Running title: CaaX processing in Arabidopsis

Corresponding author:
Shaul Yalovsky
Department of Plant Sciences
Tel Aviv University
Tel Aviv 69978
ISRAEL
Tel: 972-3-6405268
Fax: 972-3-6406933
Email: shauly@tauex.tau.ac.il
Functional analysis of Arabidopsis post-prenylation CaaX processing enzymes and their function in subcellular protein targeting

Keren Bracha-Drori†, Keren Shichrur†, Tsofnat Cohen Lubetzky² and Shaul Yalovsky*

Department of Plant Sciences, Tel Aviv University, Tel Aviv 69978, ISRAEL
Footnotes

This research was supported by Israel Science Foundation, ISF 312/07, US-Israel Binational Agricultural Research and Development BARD 4032/07 and The German Israel Foundation GIF 834/2005 grants to SY.

Current Address: \(^1\) Clontech Inc., CA, USA, Email: keren_drori@clontech.com; \(^2\) Eldan Inc., Petach Tikvah, Israel, Email: tsofnat@eldan.biz

\(^\dagger\) These authors have equally contributed to this work.

*Corresponding author: Shaul Yalovsky, shauly@tauex.tau.ac.il
Abstract

Prenylation is a posttranslational protein modification essential for developmental processes and response to abscisic acid. Following prenylation, the three C-terminal residues are proteolitically removed and in turn the free carboxyl group of the isoprenyl cysteine is methylated. The proteolysis and methylation, collectively referred to as CaaX processing, are catalyzed by Ste24 or Rce1 endoproteases and by an isoprenyl cysteine methyltrasnferase, ICMT. Arabidopsis contains single STE24 and RCE1 and two ICMT homologues. Here we show that in yeast AtRCE1 promoted a-mating factor secretion and membrane localization of a ROP GTPase. Furthermore, GFP fusion proteins of AtSTE24, AtRCE1, AtICMTA and AtICMTB are co-localized in the ER, indicating that prenylated proteins reach this compartment and that CaaX processing is likely required for subcellular targeting. AtICMTB can process yeast a-factor more efficiently than AtICMTA. Sequence and mutational analyses revealed that the higher activity AtICMTB is conferred by five residues, which are conserved between yeast Ste14p, human ICMT and AtICMTB but not in AtICMTA. Quantitative real-time RT-PCR and microarray data show that AtICMTA expression is significantly lower compared to AtICMTB. AtICMTA null mutants have a wild type phenotype indicating that its function is redundant. However, AtICMT RNAi lines had fascinated inflorescence stems, altered phylotaxis and developed multiple buds without stem elongation. The phenotype of the ICMT RNAi lines is similar to farnesyltrasnferase β-subunit mutant era1 but is more subtle. Collectively, the data suggest that AtICMTB is likely the major isoprenyl carboxy methyltransferase and that methylation modulates activity of prenylated proteins.
Introduction

Prenylation is a posttranslational protein modification essential for the function of diverse proteins. Plant mutants lacking prenyltransferases function have pleotropic phenotypes including, enlargement of the shoot apical meristem, hypersensitivity to ABA, retarded growth rate and delayed flowering (Cutler et al., 1996; Pei et al., 1998; Running et al., 1998; Bonetta et al., 2000; Yalovsky et al., 2000; Ziegelhoffer et al., 2000; Allen et al., 2002; Running et al., 2004; Johnson et al., 2005).

Prenylation involves the attachment of either the 15-carbon farnesyl or the 20-carbon geranylgeranylgeranyl isoprenoid moieties to conserved CaaX box or double cysteine C-terminal sequence motifs. Protein farnesyltransferase (PFT) attaches the farnesyl group from farnesyl diphosphate (FPP) prenyl group donor via a thioether linkage to the cysteine of a CaaX box sequence motif (C, cysteine; a, usually an aliphatic amino acid; X, can be any amino acid but commonly serine, methionine, cysteine, alanine, or glutamine). A second protein prenyltransferase, geranylgeranyltransferase type I (PGGT), uses geranylgeranyl diphosphate (GGPP) to modify CaaX box containing proteins in which X residue is usually a leucine. A third protein prenyltransferase Rab geranylgeranyltransferase (PRGGT) uses GGPP to modify C-terminal double cysteine motifs in Rab proteins (Zhang and Casey, 1996; Yalovsky et al., 1999; Galichet and Gruissem, 2003; Maurer-Stroh et al., 2003).

Prenylation is followed by the proteolytic cleavage of the last three amino acids of the protein (aaX), leaving an isoprenyl carboxylate group at the C-terminal end of the protein. The prenylated isoprenylcysteine is in turn methylated (supplemental data Fig. S1). The proteolytic cleavage and methylation of prenylated proteins are collectively referred to as CaaX processing (Young et al., 2000). The prenyl-dependent endoproteolysis is catalyzed by either of the two endoproteases, designated Rce1 and Ste24 (Afc1) (Boyartchuk et al., 1997; Young et al., 2000). The methylation of the newly exposed isoprenyl carboxylate group is catalyzed by an Isoprenyl Carboxyl Methyltransferase (Icmt) also known as Ste14 in yeast (Clarke, 1992; Romano et al., 1998; Young et al., 2000). In yeast and animal cells, the CaaX proteases and methyltransferase are localized in the endoplasmic reticulum (ER) membranes, unlike the
prenyltransferases, which are soluble enzymes (Dai et al., 1998; Romano et al., 1998; Schmidt et al., 1998). This indicates that CaaX proteins are prenylated in the cytoplasm and then processed in the endo membrane system. In animals, CaaX processing affects fundamental processes. In fibroblasts derived from Rce1 or Icmt knockout mice farnesylated Ras but not Rho proteins were miss localized from the plasma membrane to the endo membrane (Kim et al., 1999; Bergo et al., 2001; Michaelson et al., 2005). Rce1 and Icmt knockout mice die during embryogenesis (Kim et al., 1999; Bergo et al., 2001) and the mutations affect oncogenic Ras transforming ability (Bergo et al., 2002; Bergo et al., 2004). Disruption of Ste24 induces premature aging, a condition known as Hutchinson-Gilford Progeria syndrome resulting from accumulation of prenylated unprocessed prelamin A (Bergo et al., 2002; Pendas et al., 2002; Young et al., 2005).

In Arabidopsis, CaaX proteolysis is catalyzed by conserved AtSTE24 and AtRCE1/AtFACE-2 (Bracha et al., 2002; Cadinanos et al., 2003). AtSTE24 was demonstrated to localized in the ER (Bracha et al., 2002). However, the subcellular localization of AtRCE1/AtFACE-2 has not been determined nor its effect on subcellular localization of prenylated plant proteins. Plant protein extracts have been shown to contain ICMT activity (Crowell et al., 1998; Rodriguez-Concepcion et al., 2000), and two genes encoding ICMT have been cloned (Rodriguez-Concepcion et al., 2000; Crowell and Kennedy, 2001; Narasimha Chary et al., 2002). Following the identification of the second ICMT gene in Arabidopsis, the two genes were designated AtICMTA/AtSTE14A and ICMTB/AtSTE14B (Narasimha Chary et al., 2002). It was shown that AtICMTA and AtICMTB are differentially expressed and that in vitro AtICMTB had higher catalytic activity (Narasimha Chary et al., 2002). Treatment of plants with high concentration of the methyltransferase inhibiter Acetyl Farnesyl Cysteine (AFC) induced ABA hypersensitivity (Narasimha Chary et al., 2002), reminiscent of the ABA hypersensitivity of enhanced response to ABA-1 (era1) PFT β-subunit, PGGT-I β-subunit and pluripetala (plp) PFT and PGGT-I α-subunit mutants (Cutler et al., 1996; Pei et al., 1998; Running et al., 2004; Johnson et al., 2005).

