The *Arabidopsis* AtSTE24 Is a CAAAX Protease with Broad Substrate Specificity*

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A number of eukaryotic proteins that terminate with a CXXX motif sequence are subjected to a series of post-translational modifications essential to their targeting (1–5). The CXXX domain is usually referred to as a “CAAX box,” where the C is a cysteine, the two A residues are aliphatic amino acids, and the X can be one of several amino acids (6–8). The CXXX proteins undergo three sequential, enzymatic, post-translational modifications. First, the proteins are prenylated by one of two prenyltransferases called farnesyltransferase and geranylgeranyltransferase-I (6–8). Prenylation results in the covalent attachment of either farnesyl or geranylgeranyl isoprenoid lipids to the cysteine in the CAAX box motif (6–8). In yeast and animal cells, prenylation is followed by proteolytic removal of the last three amino acids of the protein (AAX) by either of two endoproteases, RCE1 and STE24 (AFC1) (9, 10). This step is prenylation-dependent and is thought to take place on the cytoplasmic surface of the endoplasmic reticulum (ER) (11). Finally, the newly exposed carboxylate group of the isoprenylcysteine is methylated by an ER-associated prenyl-dependent carboxylmethyltransferase (PCM) (10, 12–14).

The CAAAX proteases STE24 (AFC1) and RCE1 were first identified in a genetic screen in yeast for mutants defective in the production of a biologically active α-mating pheromone (9). Farnesylation of the mating pheromone α-factor is the first in a series of modifications that convert the 36-amino acid precursor into a 12-amino acid farnesylated mature α-factor (Fig. 1). Farnesylation, trimming of the AAX (VIA) moiety and carboxylmethylylation are all required for the production of mature α-factor (9, 15, 16). In *Saccharomyces cerevisiae*, STE24 (AFC1) and RCE1 act redundantly in processing the α-factor. Only RCE1, however, is responsible for processing RAS2, whereas the only known substrate of STE24 (AFC1) is the α-factor (9, 17). Fibroblasts isolated from *rce1*Δ/Δ knockout mice were unable to process Ras proteins (18), whereas fibroblasts isolated from *zmpste24*Δ/Δ knockout mice were able to process both Ras and other proteins (19). These data suggest that RCE1 may be the major functional CAAAX protease in animal cells. In mice, ZmSte24 is responsible for trimming the 15 C-terminal residues of prenylated prelamin A (20). However, it has not been established whether ZmSte24 also functions as a CAAAX protease of prelamin A.

STE24 was initially identified as the gene responsible for processing the seven N-terminal amino acid residues of the α-factor precursor (21). Surprisingly, STE24 and AFC1 were found to be the same gene. Further studies established that STE24/AFC1 has dual functions in α-factor maturation (22, 23). AFC1 was named after first being identified as a CAAX protease. However, it was recently decided that the official name of this protein in *S. cerevisiae* and humans should be STE24 and Zmpste24, respectively. Thus, throughout this paper the protein will be referred to as STE24, and the *Arabidopsis* homologue that we identified will be referred to as AtSTE24.

The prenyltransferases are soluble enzymes that are localized in the cytoplasm (7, 8). In yeast and animal cells, STE24, RCE1, and PCM are localized in ER membranes (11, 13, 24). These data suggest that CAAAX proteins are prenylated in the cytoplasm and then further processed in the endomembrane system. Furthermore, in animal cells, Ha-Ras is targeted to the plasma membrane via the secretory pathway, whereas membrane targeting of K-Ras4B takes a different route (1, 3).

Plant protein extracts were shown to contain PCM activity

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1 The abbreviations used are: ER, endoplasmic reticulum; PCM, prenyl-dependent carboxylmethyltransferase; GFP, green fluorescent protein; MES, 4-morpholinoleneethanesulfonic acid; RT, reverse transcription; wt, wild type; CFP, cyan fluorescent protein.
The a factor processing

The STE24 Zn²⁺ metalloprotease catalyzes two independent endoproteolytic events in maturation of the yeast a-mating pheromone. The yeast a-mating pheromone is synthesized as a 36-amino acid precursor and undergoes a series of processing events to become a 12-amino acid mature secreted mating factor. Three initial processing steps, which are obligatory to the formation of the secreted a-factor, occur at the C-terminal end of the protein. These steps involve farnesylation by RAM1 (farnesyltransferase β) and RAM2 (farnesyltransferase α) gene products, followed by proteolytic processing of the VIA moiety by STE24 (STE24) or RCE1 endoproteases, and methylation of the isopropenyl cysteine by PCM (the STE14 gene product). The prenylated precursor then undergoes two additional endoproteolytic events. First, STE24 cleaves the protein seven amino acids downstream of the N-terminal methionine, and then either AXL1p or STE23p cleaves 14 amino acids downstream toward the C-terminal end. The amount of biologically active mature a-factor is directly correlated with the activity of proteins involved in its processing (10).

