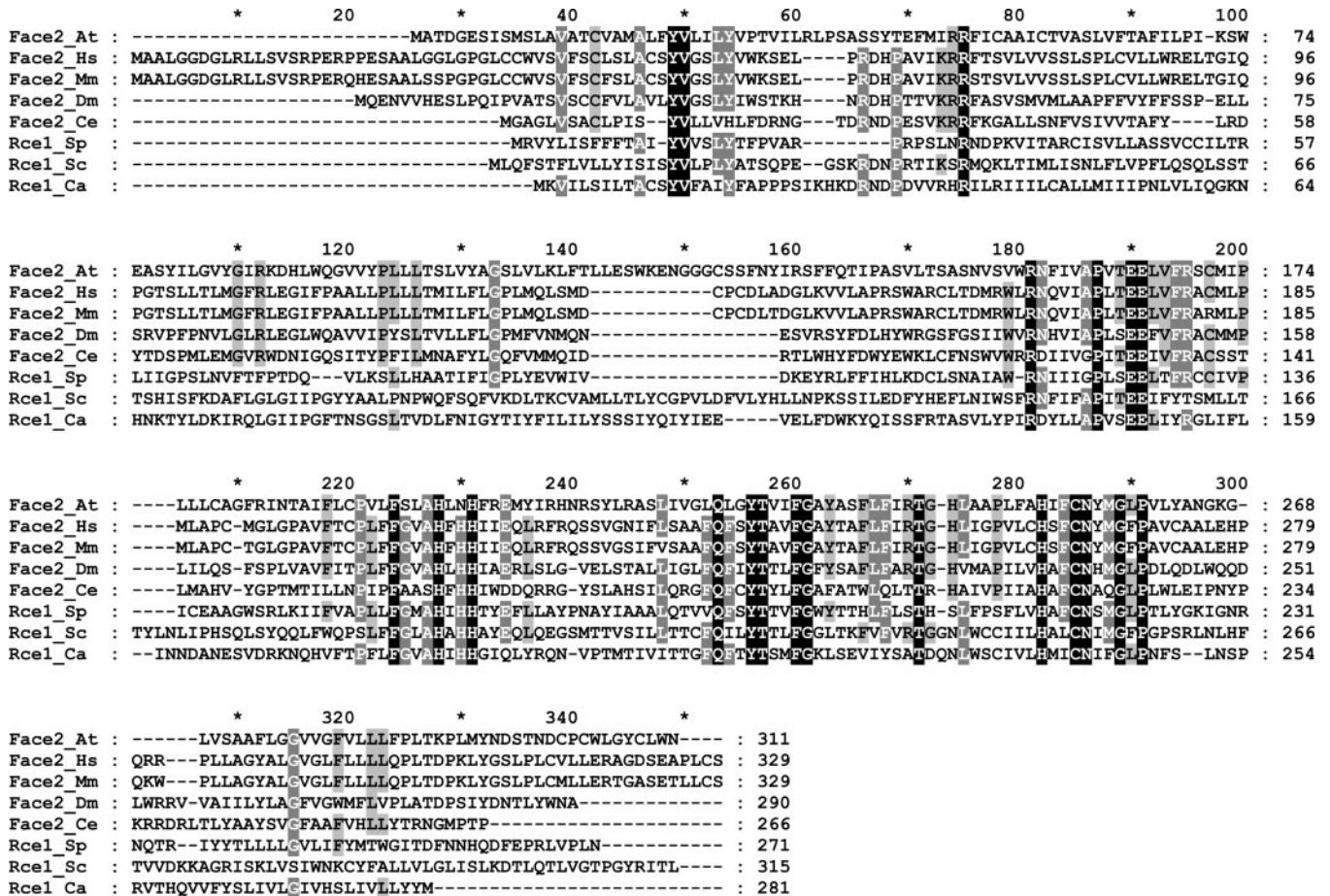


FIG. 1. Comparison of AtFACE-2 to related proteins from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *C. elegans*, *Schizosaccharomyces pombe*, *S. cerevisiae*, and *C. albicans*. Black boxes show residues identical in all of the sequences analyzed. Residues conserved in some but not all of the sequences are highlighted in gray boxes.