CaaX processing has been suggested to further increase protein affinity to cell membranes (Hancock et al., 1991; Kato et al., 1992; Ghomashchi et al., 1995; Parish et al., 1995), protect proteins from degradation (Backlund, 1997) and facilitate functional...
interactions with other proteins (Higgins and Casey, 1994; Rosenberg et al., 1998). More recently it has been shown that CaaX processing is required for subcellular targeting of prenylated proteins. Consistent with the ER localization of CaaX processing enzymes (Dai et al., 1998; Romano et al., 1998; Schmidt et al., 1998), Ras proteins accumulate in the endo membrane system in Rce1 and Icmt mutant cells (Michaelson et al., 2005).

In plants treated with the ICMT inhibitor, AFC, targeting of the prenylated calmodulin CaM53 to the plasma membrane was compromised and it accumulated in endo membranes (Rodriguez-Concepcion et al., 2000).

The present work highlights the conservation and uniqueness of CaaX processing in plants. We show that all the CaaX processing enzymes are localized in the ER and can complement respective yeast mutants. Uniquely, Arabidopsis has two ICMT horologs, ICMTA and ICMTB. In yeast cells ICMTA is less active than ICMTB. Sequence and mutational analyses revealed the residues responsible for this activity difference. Real-time RT-PCR and whole genome expression analysis show that ICMTA expression levels are significantly lower than ICMTB. A T-DNA mutant analysis shows that ICMTA function is redundant but that ICMT RNAi plants display subtle phenotypes reminiscent of era1 PFT β-subunit and plp PFT and PGGT-I shared α-subunit mutants.

Results

*Arabidopsis AtRCE1 is functionally conserved*

RCE1 is considered as the major CaaX protease. The function of AtRCE1 was tested by complementation in yeast using pheromone diffusion halo assays (Fig. S2 and (Bracha et al., 2002)). This assay can be used to quantitatively evaluate CaaX processing efficiency in vivo since the size of the growth inhibition halos correspond to the efficiency of α-factor processing (Tam et al., 1998; Schmidt et al., 2000; Trueblood et al., 2000). AtRCE1 complemented α-factor secretion in ste24Δ rce1Δ double mutant yeast strain (JRY6959) when expressed from a 2µ high-copy number plasmid (Fig. 1). Unlike Ste24, Rce1 can only catalyze CaaX proteolysis but not the additional proteolytic step required for maturation and of α-factor and its efficient secretion (supplemental data Fig.
S2 (Boyartchuk and Rine, 1998; Tam et al., 1998)). The halos that formed around the AtRCE1 expressing \textit{ste24Δ rce1Δ} cells (SYY563) were smaller compared to the halos that formed around the SYY500 AtSTE24 expressing \textit{ste24Δ rce1Δ} cells (Fig. 1B and C), indicating that \(\alpha\)-factor secretion was indeed less efficient. The halos that formed around \textit{Ste24 Rce1} wild type cells (JRY6958) were bigger than either AtSTE24 or AtRCE1 complemented cells (Fig. 1A), indicating that \(\alpha\)-factor processing by neither plant CaaX proteases was as efficient as by yeast Ste24. These data corroborated functional analysis of AtRCE1 function in vitro (Cadinanos et al., 2003).

To determine whether AtRCE1 can process plant target proteins we created a yeast strain in which AtRCE1 and GFP-AtROP9 (GFP-AtRAC7) were co-expressed in \textit{ste24Δ rce1Δ} mutant background (SYY569) (Fig. 2). AtROP9 (AtRAC7) is a type-II ROP/RAC GTPase which is farnesylated in yeast (Lavy et al., 2002). In wild type \textit{Ste24 Rce1} background (SYY538) GFP-AtROP9 was localized in the plasma membrane and endo membrane (Fig. 2A). In contrast, in \textit{ste24Δ rce1Δ} double mutant background (SYY542), GFP-AtROP9 was only localized in the endo membrane fraction (Fig. 2B). Co-expression of AtRCE1 (SYY569) restored plasma membrane localization of GFP-ROP9, confirming that AtRCE1 processed AtROP9 (Fig. 2C). Non-prenylated GFP:Atrop9mS mutant in which the prenyl-acceptor cysteine was mutated to serine was dispersed in the cytoplasm following expression in \textit{Ste24 Rce1} wild type cells (SYY539) (Fig. 2D). These data show that prenylation promotes association of proteins with the endo-membrane system while CaaX processing is required for subcellular targeting.

\textit{Subcellular localization of CaaX processing enzymes}

We have previously demonstrated that AtSTE24 is localized in the ER (Bracha et al., 2002) and that ICMTA localized in endo membranes (Rodriguez-Concepcion et al., 2000). If in plants too prenylated proteins reach the ER where they undergo CaaX processing, AtRCE1, ICMTA and ICMTB should be also localized in the ER. To test this assumption, the subcellular localization of AtRCE1, AtICMTA and AtICMTB were determined.

Following transient expression AtICMTA, AtICMTB and AtRCE1 GFP fusion proteins had typical to ER reticulate and perinuclear subcellular distribution pattern (Fig. 3A, E...
and I and supplemental data, Fig. S3B, D, E and G). Co-localization analysis of AtICMTA, AtICMTB and AtRCE1 together with the ER marker ER-mCherry (Nelson et al., 2007) were carried out to confirm the ER localization. The results (Fig. 3B-D, F-H and J-L) show that fusion proteins of all three CaaX processing enzyme were co-localized with the marker confirming their ER localization. Co-localization of CFP-AtICMTA with YFP-AtSTE24 and of YFP-ICMTA with CFP-AtRCE1 was used to further confirm the ER co-localization of the CaaX processing enzymes in the ER (supplemental data Fig. S3A to F).

**ICMTB is likely the major carboxymethyl isoprenyl transferase in Arabidopsis**

Based on its reversibility the isoprenyl carboxy methylation step has been suggested to play a regulatory role (Young et al., 2000). Recently, a prenyl cysteine methylesterase has been identified in Arabidopsis (Deem et al., 2006) suggesting that methylation/demethylation cycles of prenylated proteins may regulate their function. This has prompted us to study the role of prenylcysteine methyltransferases and their function in Arabidopsis.

In vitro, AtICMTB/AtSTE14B (At5g08335) was shown to be more active than AtICMTA/AtSTE14A (At5g23320) (Narasimha Chary et al., 2002). Sequence comparison highlighted five amino acids that are conserved between AtICMTB and yeast Ste14p and differ in AtICMTA (Fig. 4A, red rectangles). These residues include: K\textsuperscript{154} and K\textsuperscript{155} in Ste14p and R\textsuperscript{111} and R\textsuperscript{112} in AtICMTB, D\textsuperscript{208} in Ste14p and E\textsuperscript{165} in AtICMTB, N\textsuperscript{230} and K\textsuperscript{231} in Ste14p and Q\textsuperscript{187} and R\textsuperscript{188} in AtICMTB. Interestingly, these residues are also structurally conserved in human (HsICMT) and the moss Physcomitrella patens (PpICMT) ICMT proteins (Fig. 4A). Hydropathy plot analysis showed that AtICMTB and AtICMTA have 6-8 transmembrane helixes and that their overall topology is similar to Ste14p (Fig. 4B). Interestingly, all the five residues that are conserved between AtICMTB and Ste14p, HsICMT and PpICMT and differ in AtICMTA are located on hydrophylic domains (Fig. 4B, asteriks), which were shown to be essential for Ste14p activity (Romano and Michaelis, 2001).