(4, 25), and a plant gene encoding PCM (AtPCM) was cloned (4). Transiently expressed GFP-AtPCM fusion protein accumulated in a subfraction of the endomembrane system and inhibition of the methyltransferase activity resulted in accumulation of CaM53, a prenylated plant protein, in the same subcellular fraction (4). Because proteolysis of the AAX moiety is a prerequisite for methylation, it was expected that CaAXX proteases would be found in plants as well. However, the existence of plant homologues to either STE24 or RCE1 has yet to be reported. An important major unresolved issue in protein prenylation and related modifications in plants concerns the identification and characterization of the proteases or proteases that trim the AAX group.

This paper reports on the cloning and characterization of AtSTE24, an Arabidopsis homologue of the CAAX protease STE24. AtSTE24 complemented STE24 function in mutant yeast cells and processed a number of prenylated proteins, including a plant substrate. Searches for a homologue of RCE1 in plants, however, has so far been unsuccessful, suggesting that AtSTE24 may be the only CAAX protease in plants or that plants may have an additional as yet unknown CAAX protease. The subcellular localization of GFP-AtSTE24 was also determined. These results provide new insight into protein prenylation and the related CAAX processing events that follow it. 

**Experimental Procedures**

**Plasmid Construction and Cloning**—All of the plasmids used in this study are listed in Table I. AtSTE24—The protein data base was searched for a plant homologue of STE24 using the yeast and human STE24 amino acid sequences. A genomic sequence from Arabidopsis thaliana (GenBank™ accession number AF007269), encoding for a putative protein showing 50% homology to both yeast and human STE24 proteins, was identified. A 1275-bp fragment was isolated from a flower cDNA library using oligonucleotide primers P1 (5'-TAGAGGATCCAGATGGCGGCTTTCATGGAACACC-3') and P2 (5'-AACCCTGCTAAGCTTGTAAGTCTCTGCTTCCTC-3'). The amplified fragment was subcloned into pGEM (Table I) to create pSY10. pSY16 was then used to fully sequence AtSTE24 cDNA.

To express the GFP-AtSTE24 fusion protein in plants, a BanHI-XhoI fragment containing a full-length AtSTE24 cDNA sequence was cloned into pGFP-MRC (Table I) in frame with the C terminus of GFP to create pSY26. Subsequently, pSY26 was digested with SpfI to isolate a 3.5-kb fragment containing the cauliflower mosaic virus (CaMV35S) promoter, GFP-AtSTE24, and NOS 3'-end, which was subcloned into the plant binary vector, pCAMBIA2300, to create pSY27 (Table I). To express AtSTE24 in S. cerevisiae, a BamHI-SacI fragment of AtSTE24 was subcloned into the yeast plasmids, pJR1133 and pJR1131 (Table I), to create plasmids pSY24 and pSY25, respectively. 

**RacU88402**—An expression vector of the Rac-like U88402 gene (GenBank™ accession number U88402) was identified by data base searches for CAAX motif plant proteins. Clone number D290T, containing RacU88402, was obtained from the Arabidopsis Biology Resource Center. Two oligonucleotide primers were designed to place appropriate restriction sites on the 5'- and 3'-ends: P129 (5'-CGGACTGTTACATTCTTGAGAGGGCTGTAATGGT-3') and P132 (5'-CCGGTCGAGTTAGTTACCAAAATGCGGTTCG-3') of oligonucleotide primers. The resulting fragment was cloned into pGEM to create pSY106. A racU88402Ms mutant in which the prenyl acceptor cysteine residue had been changed to serine was created by PCR using P129 and p125 (5'-CCGCTGATGTCATTCTTGAGAGGGCTGTAATGGT-3'). The amplified fragment was amplified by PCR and cloned into pGEM to create pSY106. A racU88402Ms gene product. The plant binary vector, pCAMBIA2300, to create pSY27 (Table I). In frame with the C terminus of GFP to create pSY110 and pSY99, respectively. To express GFP-U88402 and GFP-u88402Ms in S. cerevisiae, pSY99 and pSY110 were digested with XhoI and XbaI to isolate GFP-U88402 and GFP-u88402Ms fragments, which were subsequently blunt-ended with Klenow DNA polymerase and subcloned into pJR1138 (Table II).