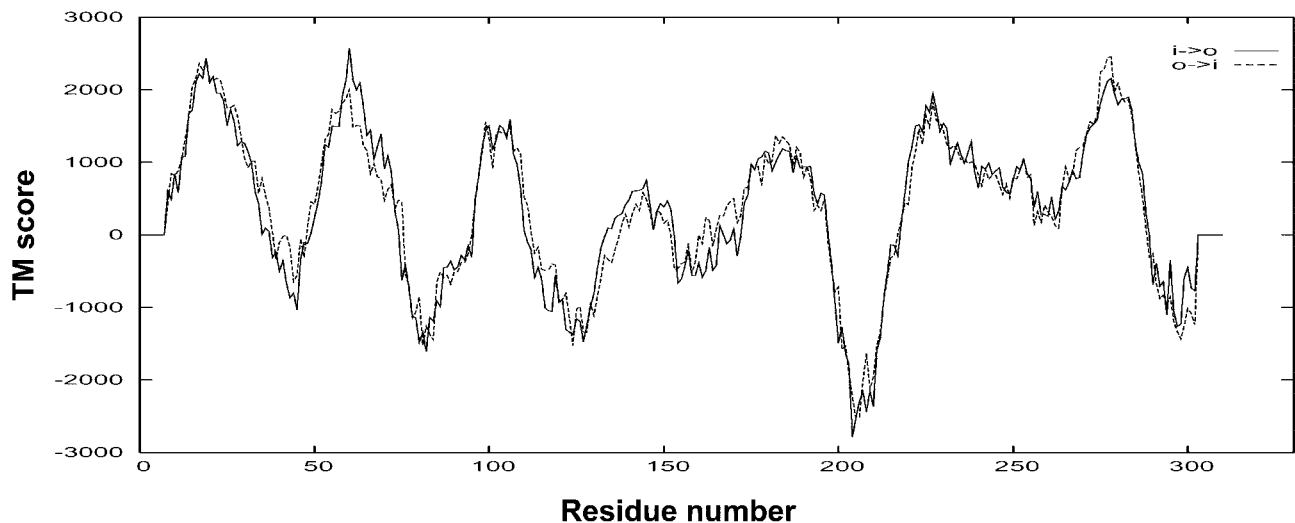


FIG. 2. Predicted membrane spans of AtFACE-2 protein. Hypothetical membrane-spanning regions were predicted with the program TMpred ([www.ch.embnet.org/software/TMPRED\\_form.html](http://www.ch.embnet.org/software/TMPRED_form.html)). The continuous and the broken lines represent the predictions assuming cytoplasmic or endoplasmic reticulum-luminal positions of the N terminus of the polypeptide, respectively. *TM*, transmembrane.

(13). For this study, we transformed a *rce1Δ ste24Δ* yeast strain (8) with expression plasmids encoding AtFACE-2 and evaluated the ability of these plasmids to promote the production of the farnesylated mating pheromone **a**-factor. The production of **a**-factor was followed by two *in vivo* methods. One was the halo test, which is based on the growth inhibition of a *MAT $\alpha$  sst2* cells that are supersensitive to mature extracellular **a**-factor

(Fig. 4A) (27, 28). When the AtFACE-2-expressing *MAT $\alpha$*  yeast strains were examined, a clear halo surrounding the *MAT $\alpha$*  cells was evident, indicative of growth inhibition and indirectly indicative of a functional CAAX protease. The strains transformed with a centromeric (low copy) plasmid carrying the AtFACE-2 cDNA either in native (yWS148) or HA-tagged form (yWS149) under the control of the natural yeast *RCE1* regula-

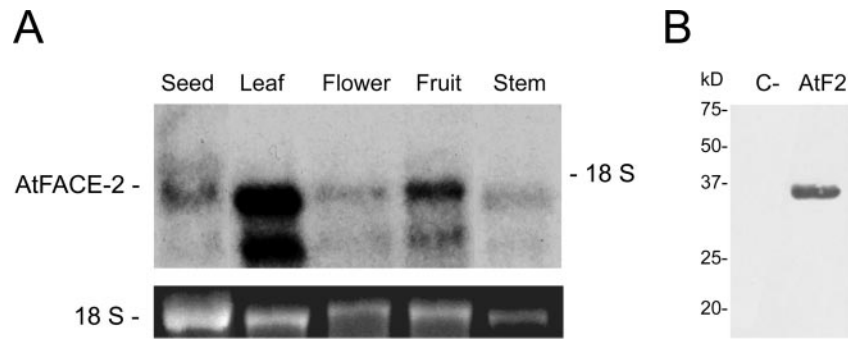


FIG. 3. **Expression analysis of *AtFACE-2* gene.** A, Northern blot analysis of RNA (approximately 20  $\mu$ g) from different plant tissues using a probe against *AtFACE-2*. The position of 18 S *A. thaliana* ribosomal RNA is indicated. The amount of 18 S rRNA is shown as a loading control. B, Western blot analysis using an anti-HA monoclonal antibody of membranes (15  $\mu$ g of protein) from yeast transformed with either an empty vector (SM3650, C-) or a plasmid encoding the HA-tagged cDNA of *AtFACE-2* (yWS163). The positions of protein standards are shown.