Pheromone diffusion halo assays were carried out to compare the activities of AtICMTA and AtICMTB in yeast and whether differences their ability to process a-
factor correspond to the structural conservation between AtICMTB and Ste14p (Fig. 5). The halos that formed around ste14Δ mutant cells that expressed AtICMTB from a 2μ high copy number plasmid (SYY564) were larger than the halos that formed around ste14Δ cells that expressed AtICMTA (SYY526) that and comparable in size to the halos that formed around wild type Ste14 cells (Fig. 5A to C). When AtICMTB was expressed from a single copy CEN plasmid (SYY565) the halo size was smaller indicating that methylation of a-factor by AtICMTB was less efficient then by yeast Ste14. An AtICMTA mutant, AticmtAmR111E112R187Q188, harboring point mutations that converted five amino acids to their corresponding residues in AtICMTB (N111/R, Y112/R, Q165/E, E187/Q and S188/R) was created. The halos that formed around ste14Δ cells that expressed AticmtAmR111E112R187Q188 (SYY579 and SYY578) were comparable to the halos that formed around AtICMTB expressing cells (Fig. 5D and F). Halo sizes were calculated relative to WT Ste14 cells, which average size, was taken as 1. The average halo size around AtICMTA complemented cells (when expressed from a 2μ plasmid) was 0.57 of WT Ste14 cells with a SE of +/- 0.09. The average halo sizes around AtICMTB and AticmtAmR111E112R187Q188 complemented cells (expressed from 2μ plasmids) were 1.05 +/- 0.07 and 0.96 +/- 0.06, respectively. These results indicated that the a-factor was processed at similar efficiencies by AtICMTB and the mutant AticmtAmR111E112R187Q188 and confirmed that the reduced activity of AtICMTA results from changes in structurally conserved residues rather than technical problems of the experimental setup. Interestingly, activity of AticmtAmE165 single mutant and the AticmtAmR111E112E165 triple mutant was similar to WT AtICMTA (data not shown), suggesting that all five residues are critical for efficient ICMT activity.

Real-time quantitative RT-PCR (Q-PCR) experiments showed that AtICMTB was expressed at significantly higher levels compared to AtICMTA (Fig. 6A). The Q-PCR data were corroborated by examining microarray expression data using Genevestigator (Zimmermann et al., 2004; Laule et al., 2006) (Fig. 6B and supplemental Fig. S4). The microarray data show that expression of ICMTA is low in most tissues and throughout development and that in all cases the expression of ICMTB is higher. The Q-PCR analyses showed that AtRCE1 is expressed at similar levels to AtICMTB (Fig. 6A). Unfortunately, AtRCE1 (At2G36305) is currently not represented on the ATH1
Affymetrix® microarray chips and thus whole genome analysis of its expression is currently unavailable. The high sensitivity of the Q-PCR enabled detection of slightly higher relative expression levels of *AtRCE1* and *AtICMTB* in flowers and leaves relative to roots and seedlings.

The activity and expression data suggested that *AtICMTA* has a minor or restricted function and that *AtICMTB* is likely the major isoprenyl methyltransferase in Arabidopsis. Mutant analyses were carried out to test this assumption.

*The function of ICMTA and ICMTB in Arabidopsis*

An Arabidopsis line (P65H9) with a T-DNA insertion three nucleotides upstream of the termination TGA codon of *AtICMTA* (supplemental data Figs. S5 and S6) was identified in screens of the University of Wisconsin Biotechnology Center T-DNA collection (www.biotech.wisc.edu). No RNA was detected in *AticmtA*−/− homozygote mutant plants by RT-PCR with *AtICMTA* gene-specific primers (supplemental data, Figs. S5, S7 and Table 1), indicating the mutant is a null. An RT-PCR reaction on the same RNA template using *AtICMTB*-specific primers (supplemental data, Figs. S5, S7 and Table 1) showed that the mutation had no effect on its expression (supplemental data, Fig. S5). Cloning and sequencing showed that the weak band that was amplified by the *AtICMTA*-specific primers was *AtICMTB*. The *AticmtA*−/− plants had no visible phenotype including increased sensitivity to ABA, indicating that *AtICMTA* function is redundant with *ICMTB*.

Unfortunately, no mutant with compromised *AtICMTB* expression has been identified. To further explore the function of isoprenyl cysteine methylation in plants we generated *ICMT* RNAi plants (*Aticmtsil*). These plants expressed two 380 bp fragments of the *ICMTA* coding region in reverse orientation. *ICMTA* and *ICMTB* show approximately 80% identity at the nucleotide level (supplemental data, Fig. S7) making it highly likely that the RNAi construct would silence both *ICMT* genes. One hundred and ten independent transgenic lines were isolated. Of which, a total of sixty three (70%) had a similar abnormal phenotype (Fig. 7). Six independent T1 lines were selected for further analysis.
Aticmtsil plants had altered phyllotaxis (Fig. 7A to C, E and F). Flowers developed from the same region of the inflorescence stem with minimal or no elongation of the internodes (Fig. 7A to C and F). Occasionally, secondary axillary flowers developed within flowers indicating partial conversion of flowers into inflorescence (Fig. 7C). Siliques developed without internode elongation (Fig. 7F). Multiple buds developed in the axil of cauline leaves (Fig. 7D). All the plants developed fasciated bifurcated stems (Fig. 7E). Interestingly, altered phyllotaxis, fasciated stems and development of axillary flowers were also observed in the era1 PFT β-subunit mutant plants (Fig. 7H), suggesting functional relatedness of the two genes. Unlike in era1 and plp plants (Running et al., 1998; Yalovsky et al., 2000; Running et al., 2004), Aticmtsil flowers did not have extra flower organs or additional rosette leaves and were not late flowering and partially male sterile.

It was difficult to observe consistent reduction in mRNA levels in the Aticmtsil plants, suggesting a posttranscriptional silencing mechanism. To prove that the phenotype of the plants was associated with the ICMT RNAi we monitored the co-segregation of the mutant phenotype and the kanamycin resistant selection marker. To this end, approximately one hundred seedlings from each of three independent transgenic lines were grown on soil without selection and their phenotypes were scored. Plants were allowed to self, their seeds were harvested, germinated on a kanamycin-containing selection medium and the ratios between resistant and sensitive seedlings were determined. In turn, plants were transferred from the selection plates to soil and their phenotypes were scored again. In non-segregating populations in which all the plants had a mutant phenotype 100% of the progeny were kanamycin resistant. The progeny of plants without a mutant phenotype were all kanamycin sensitive. The progeny of plants heterozygote to the RNAi segregated in a 3:1 ratio with respect to the selection marker. All the kanamycin resistant plants showed a mutant phenotype after transferring to soil. In contrast, rescued kanamycin-sensitive plants that were transferred to soil did not show the mutant phenotype. The segregation analysis confirmed that the abnormal phenotype of the Aticmtsil plants were associated with the AtICMT RNAi transgene.
Discussion

In this and a previous work (Bracha et al., 2002) we showed that the plant CaaX processing machinery is evolutionarily conserved. The Arabidopsis CaaX proteases AtSTE24 and AtRCE1 can complement corresponding yeast mutants promoting α-factor processing (Fig. 1 and (Bracha et al., 2002)). Proteolysis of plant prenylated proteins by either AtRCE1 or AtSTE24 was sufficient to promote their plasma membrane localization in yeast cells ((Fig. 2 and (Bracha et al., 2002)). Both AtRCE1 and AtSTE24 are expressed in most tissues (Figs. 6 and S4 and (Bracha et al., 2002)). All CaaX processing enzymes are localized in the ER (Fig. 3), like their homologues in yeast and mammalian cells. These findings strongly suggests that following prenylation proteins are targeted to the ER where they undergo CaaX processing, which in turn is likely required for their subcellular targeting. Our expression, activity and mutant analyses suggests that AtICMTB is likely the major prenyl-dependent carboxy methyltransferase in Arabidopsis and that AtICMTA function is most likely redundant (Fig. 4-7). The similarity of the Aticmt<sup>sil</sup> lines to era1 and plp suggests that isoprenyl cysteine methylation modulates the function of prenylated proteins.