Although Rac88402 was obtained from the Arabidopsis Biology Resource Center as an expressed sequence tag clone, no genomic homologue could be identified in the Arabidopsis data base. All our attempts to amplify Rac88402, either directly from Arabidopsis cDNA libraries or by RT-PCR, proved fruitless. Rac88402 shows the highest homology to a Rac2 protein of Dictyostelium discoideum, and curators at the Arabidopsis data base have suggested that its likely origin is contamination of the expressed sequence tag library with foreign RNA. All of the clones were fully sequenced to confirm that no PCR and cloning-generated errors had been introduced.

**Generation of Yeast Strains**—All of the yeast strains used in this study are listed in Table II. Yeast transformation was carried out with a standard lithium acetate transformation protocol (26).

**Growth Arrest Pheromone Diffusion (Halo) Assays**—The assays were carried out essentially as described previously (17) with the following modifications. Three μl of MATα cell slurry (~10⁷ cells) were spotted onto a solid, rich medium (YPD) plate containing 0.1% Triton X-100 that had been spread with a lawn (~2 x 10⁶ cells) of the MATα sst2 cells (JRY3443). After 1 day of growth at 28 °C, the size of the halo was measured. 

**Plant Material**—Nicotiana benthamiana plants were grown in 10-cm pots. The seeds were sown on a mix of 70% soil with vermiculite (Avi Garden, Ltd.) and seeds were irrigated from below. The plants were grown in an environmental growth chamber under long days (16 h light/8 h dark cycles) at 27 °C. Light intensity was 100 μmol m⁻² s⁻¹.

**Leaf Injections**—Young leaves from N. benthamiana were injected with Agrobacterium tumefaciens GV3101mp90 strains harboring plasmids pSY27 and pSY99.Transient cultures were grown overnight at 28 °C in LB liquid medium with kanamycin (50 μg/ml). The next day, cultures were harvested by centrifugation and resuspended in 4 volumes of induction medium (50.78 mM MES, 0.5% glucose, 1.73 mM NaH₂PO₄, 0.2 mM acetylserine, and 5% 20X-AB mix (20X-AB mix comprised 373.9 mM NH₄Cl, 24.34 mM MgSO₄, 40.23 mM KCl, 1.36 mM CaCl₂, 0.18 mM FeSO₄·7H₂O). The cells were grown for an additional 6 h until A₆₀₀ = 0.2–0.8. The cultures were then diluted with induction medium to A₆₀₀ = 0.2 and injected into the abaxial leaf side using a 1-ml syringe (without a needle). To inject, the syringe was held against the surface of a leaf while applying counter-pressure with a finger to the opposite (adaxial) surface. The leaves were observed for GFP fluorescence at 12–24 h post-injection.

**Fluorescence Imaging**—Wide field fluorescence imaging was carried out on a Zeiss Axioplan-2 Imaging fluorescent microscope, with an AxioCam cooled CCD camera, a Zeiss filter set 40, and the appropriate Planck set filters. Confocal laser fluorescence imaging was carried out using a Zeiss R510 confocal laser scanning microscope. Excitation was carried out with an Argon laser set to 488 nm. Emission was detected with a 525 ± 15 nm band path filter. Zeiss Axiosvision, Zeiss CLSM-5, and Adobe Photoshop 6.0 were used for image analysis.
Real Time RT-PCR—Real time RT-PCR was performed in a fluorescence temperature cycler (LightCycler; Roche Molecular Biochemicals). Total RNA was isolated from various tissues using an SV total RNA isolation Kit (Promega). cDNA first strand synthesis was performed as previously described (28). cDNA corresponding to 50 ng of RNA served as a template in a 20-μl reaction containing 4 mM MgCl₂, 0.5 mM gene specific primers, and 2 μl of LightCycler-FastStart DNA Master SYBR Green-I mix (Roche Molecular Biochemicals). The samples were loaded into capillary tubes and incubated in the fluorescence thermocycler (LightCycler) for an initial denaturation at 95 °C for 10 min. The PCR reaction consisted of 45 cycles of 15 s at 95 °C, 10 s at 55 °C, and 55 s at 72 °C. The amount of PCR products was estimated by measuring SYBR Green-I fluorescence at the end of each cycle. To confirm amplification of specific transcripts, melting curve profiles were produced at the end of each run. These melting curves were produced by measuring the fluorescence of samples cooled to 65 °C for 25 s and then reheated to 95 °C at increments of 0.1 °C/s. A 1273-bp fragment of AtSTE24 was amplified with primers p1 (5′-TAGAGGATCCAGGCGATGCTTTC-3′) and p2 (5′-ACCGTCTGAGTTTAGTATATGCTTTC-3′). A 520-bp fragment of ubiquitin was amplified with primers: CGATTACTCTTGAGGTGGAG and AGACCA-TCATGGAAACC-3′) and Ref. 29.