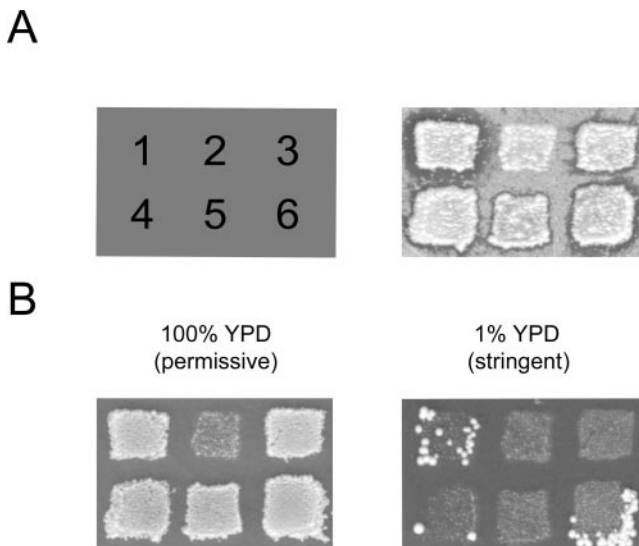


FIG. 4. **Complementation of the mating defect of *S. cerevisiae* lacking *RCE1* and *STE24* by *AtFACE-2*.** A, Halo test. The indicated *MAT $\alpha$*  yeast strains were grown as patches on selective medium for 2 days then replica-transferred onto a plate containing a lawn of super-sensitive *MAT $\alpha$  sst2* cells. The strains used are SM3041 (wild type) (1) or plasmid transformants of SM3614 (*rce1 $\Delta$  ste24 $\Delta$* ) (2–6). Respectively, strains identified as 2–6 are SM3650 (pRS316), yWS122 (*RCE1-HA*), yWS148 (*AtFACE-2*), yWS149 (HA-*AtFACE-2*), and yWS163 (P<sub>PGK</sub>-HA-*AtFACE-2*). The formation of a zone of growth inhibition surrounding the spotted cells is indicative of **a**-factor production by the cells. B, mating test. The same strains as in A were replica-transferred onto a lawn of *MAT $\alpha$  lys2* cells suspended in the indicated medium prior to preparation of the lawn. The growth of diploids is indicative of mating.

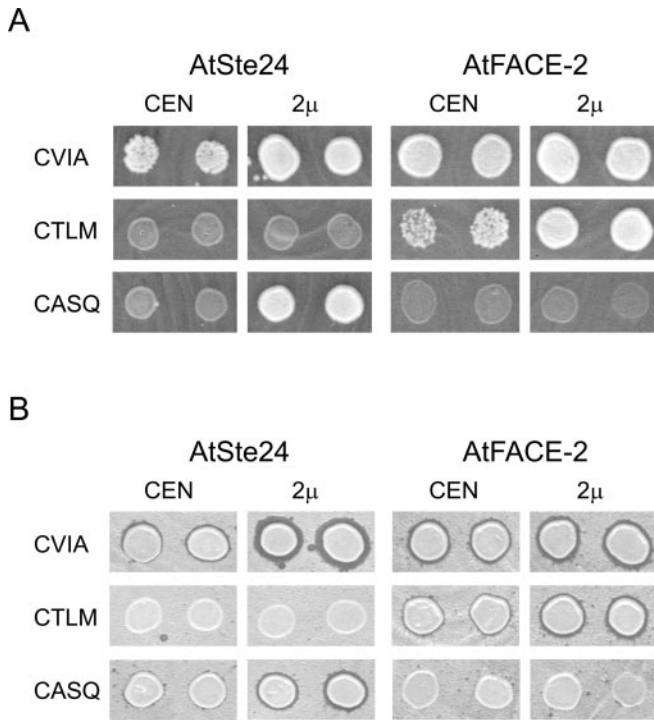
tory sequences reproducibly produced halos of a magnitude similar to those produced by cells transformed with a similar vector containing HA-tagged yeast *Rce1p* (yWS122). These results demonstrate the ability of *AtFACE-2* to participate in the maturation process of **a**-factor. Of note, we observed that the halos produced by these strains, which only contained a *FACE-2/Rce1p*-like CAAX protease, were significantly smaller than those produced by wild-type cells. We found that *AtFACE-2* dependent **a**-factor production could be improved by the overexpression of HA-tagged *AtFACE-2*, *i.e.* by controlling expression with the *PGK* promoter in a multi-copy plasmid. A strain overexpressing *AtFACE-2* produced halos of similar size to those obtained with wild-type cells. This phenomenon has been previously observed for *FACE-2/Rce1* proteases from yeast and nematode. The reduced production of **a**-factor has been attributed to the absence of the *FACE-1/Ste24p*-type enzyme, which is required for a separate step in **a**-factor production (13, 14).