Evaluation of CaaX processing activity by plant CaaX proteases in yeast

Both Ste24 and Rce1 can carry out the CaaX processing cleavage of prenylated α-factor. The processed α-factor is then methylated and in turn cleaved again by Ste24 at internal N-terminal site and then by Ste23 to yield the mature secreted α-factor (Fig. S2) (Boyartchuk and Rine, 1998; Tam et al., 1998)). The Arabidopsis AtRCE1 homologue has been previously identified and characterized biochemically (Cadinanos et al., 2003). When expressed in rce1Δ ste24Δ mutant cells, AtRCE1 was able to restore α-factor production (Fig. 1), demonstrating its functionality as a CaaX protease in vivo in yeast. The yeast Ste24 and Rce1 enzymes are known to have partially overlapping substrate specificity (Trueblood et al., 2000). It was not known whether this specificity pattern extends to other organisms. Our results indicate that this may be the case. We found that the substrate specificity of AtRCE1 is partially overlapping with that of AtSTE24 as demonstrated by their ability to process both the Arabidopsis AtROP9 (Fig. 2C and
(Bracha et al., 2002)) and the yeast a-factor. The halos that formed around the AtRCE1 complemented ste24Δ rce1Δ cells were smaller than the halos formed around cells that were complemented with AtSTE24. The differences in halo sizes indicate a-factor processing by AtRCE1 was less efficient than by AtSTE24, likely owing the dual role of AtSTE24 in a-factor processing.

The subcellular localization of the farnesylated GFP-AtROP9 in wild type, rce1Δ ste24Δ and rce1Δ ste24Δ cells complemented with AtRCE1 revealed a role for CaaX proteolysis in targeting of prenylated protein to the plasma membrane. Consistently, in fibroblasts derived from rce1 or icmt knockout mice farnesylated Ras accumulated in the endomembrane (Michaelson et al., 2005). Likewise, the geranylgeranylated petunia calmodulin CaM53 (Rodriguez-Concepcion et al., 1999; Caldelari et al., 2001) accumulated in the endomembranes following transient expression N. benthamiana leaf epidermal cells treated with the carboxymethylation inhibitor Acetyl Farnesyl Cysteine (AFC) (Rodriguez-Concepcion et al., 2000).

Subcellular targeting of prenylated proteins

Our results suggest the following targeting pathway for CaaX proteins in plant cells. Nascent CaaX proteins are synthesized in the cytosol where they encounter one of two protein prenyltransferases that modify the CaaX cysteine by thioether linkage of a C-15 farnesyl or C-20 geranylgeranyl lipid. Prenylated CaaX proteins have high affinity for the ER where they encounter a prenyl-CaaX specific protease, either AtSTE24 or AtRCE1 that removes the -aaX amino acids. The newly exposed C-terminal isoprenylcysteine then becomes a substrate for ER-localized ICMT that methylesterifies the α-carboxyl group of the isoprenylcysteine. The end product of this three step posttranslational modification process contains a hydrophobic domain at the C-terminus of an otherwise hydrophilic protein. It remains to be established whether the fully modified proteins in plant cells arrive at their final location via the secretory pathway or through a different route.
The function of ICMTA and ICMTB

AtICMTB/AtSTE14B had lower $K_M$ and a higher catalytic activity compared to AtICMTA/AtSTE14A in carboxy methylation assays in vitro. Expression analysis demonstrated that AtICMTB/AtSTE14B is more widely expressed than AtICMTA/AtSTE14. Based on these observations it has been suggested that AtICMTB/STE14B is the major prenyl-dependent carboxy methyltransferase in Arabidopsis (Narasimha Chary et al., 2002). In an extension to this work we show that AtICMTB also has higher activity in yeast. Analysis of the sequences differences between AtICMTA and AtICMTB revealed five amino acids conserved between AtICMTB, yeast Ste14p, human and moss ICMT proteins that differ in ICMTA (Fig. 4). Our results suggest that these five amino acids are responsible for the different activities of AtICMTA and AtICMTB (Fig. 5).

Based on a topology model, Ste14p contains six membrane spans, two of which form a helical hairpin. According to this model most of the Ste14p hydrophilic regions are located in the cytosol (Romano and Michaelis, 2001). Hydropathy analysis suggests that the overall topology of Ste14p is preserved among all members of the ICMT family, (Romano and Michaelis, 2001). AtICMTB and AtICMTA have similar topology and contain between 6-8 transmembrane helixes (Fig. 4B). In contrast to their differing N-terminal topologies, ICMT proteins from different organisms share similar C-terminal hydropathy profiles (Romano and Michaelis, 2001).

Sequence analysis revealed that four of the five amino acids unique to AtICMTA (N$^{111}$, Y$^{112}$, Q$^{165}$ and S$^{188}$) introduce neutral or hydrophobic residues into cytosolic spans, which may disrupt the topology or structure of AtICMTA. It is noteworthy that Q$^{165}$ is located in region close to the end of the transmembrane span and the beginning of the cytosolic part (Fig 4B). The charged amino acid E$^{187}$ in AtICMTA is represented by the neutral residues Q$^{187}$ and N$^{230}$ in AtICMTB and Ste14p, respectively. Our findings that activity of AtICMTA is reduced compared to AtICMTB and that substituting all five residues to AtICMTB-like restore activity in yeast (Fig. 5) are consistent with the hypothesis that residues in the C-terminal hydrophilic domains play a critical role in the function of ICMT (Romano and Michaelis, 2001).
Interestingly, querying of plant sequence databases revealed small families of ICMT proteins in monocot and dicot of plant species. For example, we identified four putative ICMT proteins in rice (supplemental material Fig. S8) and in grape vine (data not shown). The sequence alignment of ICMT protein from Arabidopsis, poplar, rice, Physomitrella, yeast and human shows that while AtICMTA contain a serine residue at position 188 all the ICMT protein have positively charged lysine or arginine residues at this position (Supplemental material Fig. S8). As noted above, activity of AticmtAm$^{E165}$ single mutant and the AticmtAm$^{R111R112E165}$ triple mutant was similar to WT AtICMTA (data not shown). Taken together these data suggest that a positively charged residue at position 188 is critical for catalytic activity of ICMT. In contrast the charged residues at positions 111 and 112 are less conserved (Supplemental material Fig. S8). The existence of small ICMT protein families in different plant species and identity between AtICMTA and poplar ICMT at positions 111 (N) and 165 (Q) and between AtICMTA and rice ICMT protein at position 112 (Y) may indicate that the ICMT proteins have diverged early during the evolution of higher plants.

The Q-PCR analysis showed that AtICMTB is expressed at significantly higher levels and compared to AtICMTA (Fig. 6), confirming earlier finding (Narasimha Chary et al., 2002). We further extended the expression analysis using microarray data available on the web (Zimmermann et al., 2004; Laule et al., 2006). This analysis confirmed the Q-PCR experiments and demonstrated that in all tissues, developmental stages and under any stimuli the expression of AtICMTB is higher (Fig. 6 and supplemental data Fig. S4).