TABLE I

| Plasmid name | Description | Source or reference |
|--------------|-------------|---------------------|
| pJR1131 | Yeast low copy CEN shuttle vector containing a URA3 marker, a glycosyl phosphatidyl inositol (GPI) anchor signal sequence, and bacterial origin of replication | Rine laboratory and Ref. 29 |
| pJR1133 | Yeast high copy 2μ shuttle vector identical to pJR1131 | Rine laboratory |
| pJR1138 | Yeast high copy 2μ shuttle vector identical to pJR1131 | Rine laboratory |
| pGFP-MRC | 35S :: GFP-NOS 3′ end, AmpR | Ref. 32 |
| pECPF-N1 | CFP vector | Clontech |
| pSY55 | 35S :: CFP-NOS 3′ end, AmpR | This study |
| pCAMBIA 2300 | Plant T-DNA-based binary vector, KanR | CAMBIA |
| pDNR-1 | Creator donor vector, AmpR | Clontech |
| pGEM | PCR product TA cloning vector, AmpR | Promega |
| pYS16 | pGEM-AstE24 | This study |
| pYS25 | pJR1133-AstE24 | This study |
| pYS26 | pGFP-MRC-AstE24 | This study |
| pYS27 | pCAMBIA 2300-GFP-AstE24 | This study |
| pYS58 | pGFP-AstE24 | This study |
| pYS59 | pCAMBIA 2300-CFP-AstE24 | This study |
| pYS77 | pDNR-1-AstE24 | This study |
| pYS78 | pDNR-1-Ast24mA-254 | This study |
| pYS82 | pJR1133-Ast24mA-254 | This study |
| pYS99 | pGFP-Rac88042mS | This study |
| pYS10 | pGFP-Rac88042 | This study |
| pYS29 | pJR1138-GFP-Rac88042 | This study |
| pYS30 | pJR1138-GFP-Rac88042 | This study |
| pYS56 | pGFP-AstBDCaM53 | Ref. 4 |
| pYS6 | pJR1138-GFP-AstBDCaM53 | This study |
| pSY106 | pGEM Rac88042 | This study |
| pSY107 | pGEM Rac88042mS | This study |
| p35S-ACA2-GFP | Plant T-DNA binary vector expressing the ER marker ACA2p | This study |

TABLE II

| Strain | Genotype | Source or reference |
|--------|----------|---------------------|
| JRY 6958 | MATα his3 leu2 met15 ura3 pep4Δ : KanMX | Rine laboratory |
| JRY 6959 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 | Rine laboratory |
| JRY 6961 | MATα rec1Δ : TRP his3 leu2 lys2 met15 ura3 pep4Δ : KanMX | Rine laboratory |
| JRY 6962 | MATα ste24Δ : HIS his3 leu2 met15 ura3 | Rine laboratory |
| JRY 3443 | MATα ste24Δ : HIS his3 leu2 met15 ura3 can1 | Rine laboratory |
| SYY 500 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6959 |
| SYY 501 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pJR1133 | Transformant of JRY 6959 |
| SYY 502 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY25 | Transformant of JRY 6959 |
| SYY 503 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pJR1131 | Transformant of JRY 6959 |
| SYY 504 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY20 | Transformant of JRY 6958 |
| SYY 505 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY20 | Transformant of JRY 6958 |
| SYY 506 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY30 | Transformant of JRY 6958 |
| SYY 507 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 508 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 509 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 510 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 511 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 512 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 513 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 514 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 515 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 516 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 517 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 518 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 519 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |
| SYY 520 | MATα ste24Δ : HIS rec1Δ : TRP his3 leu2 lys2 met15 ura3 + pSY24 | Transformant of JRY 6958 |