The ability of *AtFACE-2* to process yeast **a**-factor *in vivo* was confirmed using a second **a**-factor production test (Fig. 4B). For

the patch-mating test, the *MAT $\alpha$*  yeast strains described in Fig. 4A were replica-plated onto a lawn of *MAT $\alpha$  lys2* cells that had been previously spread on selective solid media. In this test, **a**-factor production is assessed by the generation of prototrophic diploids that form as a result of productive mating events. These diploid cells and not the haploid parents are able to grow on minimal medium. We find that both native and HA-tagged *AtFACE-2* cDNAs are able to revert the mating defect of the *rce1 $\Delta$  ste24 $\Delta$*  mutant cells as efficiently as the yeast CAAX protease *Rce1p*, paralleling perfectly the results obtained in the halo assay. To address the relative activity of *AtFACE-2*, the patch-mating test was also carried out under stringent mating conditions (36). The results show that mating competence is correlated with increased expression of *AtFACE-2* (compare low copy *versus* overexpression conditions). Combined, both **a**-factor production tests reveal that *AtFACE-2* is a functional CAAX protease.

*Distinct Substrate Specificity of A. thaliana CAAX Proteases*—The yeast CAAX proteases have partially overlapping substrate specificity (6, 9). To compare the substrate specificity of *AtFACE-2* and the previously described *AtSte24*, we tested the ability of these proteases to promote the processing of three different CAAX variants of *S. cerevisiae* **a**-factor using the bioassays described above. For this purpose, we generated a yeast strain lacking the **a**-factor genes and endogenous CAAX proteolytic activity that could be co-transformed with plasmids encoding an individual CAAX protease (*AtSte24p* or *AtFACE-2*) and an **a**-factor CAAX variant (CVIA, CTLM, or CASQ). The wild-type CAAX motif of **a**-factor (CVIA) is recognized by both yeast *Rce1p* and *Ste24p*. One of the variants ends with the CTLM sequence that is present in *Ste18p* (a  $\gamma$ -subunit of a yeast heterotrimeric G protein). This motif has been shown to be yeast *Rce1p*-specific. The other variant contains the CASQ sequence that is derived from the yeast *DnaJ*-like chaperone *Ydj1p/Mas5p*. This motif is yeast *Ste24p*-specific. The specificity of the CAAX protease from other organisms has not yet been addressed.

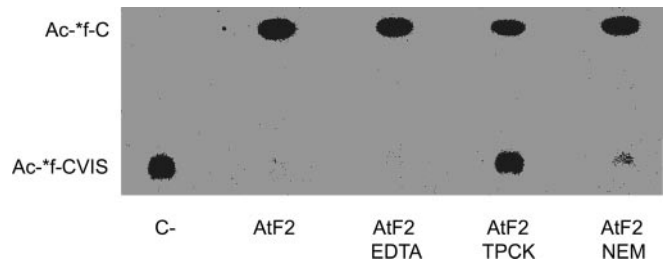
Assays based on **a**-factor production are convenient indirect methods to evaluate the ability of a CAAX protease to cleave the **a**-factor CAAX motif. Using such assays, we have evaluated the specificity of the *A. thaliana* CAAX proteases and found that both proteases are active against the wild-type pheromone (CVIA) as judged by the ability of the protease to promote mating and the formation of diploids (Fig. 5A). Consistent with the reported specificity of the yeast enzymes, *AtFACE-2* but not *AtSte24* is able to promote mating of a strain expressing the CTLM variant. Conversely, only *AtSte24p* has the ability to promote cleavage of the CASQ motif. These results are paralleled by those obtained in a halo assay (Fig. 5B), confirming the different substrate specificity of *AtSte24* and *AtFACE-2*. Al-