The activity and expression analysis strongly suggested that AtICMTB is the major isoprenyl carboxymethyltransferase in Arabidopsis. The lack of noticeable developmental abnormalities and ABA hypersensitivity in the AtICMTA knockout mutant supports this conclusion. In contrast, Aticmt$^{all}$ plants had distinguishable developmental alterations implicating that likely function of both ICMTA and ICMTB genes has been compromised. While unique AtICMTA activities can not be ruled out at this stage it is questionable whether it has any essential function.
The phenotype of Aticmtsil plants

The fascination and bifurcation of the stem and the altered phyllotaxis of the Aticmtsil plants suggested that organization of the shoot apical meristem was compromised. A similar phenotype was observed in the era1-2 PFTB and plp PFTA/PGGTA mutants (Yalovsky et al., 2000; Running et al., 2004). Thus, prenylation and CaaX processing are required for proper function of the shoot apical meristem. Interestingly, the Aticmtsil plants were not ABA hypersensitive (data not shown).

The relatively mild phenotype of the Aticmtsil plants could have been a result of incomplete silencing of AtICMTA and AtICMTB expression. Alternatively, the mild phenotype could be due the partial function of prenylated but non-methylated proteins. Although it was difficult to observe consistent reduction in RNA levels of AtICMTA and AtICMTB in the RNAi lines, the presence of the mutant phenotype in 70% of the T1 lines and the strict co-segregation of the abnormal phenotype with the RNAi construct indicates that the expression of the ICMT genes was very likely affected. The absence of similar phenotypes in the icmtA−/− T-DNA knockout plants and the 80 percent sequence identity at the nucleotide level between AtICMTA and AtICMTB further supports the notion that the RNAi affected expression of both genes.

It is noteworthy, that the relatively subtle phenotype of the Aticmtsil plants is consistent with findings on the function of ICMT yeast and mammals. Saccharomyces cerevisiae rce1Δ ste24Δ and ste14Δ mutants cannot mate but their growth rate is normal. In contrast, ram1Δ (FTβ− subunit mutant) cells display a retarded growth rate due to malfunction of Ras1p and Ras2p and cdc43Δ (GGT-Iβ subunit) mutant cells are non-viable, primarily due to the malfunction of Cdc42p (Trueblood et al., 1993). Thus, in yeast prenylated non-methylated Ras and Cdc42 proteins are still functional but the α-factor is not processed and secreted. Rce1 and Icmt knockout mice die during embryogenesis but fibroblasts obtained from these embryos can be maintained in culture (Michaelson et al., 2005), indicating that cell division was not completely compromised. Future analysis will be required to determine whether in plants many prenylated non-methylated proteins are still functional.

The importance of α-carboxyl methylation of prenylated proteins is illustrated by the fact that this is the final and the only potentially reversible step during prenylation and
CaaX processing. Consequently, the targeting and function of prenylated plant proteins may be modulated by the methylation/demethylation status of the proteins. Consistent with this hypothesis, specific prenylcysteine methyl ester hydrolase activities have been described in mammals and more recently also in plants (Perez-Sala et al., 1991; Tan and Rando, 1992; Valentijn and Jamieson, 1998; Van Dessel et al., 2001; Deem et al., 2006).

Materials and Methods

Molecular cloning
All plasmids used in this study are listed in supplementary materials Table 2. Plasmids were generated using standard protocols. Oligonucleotide primers used in PCR reaction to generate appropriate restriction sites, site directed mutagenesis and sequencing are listed in supplementary data Table 1. All fragments and construct were sequenced to verify that no PCR-generated errors were introduced and that relevant fragments are cloned in the correct frame. *E. coli DH5α* was used for all recombinant plasmids constructions.

Plasmid production for routine DNA manipulations and sequencing was prepared with "QIAGENE mini prep purification kit" according to the manufacturer protocol.

Yeast expression vectors were generated by cloning the corresponding cDNAs into the polycloning sites of high copy (2µ) or low copy (CEN) yeast vectors. The yeast vectors contain a glyceraldehydes-3-phosphate dehydrogenase gene (GPD) promoter and phosphoglycerate kinase gene (PGK) terminator, separated by restriction sites.

Plant expression vectors were generated by cloning a SphI, HindIII or NotI cassettes containing the cauliflower mosaic virus (CaMV35S) promoter, gene of interest, and NOS 3’-end, into plant binary vectors, pCAMBIA2300 (SphI, HindIII).

Fusion protein to the C terminus. A fragment containing a full-length cDNA sequence of the gene of interest, without the ATG and with a stop codon, was cloned in frame with the C terminus of plasmids containing the GFP, CFP, YFP.
**Fusion protein to the** N** terminus.** A fragment containing a full-length cDNA sequence of the gene of interest, with the ATG and without stop codon, was cloned in frame with the N terminus of plasmids containing the GFP, CFP, YFP.

**Generation of AticmtAmR^{111}E^{165}Q^{187}R^{188} mutant.** pSY60 was used as the template together with primers SYP50 and SYP51 to mutate AtICMTA Q^{165}E to E^{165} using a “QuikChange” site directed mutagenesis kit (Stratagene) to create pSY915. AticmtAmE^{165} was sequenced to verify that no PCR generated errors were introduced. pSY915 was used as the template together with primers SYP54 and SYP55 to mutate AticmtAmE^{165} N^{111}Y^{112} to R^{111}R^{112}, respectively using a QuikChange (Stratagene) to create pSY902. pSY902 was used as the template together with primers SYP52 and SYP53 to mutate AticmtAmE^{165}Q^{187}R^{188} E^{187}S^{188} to Q^{187} and R^{188} using a QuikChange (Stratagene) to create pSY904. AticmtAmR^{111}R^{112}E^{165}Q^{187}R^{188} was sequenced to verify that no PCR generated errors were introduced.

**Construction of AtICMTA-RNAi plasmid.** A fragment from position 1 to 380 of AtICMTA was amplified with oligonucleotide primers SYP304 and SYP305. The resulting fragment was purified, digested with NcoI and XhoI and cloned into pGFP-MRC to generate pSY302. An identical fragment was amplified with oligonucleotide primers SYP306 and SYP307, purified, digested with XbaI and SacI and cloned into pSY302 to generate pSY303. Cloning was such that both fragments were flanking GFP in a reverse orientation to one another. Next, pSY303 was digested with HindIII to isolate a cassette containing the 35S promoter, reverse oriented AtICMTA fragments flanking GFP and NOS transcriptional terminator. This cassette was subcloned into pCAMBIA2300 to generate pSY304

**PCR**

PCR was used for gene amplification, gene detection and cloning. Unless mentioned, reaction conditions were as follows: 5-100ng DNA, 0.05mM dNTPs, 7.5µM of each primer, 1x reaction buffer (10mM Tris pH-8.8, 50mM KCl, 0.08% Nonidet P40), 1.5mM MgCl₂, 0.1mg/ml BSA and 0.125-0.25 µl Taq DNA polymerase or Pfu DNA polymerase (Promega) that was added in a hot-start fashion following the annealing step of the first cycle, template in a final volume of 25µl. The reaction conditions were: 2 min at 94°C followed by 30
repeats of 30 sec at 94°C; 30 sec at 55°C; 1 min per 1KB at 72°C and an additional step of 3 min at 72°C.

**Yeast work**

All yeast strains used in this study are listed in supplementary data Table 3. Yeast transformation was carried out with a modified lithium acetate transformation protocol (Ito et al., 1983; Yalovsky et al., 1997).

*Subcellular localization of GFP-AtROP/AtRAC7 in yeast.* AtROP9 and Atrop9mS were subcloned in a yeast expression vector pJR1138-GFP to obtain plasmids pSY32, and pSY31, respectively (Table 2) (Lavy et al., 2002). *Saccharomyces cerevisiae* (*S. cerevisiae*) strains JRY6959, JRY6958 and SYY500 (Table 3) were transformed with the resulting plasmids to obtain yeast strains SYY535, SYY538, SYY539 and SYY542. Subcellular localization of the GFP fusion proteins was determined using a Confocal Laser Scanning Microscope (CLSM) following overnight growth of cells at 30°C on rich media plates. To prevent cell motion and obtain sharp images cells were loaded onto slides in 0.1% low melting agarose.