Yeast strains used in this study were monitored and analyzed using LightCycler Software (Roche Molecular Biochemicals). The software first normalizes each sample by detecting the background fluorescence present in the initial cycles. 5% of the full scale fluorescence threshold is then set, and the software determines the cycle number at which each sample reaches this threshold. The cycle number at which this 5% threshold is reached correlates inversely to the log of the initial template concentration. Relative levels of AtSTE24 transcript were corrected by normalization against the ubiquitin transcript levels. The specificity of the amplification products was further verified by subjecting the amplification products to electrophoresis on a 1% agarose gel.
The primary and secondary structures of AtSTE24. A, alignment of STE24 sequences from Arabidopsis (GenBank™ accession number AF353722), human (GenBank™ accession number AF064267), and S. cerevisiae (GenBank™ accession number U77137). Identical residues are denoted by black boxes. The black lines indicate sequences that formerly appeared as introns on the Arabidopsis Genome Initiative data base. The black triangle indicates the start of a 30-bp sequence that formerly appeared as an exon on the Arabidopsis Genome Initiative database. The upper box indicates an HEXXH Zn²⁺-metalloprotease signature. The lower box indicates a KXXX ER membrane retention signal. B, AtSTE24 is likely a transmembrane protein. A schematic model of AtSTE24 representing the location of its putative seven transmembrane spanning domains (black boxes). The transmembrane helixes span the following amino acid residues: helix 1, residues 1–19; helix 2, residues 68–87; helix 3, residues 111–129; helix 4, residues 157–174; helix 5, residues 180–200; helix 6, residues 294–310; and helix 7, residues 331–350.
JRY6959 (Table II) were grown in minimal medium with the appropriate supplements to $A_{opt} = 0.7$. Total protein extracts were prepared as described previously (29). Methylation assays were carried out as follows: 20 $\mu$L of SYT 515 protein extract were incubated with 20 $\mu$L of total protein extracts prepared from either SYT 506, JRY 6956, or JRY6959 in the presence of a reaction buffer containing 100 mM Hepes-KOH, pH 7.4, 5 mM MgCl$_2$, 50 $\mu$L ZnCl$_2$, 1 mM phenylmethanesulfonyl fluoride, 1 $\mu$Ci of [H]$\text{AdoMet}$ (69 Ci/mmol), and 0.5 $\mu$L AdoMet. The reactions were carried out at 30 °C for 30 min. The reactions were terminated by heat denaturation in an SDS sample buffer (30). Aliquots (15 $\mu$L) of each reaction were fractionated in turn on SDS-PAGE (30). The proteins were electrotransferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were decorated with mouse anti-GFP monoclonal antibodies (Stress-Gene) as primary antibodies, followed by blotting grade goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (Bio-Rad). The blots were developed with Super Signal Substrate kit (Pierce) and exposed to x-ray film (Kodak XAR).

Characterization of the Plant AtSTE24 CAAX Protease

**RESULTS**

**AtSTE24 Is Homologous to Yeast and Human Proteins—** Yeast and human STE24 amino acid sequences were used to search the *Arabidopsis* data base. A genomic sequence of 3097 bp from chromosome 4 (GenBank™ accession number AF007269) with a deduced 939-bp cDNA showing high homology to both yeast and human STE24 was isolated. We termed this gene *AtSTE24*. *AtSTE24* cDNA was cloned from a flower cDNA library using a PCR-based approach with oligonucleotide primers (Stratagene) to create pSY77 (pDNR-1-AtSTE24). pSY77 was used as the template together with primers p14 (5' -TGTCGAGTTTTTGCTGC-3') and p15 (5' -CCAGGATCCGTCTCTCAG-3') to mutate AtSTE24 His$^{294}$ to alanine using a QuikChange site-directed mutagenesis kit (Stratagene) to create pSY78. Atste24mA$^{294}$ was sequenced to verify that no PCR generated errors were introduced. pSY78 was digested with BamHI and XhoI to isolate a Atste24mA284 fragment, which was blunt-ended with Klenow fragment DNA polymerase and subcloned into pJR1133 to create pSY62.

**Site-directed Mutagenesis—** A BamHI-HindIII fragment containing a full-length AtSTE24 DNA sequence was subcloned into pDNR-1 in frame with the lox-p site to create pSY77 (pDNR-1-AtSTE24) (Table I). pSY77 was used as the template together with primers p14 (5' -TGTCGAGTTTTTGCTGC-3') and p15 (5' -CCAGGATCCGTCTCTCAG-3') to mutate AtSTE24 His$^{294}$ to alanine using a QuikChange site-directed mutagenesis kit (Stratagene) to create pSY78. Atste24mA$^{294}$ was sequenced to verify that no PCR generated errors were introduced. pSY78 was digested with BamHI and XhoI to isolate a Atste24mA284 fragment, which was blunt-ended with Klenow fragment DNA polymerase and subcloned into pJR1133 to create pSY62.

**Expression pattern of AtSTE24.** The expression pattern of AtSTE24 was determined by real time RT-PCR. A, relative levels of AtSTE24 RNA in leaf, stem, and flower tissues, normalized against the level of ubiquitin, showing slightly higher expression level in flowers. B, melting curves of real time PCR products. The single pick indicates that only one product was amplified in the reaction. C, final reaction products of the real time PCR after 35 cycles. Left lane, DNA ladder mix (Fermentas), H$_2$O-negative control. The reactions were carried out in duplicate.