**FIG. 5. Differential substrate specificity of AtSte24 and AtFACE-2.** *A*, mating test of *MATa* yeast strains expressing wild-type (CVIA) or CAAX variants of *a*-factor (CTLM and CASQ). The strains also contain either AtSte24 or AtFACE-2 encoded in low copy (*CEN*) or multi-copy (*2μ*) plasmids. The strains were grown to saturation, harvested, and washed with sterile  $H_2O$ , and a portion of the resuspended cells was spotted on a lawn of *MATα lys2* cells. The growth of diploids is indicative of mating. *B*, Halo test of the strains described in *A*. These strains were grown and harvested as above and spotted on a lawn of *MATα sst2* cells that are supersensitive to pheromone. The formation of a zone of growth inhibition surrounding the spotted cells is indicative of *a*-factor production by the cells. The strains evaluated were created by co-transforming *yWS164* with an *A. thaliana* CAAX protease, AtSte24 (pWS413 or pWS450) or AtFACE-2 (pWS400 or pWS448), and an *a*-factor-encoding plasmid, CVIA (pWS438), CASQ (pWS440), or CTLM (pWS441).

though the halo assay is more sensitive measure of *a*-factor production, no cleavage of the nonspecific CAAX motif is observed, even when the proteases are overexpressed. Combined, the results indicate that the *A. thaliana* CAAX proteases have the ability to differentiate between target substrates, provided that these targets have CAAX motifs that are specific for one or the other protease (e.g. CTLM and CASQ).

**In Vitro Activity of AtFACE-2**—To test *in vitro* the enzymatic activity of AtFACE-2, we prepared membrane extracts from yeast cells expressing AtFACE-2 as the only CAAX protease and assayed the membranes for proteolytic activity against a farnesylated tetrapeptide (Fig. 6). Membrane-enriched extracts were prepared from *rce1Δ ste24Δ S. cerevisiae* cells overexpressing HA-tagged AtFACE-2. The membranes were incubated with the radiolabeled peptide Ac-farnesyl-Cys-Val-Ile-Ser, and proteolytic activity was assessed by the appearance of Ac-farnesyl-Cys according to previously described methods (33). This analysis finds that membranes from yeast expressing AtFACE-2 can cleave the farnesylated tetrapeptide very efficiently. Control membranes isolated from non-transformed *rce1Δ ste24Δ* cells failed to show detectable activity in this assay. Further characterization of the CAAX proteolytic activity of AtFACE-2 reveals that its activity is sensitive to TPCK and *N*-ethylmaleimide and resistant to chelating compounds such as EDTA. Thus, the enzymatic characteristics of AtFACE-2 are similar to those reported for FACE-2-type pro-



**FIG. 6. *In vitro* CAAX proteolytic activity of AtFACE-2.** 25 pmol of the radioactive farnesylated peptide Ac-[ $^3H$ ]farnesyl-CVIS (*Ac-f-CVIS*) were treated with 5  $\mu$ g of membranes derived from the yeast strain SM3650, which lacks both Ste24p and Rce1p (*C-*) or *yWS163*, which expresses AtFACE-2 (*AtF2*) as the only CAAX protease. Where indicated, membranes were preincubated with 50 mM EDTA, 200 mM TPCK, or 1 mM *N*-ethylmaleimide (*NEM*). The substrate *Ac-f-CVIS* and the product *Ac-f-C* of the reaction were separated by TLC.

teases from yeast (6, 9, 35), mammals (12), and invertebrates (13). These findings, together with its sequence similarity and expression pattern, strongly suggest that AtFACE-2 is a plant ortholog of the FACE-2/Rce1 enzyme family.

#### DISCUSSION

Isoprenylation of plant proteins has been reported to be relevant in cell cycle control, signal transduction, cytoskeletal organization, and intracellular vesicle transport, thus playing a critical role in plant physiology and development (37). Accordingly, plant protein prenyltransferases have been identified and mutation of the corresponding genes have been shown to cause severe developmental abnormalities, apparently because of aberrant abscisic acid signaling (38, 39). Also, two distinct prenylcysteine  $\alpha$ -carboxyl methyltransferases from *A. thaliana* have been identified, both of which are capable of complementing the lack of this enzymatic activity in yeast (40).