*Growth arrest pheromone diffusion (halo) assays.* Assays were carried out essentially as previously described (Trueblood et al., 2000), with the following modifications. Three µl of *MATa* cell slurry (approximately 10^7 cells) were spotted onto a solid, rich medium (YPD) plate containing 0.01% Triton X-100 that had been spread with a lawn (approximately 2x10^6 cells) of the *MATα sst2α* cells (JRY3443). After 1 day of growth at 28°C, the relative amounts of a-factor produced by each *MATa* strain were evident from the size of the growth inhibition zone (halo) surrounding the *MATa* cells. The relative halo sizes were directly proportional to the amount of a-factor exported from the *MATa* cells (Ito et al., 1983). Experiments were repeated 3 times with 4-5 replicates.

**Plant material.**

*Nicotiana benthamiana* (*N. benthamiana*) and Arabidopsis (*Col-0 and Ws*) plants were grown as previously described (Bracha et al., 2002; Lavy et al., 2002).
Transient expression assays

Young leaves of *N. benthamiana* were injected on the abaxial side with *Agrobacterium tumefaciens* GV3101/pMP90 strains harboring appropriate plasmids as previously described (Bracha et al., 2002; Lavy et al., 2002). ER-localization was determined by co-expression with ER-mCherry, which is composed of the N-terminal signal peptide of AtWAK2 (Wall Associated Kinase 2) and a C-terminal ER-retention signal (His-Asp-Glu-Leu) (Nelson et al., 2007) or with GFP-AtSTE24 (Bracha et al., 2002). Leaves were observed for GFP and mCherry fluorescence at 24 to 48 hours post-injection.

Arabidopsis transformation

Stable Arabidopsis transgenic plants were obtained using the floral dip method (Clough and Bent, 1998). Transgenic plants were selected by either growth on kanamycin containing 0.5 X Murashige Skoog (MS) medium or bar spraying soil-grown plants with BASTA. *AtICMTA* plants were also analyzed by PCR to reveal transgene existence.

Plant DNA and RNA isolation and quantitative real-time RT-PCR (Q-PCR)

*DNA isolation.* Leaves were grind with a mortar and pestle and 1-2 g were used for DNA isolation using GenElut Plant Genomic DNA Miniprep Kit (Sigma, Stenheim, Germany) according to manufacturer’s protocol.

*RNA isolation.* Leaves were grind with a mortar and pestle and 100-200 mg were used for total RNA isolation using SV total RNA isolation kit (Promega, Madison, WI, USA) according to manufacturer’s protocol.

*Q-PCR.* Total RNA was isolated from leaves, flowers, seedling and root using SV total isolation RNA kit (Promega, Madison, WI, USA). For reverse transcription, 1 µg of total RNA was denatured at 70°C for 5 min in the presence of 0.5 µg of oligo(dT)15 primers. The tubes were immediately chilled on ice and reverse transcribed with 200 units of MMLV-reverse transcriptase according to the manufacturer's instructions (Promega, Madison, WI, USA) in a total volume of 25 µL at 42°C for 60 min. Quantification of *AtRCE1, AtICMTA* and *AtICMTB* RNA levels were carried out by Q-PCR using an ABI Prism 7700 StepOnePlus™ Instrument (Applied Biosystems, Weiterstadt, Germany). Study samples were run in triplicate on 8-well optical PCR strips (Applied Biosystems) in
a final volume of 10 µL. Primers were designed according to Roche Universal ProbeLibrary (https://www.roche-applied-science.com/sis/rtcpr/upl/index.jsp). The following primers pairs were used: *AtRCE1* – 500 nmolL\(^{-1}\) of each SYP1008 + SYP1009 to amplify a 106 bp fragment from positions 265-371; *AtICMTA* 125 nmolL\(^{-1}\) of each SYP1006 + SYP1007 to amplify a 59 bp fragment from positions 252–311; *AtICMTB* 500 nmolL\(^{-1}\) of each SYP1004 + SYP 1005 to amplify a 120 bp fragment form positions 179–299. The PCR cycles were run as follows: 10 min initial denaturation at 95°C, followed by 40 subsequent cycles of 15 sec denaturation at 95°C followed by 1 min primer annealing and elongation at 60°C. The specificity of the unique amplification product was determined by melting curve analysis according to the manufacturer's instructions (Applied Biosystems, Weiterstadt, Germany). Relative quantities of RNA were calculated by the standard curve method (Applied Biosystems Incorporated (2001) User Bulletin #2: ABI PRISM 7700 Sequence Detection System. http://www.appliedbiosystems.com). DNA dilution series were prepared to calculate amplification efficiency coefficient for each gene. The relative levels of *AtRCE1*, *AtICMTA* and *AtICMTB* RNA were calculated according to the amplification efficiency coefficient and normalized against *ACTIN8* gene standard, which level was taken as 1. The analysis was repeated with three independent biological replicates.

**Identification and analysis of *AtICMTA* T-DNA mutant**

DNA pulls of the University if Wisconsin Biotechnology Center T-DNA mutant collection were screened with oligonucleotide primers SYP300 and JL202 or SYP301 and JL202. Southern blot of the amplified PCR fragments with an *AtICMTA* gene-specific probe was used to verify identification of potential T-DNA insertion in *AtICMTA*. In turn, the relevant seed stock, P65H9, was ordered and the T-DNA insertion site was determined by sequencing. Expression level of *AtICMTA* and *AtICMTB* in the mutant homozygote plants was determined by RT-PCR with nucleotide primers SYP304 and SYP305, SYP306 and SYP307, respectively.
Plant Imaging and confocal laser fluorescence imaging

Images of plants were obtained with Nikon Coolpix cooled coupled device (CCD) camera or with Zeiss Axiocam CCD camera mounted on a Zeiss SV11 stereomicroscope.

Confocal imaging. Imaging were carried out on Leica TCS-SL confocal laser scanning microscope using 20X multi immersion or 63X water immersion objectives with numerical apertures (NA) of 0.7 and 1.2, respectively. GFP excitation was carried out at with argon laser at 488 nm together with a 500 nm beam splitter and spectral detector set between 510-535 nm. mCherry was excited with argon laser at 514 nm together with 458/514 dichroic beam splitter and spectral detector set between 580-630. Dual imaging of GFP and mCherry was carried out in a sequential mode to avoid channel bleeding. The reticulate ER images are of single confocal sections and the co-localization images are maximum projections stacks of multiple confocal sections. Image processing was carried out with Leica LCS, Zeiss LSM browser and Adobe photoshop.

ABA germination assays

ABA germination assays were carried out as previously described (Running et al., 2004).

Acknowledgements

We thank Dr. Nir Ohad for access to the Q-PCR machine, Dr. Tali Yahalom for technical advice and members of the Yalovsky lab for their help during this project.

Literature cited

Allen GJ, Murata Y, Chu SP, Nafisi M, Schroeder JI (2002) Hypersensitivity of abscisic acid-induced cytosolic calcium increases in the Arabidopsis farnesyltransferase mutant era1-2. Plant Cell 14: 1649-1662.