**Fig. 3. Expression pattern of AtSTE24.** The expression pattern of AtSTE24 was determined by real time RT-PCR. A, relative levels of AtSTE24 RNA in leaf, stem, and flower tissues, normalized against the level of ubiquitin, showing slightly higher expression level in flowers. B, melting curves of real time PCR products. The single pick indicates that only one product was amplified in the reaction. C, final reaction products of the real time PCR after 35 cycles. Left lane, DNA ladder mix (Fermentas), H$_2$O-negative control. The reactions were carried out in duplicate.
AtSTE24 and ACA2p localize to the same subcellular fraction, CFP-AtSTE24 and GFP-ACA2p were coexpressed in the same cells. The colocalization data (Fig. 4, C–E) show that the two fusion proteins localized to the same fraction. The localization of both fusion proteins differs markedly from the localization of nonfused GFP, which can be detected dispersed throughout the cytosol and nucleus (Fig. 4F). This distribution of free GFP is typical of plant cells. Because of its size, GFP diffuses through the nuclear pores and enters the nucleus unspecifically. In addition, plant cells have large vacuoles that encompass most of the cell volume. Because of the vacuoles, in fluorescence and confocal microscope imaging, the cytoplasm often appears as a diffuse line with invaginations circumventing cells and as strands crossing the cell. These combined data suggest that, like its yeast homologue, AtSTE24 is localized in the ER (11).

**AtSTE24 Is a Functional Homologue of Yeast STE24**—To determine whether AtSTE24 is a functional homologue of the yeast STE24 protease, it was expressed in mutant *ste24Δ rce1Δ* S. cerevisiae cells. Complementation was tested by evaluating the relative levels of biologically active α-factor produced by MATa strains in pheromone diffusion halo assays (“Experimental Procedures”). α-Factor produces a growth inhibition zone known as a halo when applied to a mat of *sst2 MATα* cells, because of the growth arrest of the latter at the G1 phase of the cell cycle. The size of the halo corresponds to the concentration of α-factor and hence to the amount of active pheromone produced by the MATα cells (17). For it to be exported and functional, the 36-amino acid α-factor precursor undergoes farnesyl proteolytic trimming of the AAX(VIA) moiety by either STE24 or RCE1 carboxymethylation and two N-terminal proteolytic cleavage events, the first of which is catalyzed by STE24 (Fig. 1). Thus, the amount of active α-factor and the corresponding halo size depend directly on the STE24 activity. The halos formed around the *ste24Δ rce1Δ* mutations that expressed AtSTE24 from a 2μ high copy number plasmid (Fig. 5B) were somewhat smaller than those formed around the wild type cells (Fig. 5A). The halos were even smaller when AtSTE24 was expressed from a low copy CEN plasmid (Fig. 5C). No halos were formed around untransformed *ste24Δ rce1Δ* cells (Fig. 5D), whereas cells transformed with vectors alone (Fig. 5, E and F), and following plasmid loss (Fig. 5G). These results show that AtSTE24 is a functional homologue of the yeast STE24.

AtSTE24 contains a conserved HEXXH Zn2+ metalloprotease signature. To determine whether this motif is required for enzymatic activity, we mutated His284 to alanine to obtain a mutated protein with an AEXXXI motif. The mutant Atste24mA284 was expressed in *rce1Δ ste24Δ* yeast cells to examine complementa-
A mutation in a conserved histidine abolishes AtSTE24 activity. His284 is the first residue of the HEXXH conserved Zn$^{2+}$ metalloprotease signature motif (Fig. 2). To determine whether this conserved histidine is required for activity, mutant Atste24mA284, in which His284 had been converted into alanine, was expressed in rce1Δ ste24Δ mutant cells. Growth inhibition halos can be seen around either wild type cells (A) or mutant ste24Δ rce1Δ cells that overexpressed AtSTE24 (B). No halos can be seen around ste24 rce1 cells that overexpressed the mutant Atste24mA284 (C) or ste24Δ rce1Δ untransformed cells (D).

**Fig. 6.** A mutation in a conserved histidine abolishes AtSTE24 activity. His284 is the first residue of the HEXXH conserved Zn$^{2+}$ metalloprotease signature motif (Fig. 2). To determine whether this conserved histidine is required for activity, mutant Atste24mA284, in which His284 had been converted into alanine, was expressed in rce1Δ ste24Δ mutant cells. Growth inhibition halos can be seen around either wild type cells (A) or mutant ste24Δ rce1Δ cells that overexpressed AtSTE24 (B). No halos can be seen around ste24 rce1 cells that overexpressed the mutant Atste24mA284 (C) or ste24Δ rce1Δ untransformed cells (D).