Recently, Bracha *et al.* (19) have reported the isolation and characterization of the first plant CAAX protease, AtSte24, a FACE-1-like enzyme cloned from *A. thaliana*. This metalloproteinase has sequence homology to its animal and yeast counterparts is ubiquitously expressed and can restore *a*-factor production in *rce1Δ ste24Δ* double mutant yeast. Bracha and co-workers (19) have shown that AtSte24 can process *in vitro* a prenylated  $Ca^{2+}$ -calmodulin from petunia and can restore membrane association of a Rac-like GTPase expressed as a green fluorescent protein fusion in yeasts. Consequently, these authors (19) suggest that this enzyme might have evolved to acquire broader substrate specificity in plants, acting as the only CAAX protease in these organisms. However, heterologous expression of AtSte24 failed to restore heat shock sensitivity in *rce1Δ ste24Δ* yeasts expressing a mutant Ras2 allele, indicating that this enzyme was not able to support yeast Ras function *in vivo* (19).

We hypothesized that FACE-2 proteins might exist in plants. FACE-2 homologues can be easily overlooked because large regions of the polypeptide chains display a limited degree of sequence identity, making orthologs difficult to identify using standard sequence comparison programs (13). In this work, we describe the identification of a gene from *A. thaliana* encoding a protein with significant sequence similarity to FACE-2/Rce1 enzymes. This protein, which we have named AtFACE-2, presents all of the sequence characteristics of FACE-2/Rce1 enzymes, including several transmembrane domains and amino acid residues reported to be critical for enzymatic activity (35). When expressed in mutant yeast cells lacking both Rce1p and Ste24p, AtFACE-2 is able to restore *a*-factor production, demonstrating its functionality as a CAAX protease *in vivo*. Moreover, recombinant AtFACE-2 cleaves a farnesylated tetrapeptide *in vitro* and has a protease inhibitor profile similar to other

FACE-2 enzymes, indicating that the protein identified in this work is a *bona fide* prenylated protein protease.

When we performed a TBLASTN search of the plant subset of the expressed sequence tag data base using the AtFACE-2 sequence as query, we detected homologous sequences in at least twelve additional plant species including *Populus tremula* (aspen), *Solanum tuberosum* (potato), *Helianthus annuus* (sunflower), *Glycine max* (soybean), *Oryza sativa* (rice), *Hordeum vulgare* (barley), *Pinus taeda* (loblolly pine), *Sorghum bicolor* (broomcorn), *Pennisetum ciliare*, *Zinnia elegans*, *Lotus japonicus*, and *Physcomitrella patens*. The polypeptides encoded by these partial cDNAs are 50–60% identical to AtFACE-2 at the amino acid sequence level and present the structural features characteristic of proteases of the FACE-2/Rce1 family such as critical residues and a high content of hydrophobic residues. According to this finding, FACE-2-like enzymes appear to be of general occurrence in plants.

The yeast Ste24p and Rce1p enzymes are known to have partially overlapping substrate specificity (9). It is not known whether this specificity pattern extends outside of yeast. Our results indicate that this may be the case. We find that the substrate specificity of AtFACE-2 is partially overlapping with that of AtSte24 as demonstrated by their ability to process a-factor variants having different CAAX motifs. The substrate specificity of the *A. thaliana* CAAX proteases, uncovered by this experiment, is compatible with observations made in yeast (9) and other species,<sup>2</sup> which indicates that these enzymes are conserved throughout evolution not only in sequence and hydrophobic nature, but also in terms of their functional properties.

Our findings suggest that FACE-1 and FACE-2 enzymes are typically found in tandem in eukaryotic systems with plants not being an exception. The importance of needing two enzymes with partially overlapping function that are unrelated in sequence and enzymatic characteristics is unknown and will deserve further investigation. The fact that FACE-2-type enzymes are conserved in organisms that lack Ras GTPases demonstrates that Ras oncoproteins are not the only biologically relevant substrates of these proteases. This finding would be in agreement with the reports indicating that, on occasions, farnesylated substrates other than Ras proteins are the relevant targets of farnesyltransferase inhibitors tested for the treatment of human cancer (5). The reagents developed in this work could be useful to better understand the structural and functional characteristics of the proteases acting on prenylated proteins and to clarify their biological and clinical importance.

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<sup>2</sup> D. A. Mandel and W. K. Schmidt, unpublished results.