Backlund PS, Jr. (1997) Post-translational processing of RhoA. Carboxyl methylation of the carboxyl-terminal prenylcysteine increases the half-life of Rhoa. J. Biol. Chem. 272: 33175-33180.
Bergo MO, Ambroziak P, Gregory C, George A, Otto JC, Kim E, Nagase H, Casey PJ, Balmain A, Young SG (2002) Absence of the CAAX endoprotease Rce1: effects on cell growth and transformation. Mol Cell Biol 22: 171-181

Bergo MO, Gavino B, Ross J, Schmidt WK, Hong C, Kendall LV, Mohr A, Meta M, Genant H, Jiang Y, Wisner ER, Van Bruggen N, Carano RA, Michaelis S, Griffey SM, Young SG (2002) Zmpste24 deficiency in mice causes spontaneous bone fractures, muscle weakness, and a prelamin A processing defect. Proc Natl Acad Sci U S A 99: 13049-13054

Bergo MO, Gavino BJ, Hong C, Beigneux AP, McMahon M, Casey PJ, Young SG (2004) Inactivation of Icmt inhibits transformation by oncogenic K-Ras and B-Raf. J Clin Invest 113: 539-550

Bergo MO, Leung GK, Ambroziak P, Otto JC, Casey PJ, Gomes AQ, Seabra MC, Young SG (2001) Isoprenylcysteine carboxyl methyltransferase deficiency in mice. J. Biol. Chem. 276: 5841-5845

Bonetta D, Bayliss P, Sun S, Sage T, McCourt P (2000) Farnesylation is involved in meristem organization in Arabidopsis. Planta 211: 182-190

Boyartchuk VL, Ashby MN, Rine J (1997) Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. Science 275: 1796-1800

Boyartchuk VL, Rine J (1998) Roles of prenyl protein proteases in maturation of Saccharomyces cerevisiae a-factor. Genetics 150: 95-101

Bracha K, Lavy M, Yalovsky S (2002) The Arabidopsis AtSTE24 is a CaaX protease with broad substrate specificity. J. Biol. Chem. 277: 29856-29864

Cadinanos J, Varela I, Mandel DA, Schmidt WK, Diaz-Perales A, Lopez-Otin C, Freije JM (2003) AtFACE-2, a functional prenylated protein protease from Arabidopsis thaliana related to mammalian Ras-converting enzymes. J Biol Chem 278: 42091-42097

Caldelari D, Sternberg H, Rodriguez-Concepcion M, Gruissem W, Yalovsky S (2001) Efficient prenylation by a plant geranylgeranyltransferase-I requires a functional CaaL box motif and a proximal polybasic domain. Plant Physiol. 126: 1416-1429
Clarke S (1992) Protein isoprenylation and methylation at carboxyl-terminal cysteine residues. Annu. Rev. Biochem. 61: 355-386.

Clough SJ, Bent AF (1998) Floral dip: A simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16: 735-743

Crowell DN, Kennedy M (2001) Identification and functional expression in yeast of a prenylcysteine alpha-carboxyl methyltransferase gene from Arabidopsis thaliana. Plant Mol. Biol. 45: 469-476

Crowell DN, Sen SE, Randall SK (1998) Prenylcysteine alpha-carboxyl methyltransferase in suspension-cultured tobacco cells. Plant Physiol. 118: 115-123.

Cutler S, Ghassemian M, Bonetta D, Cooney S, McCourt P (1996) A protein farnesyl transferase involved in abscisic acid signal transduction in Arabidopsis. Science 273: 1239-1241.

Dai Q, Choy E, Chiu V, Romano J, Slivka SR, Steitz SA, Michaelis S, Philips MR (1998) Mammalian prenylcysteine carboxyl methyltransferase is in the endoplasmic reticulum. J. Biol. Chem. 273: 15030-15034.

Deem AK, Bulterma RL, Crowell DN (2006) Prenylcysteine methylesterase in Arabidopsis thaliana. Gene 380: 159-166

Galichet A, Gruissem W (2003) Protein farnesylation in plants--conserved mechanisms but different targets. Curr Opin Plant Biol 6: 530-535

Ghomashchi F, Zhang X, Liu L, Gelb MH (1995) Binding of prenylated and polybasic peptides to membranes: affinities and intervesicle exchange. Biochemistry 34: 11910-11918.

Hancock JF, Cadwallader K, Marshall CJ (1991) Methylation and proteolysis are essential for efficient membrane binding of prenylated p21K-ras(B). Embo J. 10: 641-646.

Higgins JB, Casey PJ (1994) In vitro processing of recombinant G protein gamma subunits. Requirements for assembly of an active beta gamma complex. J. Biol. Chem. 269: 9067-9073.

Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163-168.
Johnson CD, Chary SN, Chernoff EA, Zeng Q, Running MP, Crowell DN (2005) Protein geranylgeranyltransferase I is involved in specific aspects of abscisic acid and auxin signaling in Arabidopsis. Plant Physiol 139: 722-733

Kato K, Cox AD, Hisaka MM, Graham SM, Buss JE, Der CJ (1992) Isoprenoid addition to ras protein is the critical modification for its membrane association and transforming activity. Proc. Natl. Acad. Sci. USA 89: 6403-6407

Kim E, Ambroziak P, Otto JC, Taylor B, Ashby M, Shannon K, Casey PJ, Young SG (1999) Disruption of the mouse Rce1 gene results in defective Ras processing and mislocalization of Ras within cells. J. Biol. Chem. 274: 8383-8390.

Laule O, Hirsch-Hoffmann M, Hruz T, Gruissem W, Zimmermann P (2006) Web-based analysis of the mouse transcriptome using Genevestigator. BMC Bioinformatics 7: 311

Lavy M, Bracha-Drori K, Sternberg H, Yalovsky S (2002) Cell-specific prenylation independent mechanism regulates targeting of type-II RACs. Plant Cell 14: 2431-2450

Maurer-Stroh S, Washietl S, Eisenhaber F (2003) Protein prenyltransferases. Genome Biol 4: 212

Michaelson D, Ali W, Chiu VK, Bergo M, Silletti J, Wright L, Young SG, Philips M (2005) Postprenylation CAAX processing is required for proper localization of Ras but not Rho GTPases. Mol Biol Cell 16: 1606-1616

Narasimha Chary S, Bultema RL, Packard CE, Crowell DN (2002) Prenylcysteine alpha-carboxyl methyltransferase expression and function in Arabidopsis thaliana. Plant J 32: 735-747

Nelson BK, Cai X, Nebenfuhr A (2007) A multicolored set of in vivo organelle markers for co-localization studies in Arabidopsis and other plants. Plant J 51: 1126-1136

Parish CA, Smrcka AV, Rando RR (1995) Functional significance of beta gamma-subunit carboxymethylation for the activation of phospholipase C and phosphoinositide 3-kinase. Biochemistry 34: 7722-7727.

Pei ZM, Ghassemian M, Kwak CM, McCourt P, Schroeder JI (1998) Role of farnesyltransferase in ABA regulation of guard cell anion channels and plant water loss. Science 282: 287-290
Pendas AM, Zhou Z, Cadinanos J, Freije JM, Wang J, Hultenby K, Astudillo A, Wernerson A, Rodriguez F, Tryggyason K, Lopez-Otin C (2002) Defective prelamin A processing and muscular and adipocyte alterations in Zmpste24 metalloproteinase-deficient mice. Nat. Genet. 31: 94-99.

Perez-Sala D, Tan EW, Canada FJ, Rando RR (1991) Methylation and demethylation reactions of guanine nucleotide-binding proteins of retinal rod outer segments. Proc Natl Acad Sci U S A 88: 3043-3046

Rodriguez-Concepcion M, Toledo-Ortiz G, Yalovsky S, Caldelari D, Gruissem W (2000) Carboxyl-methylation of prenylated calmodulin CaM53 is required for efficient plasma membrane targeting of the protein. Plant J. 24: 775-784.