**Fig. 7.** Coupled proteolysis carboxylmethylation of the prenylated plant calmodulin CaM53. GFP-CaM53 was expressed in rce1 ste24 cells and incubated in turn with \({}^3\text{H}\)AdoMet and protein extracts from either wild type, rce1 ste24 mutants that overexpressed AtSTE24 or rce1 ste24 cells. The proteins were fractionated on SDS-PAGE, protein bands corresponding to GFP-CaM53 were excised, and the radioactivity was counted. GFP-CaM53-independent counts (10–30 cpm) were subtracted, and methylation driven by wild type protein extract was taken as 100%. All of the reactions were carried out in triplicate.

Yeasts and human STE24 proteases do not process Ras proteins in vivo (9, 18). However, mutated α-factor harboring the Ras2 CHIS CAAX box can be processed by yeast STE24 (17). These data suggested that a conserved mechanism prevents Ras proteins from being processed by STE24. Moreover, attempts to identify RCE1 homologues in plants have so far been unsuccessful, implying that AtSTE24 may be the only prenyl-CAAX protease in plants. It was therefore crucial to determine whether AtSTE24 could process Ras proteins. To do so, we tested the ability of AtSTE24 to support yeast Ras function in vivo. Activated alleles of Ras induce a heat shock sensitivity phenotype in yeast that is dependent upon post-translational processing of the Ras C terminus. AtSTE24 failed to confer heat shock sensitivity when expressed in ste24Δ rce1Δ cells that express an activated allele of Ras2 (Val19), indicating that AtSTE24 cannot substitute functionally for Rce1p, the yeast Ras CAAX protease (data not shown).

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**Fig. 8.** AtSTE24 can process a Rac-like protein with a CLLM CAAX box. Processing of the Rac GTPase U88402 was tested by determining subcellular localization of GFP-RAC88402 variants in STE24 RCE1 wild type and mutant S. cerevisiae. A wild type prenylated GFP-RAC88402 fusion protein was expressed from a high copy number plasmid pSY30 in RCE1 STE24 wild type (A), ste24Δ rce1Δ mutant cells (B), SY500 (ste24Δ rce1Δ transformed with AtSTE24) (C), rce1Δ (D), and ste24Δ (E). F. STE24 RCE1 wild type cells expressing a non-prenylated GFP-U88402mS. The cells were observed using a confocal laser scanning microscope. Bars, 5 μm. All of the plasmids and yeast strains are listed in Tables I and II, respectively.

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**AtSTE24 Can Process Different Prenylated Proteins—**Can AtSTE24 process different prenylated proteins, or is it similar to its yeast and mammalian homologues that only process α-factor? To answer this question, we examined whether AtSTE24 could process two prenylated proteins (Figs. 7 and 8). One protein is a prenylated Ca$^{2+}$ calmodulin from petunia called CaM53 (28, 32). The other protein is a Rac-like GTPase called RACU88402 (GenBankTM accession number U88402).

The processing of CaM53 by AtSTE24 was examined by coupled methylation proteolysis assays. In these assays, prenylated unprocessed CaM53 was incubated with protein extracts containing both AtSTE24 and STE14, together with \({}^3\text{H}\)AdoMet as a methyl group donor. Carboxymethylation of prenylated CaM53 could only occur after processing by a CAAX protease. Indeed, CaM53 methylation was detected following incubation with yeast protein extracts containing either RCE1 or AtSTE24 (Fig. 7). Counts obtained with protein extracts prepared from mutant rce1 ste24 cells were 3–15% (30–150 cpm) of the counts obtained with extracts prepared from wt RCE1 STE24 cells (Fig. 7). The reactions with extracts prepared from ste14Δ strain that lacks carboxymethyltransferase (CMT) gave similar counts to reactions with extracts prepared

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2 T. Lubotsky and S. Yalovsky, unpublished data.
from rce1 ste24 cells (data not shown). Taken together, these results show that AtSTE24 could process CaM53.

To further examine the substrate specificity of AtSTE24, a Rac-like GTPase termed U88402, which terminates with a CLLM CAAX box, was expressed in yeast fused to GFP (Fig. 8). In wild type STE24 RCE1 cells (SY508), GFP-U88402 fluorescence was detected in the endomembranes and plasma membranes (Fig. 8A). A GFP-U88402 expressed in ste24Δ rce1Δ double mutant cells (SY 509) was localized almost exclusively in the endomembranes (Fig. 8B). Expression of AtSTE24 in the double mutant cells (SY 510) promoted plasma membrane association of the fusion protein (Fig. 8C) similar to its distribution in STE24 RCE1 wild type cells. GFP-U88402 was localized mainly in the endomembranes when expressed in SY518, an rce1Δ strain containing the yeast STE24 (Fig. 8D). As with wt cells, the fusion protein was detected both in endomembrane and in plasma membrane when expressed in SY519 ste24Δ cells that contain RCE1 (Fig. 8E). A nonprenylatable GFP-u88402mS mutant, in which the prenyl acceptor cysteine had been changed into serine, was dispersed throughout the cytoplasm (Fig. 8F).

Collectively, these results suggest that the yeast STE24 and AtSTE24 differ in their substrate specificities. Whereas STE24 can only process α-factor, AtSTE24 is able to process different prenylated proteins both in vitro and in vivo (Figs. 7 and 8).

DISCUSSION

The Arabidopsis AtSTE24 is a structural and functional homologue of the yeast STE24 CAAX Zn2+ metalloprotease (Figs. 2, 5, and 6). Compared with STE24 from S. cerevisiae, AtSTE24 could process α-mating factor (Fig. 5, A–C). Similar to the yeast enzyme, AtSTE24 was unable to process Ras2p (data not shown). S. cerevisiae STE24, however, could process mutated α-factor harboring the Ras2 C1H3 CAAX box (17). These findings suggest that the inability of STE24 to process Ras2p may not result from the poor affinity of STE24 to the CAAX prenyl-CAAX.

In both yeast and animal cells, CAAX-processing of Ras proteins is catalyzed solely by RCE1 (10). A structural homologue of RCE1 from Arabidopsis or any other plant species has yet to be identified (33). Moreover, analyses of several plant proteins have so far failed to identify functional homology to RCE1.3 Homologues of Ras proteins have not been identified so far in plants, despite the vast amount of sequence data that has become available and the intense efforts by several laboratories to identify Ras homologues.3 It is therefore tempting to speculate that a homologue of RCE1, the Ras protein C1H3 protease, may not exist in plants. Further studies will be required to discover whether AtSTE24 is the only CAAX protease in plants or whether plants have other unrelated CAAX proteases. In this respect, it is important to note that no primary, structural homology exists between STE24 and RCE1 and therefore the existence of other classes of CAAX proteases should not be ruled out.

AtSTE24 and S. cerevisiae STE24 were found to have somewhat different substrate specificities (Figs. 7 and 8). AtSTE24 could process the petunia calmodulin CaM53, which harbors a C1H3 CAAX box (Fig. 7). Yeast STE24 could not process the mutated α-factor in which the a1 position was occupied by a threonine residue (17). The yeast RCE1 could process α-factor mutants with threonine at the a1 position, suggesting that in plants, AtSTE24 may have adapted to function in the absence of RCE1. For the same reasons, it would be interesting to examine the substrate specificity of the human STE24.

Our results (Fig. 4) suggest that AtSTE24 is localized in the ER. In yeast and animal cells, the CAAX proteases are localized in the ER (11, 13, 24). It has been shown that both Ha-Ras and K-Ras4B are initially targeted to the ER and that Ha-Ras is then transported to the plasma membrane through the secretory pathway, whereas K-Ras4B takes a different route (1, 3). It would be interesting to see whether prenylated proteins in plants are targeted to plasma membrane through the secretory pathway and whether any additional or alternative pathways exist.

The ubiquitous RNA expression pattern of AtSTE24 (Fig. 3) suggests that it plays a housekeeping role. In yeast, STE24 functions both in CAAX and N-terminal α-factor processing, and recent evidence shows that ste24 arp2 and ste24 arc10 are synthetic lethal mutants (34), suggesting a role for STE24 in cell polarity regulation. In mice, ZmSTE24 functions in prelamin A processing (20). Here we have shown that AtSTE24 can process different prenylated proteins and that it is likely to function as a CAAX protease in plants. However, AtSTE24 may also have other functions in plants. It should be noted, however, that homologues to either yeast α-mating factor or lamins have not been identified in plants.

In this study, we applied four different assays that utilize yeast mutants in an attempt to identify and characterize a plant CAAX protease. Using the same assays, namely, pheromone diffusion halo assays, suppression of the activated Ras-induced heat shock sensitivity, coupled methylation proteolysis assays, and subcellular localization of GFP fusion proteins, it should be possible to identify additional plant CAAX box processing proteins. The yeast system provides a means to characterize the contribution of specific proteins because some plantspecific factors that may influence protein targeting are absent.

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