Rodriguez-Concepcion M, Yalovsky S, Zik M, Fromm H, Gruissem W (1999) The prenylation status of a novel plant calmodulin directs plasma membrane or nuclear localization of the protein. EMBO J. 18: 1996-2007.

Romano JD, Michaelis S (2001) Topological and mutational analysis of Saccharomyces cerevisiae Ste14p, founding member of the isoprenylcysteine carboxyl methyltransferase family. Mol Biol Cell 12: 1957-1971

Romano JD, Schmidt WK, Michaelis S (1998) The Saccharomyces cerevisiae prenylcysteine carboxyl methyltransferase Ste14p is in the endoplasmic reticulum membrane. Mol. Biol. Cell 9: 2231-2247.

Rosenberg SJ, Rane MJ, Dean WL, Corpier CL, Hoffman JL, McLeish KR (1998) Effect of gamma subunit carboxyl methylation on the interaction of G protein alpha subunits with beta gamma subunits of defined composition. Cell Signal 10: 131-136.

Running MP, Fletcher JC, Meyerowitz EM (1998) The WIGGUM gene is required for proper regulation of floral meristem size in Arabidopsis. Development 125: 2545-2553

Running MP, Lavy M, Sternberg H, Galichet A, Gruissem W, Hake S, Ori N, Yalovsky S (2004) Enlarged meristems and delayed growth in plp mutants result from lack of CaaX prenyltransferases. Proc Natl Acad Sci U S A 101: 7815-7820

Schmidt WK, Tam A, Fujimura-Kamada K, Michaelis S (1998) Endoplasmic reticulum membrane localization of Rce1p and Ste24p, yeast proteases involved
in carboxyl-terminal CAAX protein processing and amino-terminal a-factor cleavage. Proc. Natl. Acad. Sci. USA 95: 11175-11180.

Schmidt WK, Tam A, Michaelis S (2000) Reconstitution of the Ste24p-dependent N-terminal proteolytic step in yeast a-factor biogenesis. J Biol Chem 275: 6227-6233.

Sorek N, Poraty L, Sternberg H, Bar E, Lewinsohn E, Yalovsky S (2007) Activation Status-Coupled Transient S Acylation Determines Membrane Partitioning of a Plant Rho-Related GTPase. Mol Cell Biol 27: 2144-2154.

Tam A, Nouvet FJ, Fujimura-Kamada K, Slunt H, Sisodia SS, Michaelis S (1998) Dual roles for Ste24p in yeast a-factor maturation: NH2-terminal proteolysis and COOH-terminal CAAX processing. J. Cell Biol. 142: 635-649.

Tan EW, Rando RR (1992) Identification of an isoprenylated cysteine methyl ester hydrolase activity in bovine rod outer segment membranes. Biochemistry 31: 5572-5578

Trueblood CE, Boyartchuk VL, Picologlou EA, Rozema D, Poulter CD, Rine J (2000) The CaaX proteases, Afc1p and Rce1p, have overlapping but distinct substrate specificities. Mol Cell Biol 20: 4381-4392.

Trueblood CE, Ohya Y, Rine J (1993) Genetic evidence for in vivo cross-specificity of the CaaX-box protein prenyltransferases farnesyltransferase and geranylgeranyltransferase-I in Saccharomyces cerevisiae. Mol. Cell. Biol. 13: 4260-4275.

Valentijn JA, Jamieson JD (1998) Carboxyl methylation of rab3D is developmentally regulated in the rat pancreas: correlation with exocrine function. Eur J Cell Biol 76: 204-211.

Van Dessel GA, De Busser HM, Lagrou AR (2001) On the occurrence of multiple isoprenylated cysteine methyl ester hydrolase activities in bovine adrenal medulla. Biochem Biophys Res Commun 284: 50-56.

Yalovsky S, Kulukian A, Rodriguez-Concepcion M, Young CA, Gruissem W (2000) Functional requirement of plant farnesyltransferase during development in Arabidopsis. Plant Cell 12: 1267-1278.
Yalovsky S, Rodr Guez-Concepcion M, Gruissem W (1999) Lipid modifications of proteins - slipping in and out of membranes. Trends Plant Sci 4: 439-445.

Yalovsky S, Trueblood CE, Callan KL, Narita JO, Jenkins SM, Rine J, Gruissem W (1997) Plant farnesyltransferase can restore yeast Ras signaling and mating. Mol. Cell. Biol. 17: 1986-1994

Young SG, Ambroziak P, Kim E, Clarke S (2000) Postprenylation protein processing: CXXX (CaaX) endoproteases and isoprenylcyteine carboxyl methyltransferase. In F Tamanoi, DS Sigman, eds, Protein Lipidation, Ed Third Vol 21. Academic Press, San Diego, pp 156-213

Young SG, Fong LG, Michaelis S (2005) Prelamin A, Zmpste24, misshapen cell nuclei, and progeria--new evidence suggesting that protein farnesylation could be important for disease pathogenesis. J Lipid Res 46: 2531-2558

Zhang FL, Casey PJ (1996) Protein prenylation: molecular mechanisms and functional consequences. Annu. Rev. Biochem. 65: 241-269

Ziegelhoffer EC, Medrano LJ, Meyerowitz EM (2000) Cloning of the Arabidopsis WIGGUM gene identifies a role for farnesylation in meristem development. Proc. Natl. Acad. Sci. USA 97: 7633-7638

Zimmermann P, Hirsch-Hoffmann M, Hennig L, Gruissem W (2004)
GENEVESTIGATOR. Arabidopsis microarray database and analysis toolbox. Plant Physiol 136: 2621-2632

Figure Legends

Figure 1
Arabidopsis AtSTE24 and AtRCE1 facilitate yeast a-factor processing. Growth inhibition halos of a-factor hypersensitive sst2α cells formed around wild type Ste24 Rcel cells (A), ste24Δ rcel1Δ expressing AtSTE24 (B) or ste24Δ rcel1Δ cells expressing AtRCE1 (C) but not around ste24Δ rcel1Δ cells transformed with empty vector control (D).
Figure 2

AtRCE1 promote plasma membrane localization of GFP:AtROP9 in ste24Δ rce1Δ double mutant yeast cells.  
A) GFP:AtROP9 detected in both plasma and endo-membranes following expression in wild type Ste24 Rce1 cells.  B) GFP:AtROP9 detected only in the endo membranes in ste24Δ rce1Δ double mutant cells.  C) AtRCE1 restored plasma membrane localization of GFP-AtROP9 in ste24Δ rce1Δ cells.  D) non-prenylated GFP:Atrop9mS mutant protein dispersed in the cytoplasm following expression in wild type Ste24 Rce1 cells.  Bars are 5 µm.

Figure 3.

Subcellular localization of AtICMTA, AtICMTB and AtRCE1 GFP fusion proteins following transient expression in Nicotiana benthamiana leaf epidermal cells.  GFP-AtICMTA (A-D), GFP-AtICMTB (E-H) and GFP-AtRCE1 (I-L) were expressed alone (A, E and I) or co-expressed with the ER-mCherry (Nelson et al., 2007) (B-D, F-H and J-L).  GFP channel (B, F and J), mCherry channel (C, G and K) and GFP/mCherry overlay (D, H and L).  Bars are 10 µm.

Figure 4.

Sequence alignment of AtICMTA, AtICMTB, S. cerevisiae Ste14p, human and Physcomitrella patens ICMT.  A) Identical residues in all five proteins are highlighted in yellow.  Identical residues between two of the proteins are highlighted in blue.  Similar residues are highlighted in green.  Red circles denote five residues that are structurally conserved between AtICMTB and Ste14p human and moss ICMTs and differ in AtICMTA.  B) Kyte and Doolittle hydropathy plot of ICMTB with a 9-amino acids window size. Hydrophobic residues appear above the mid-line and hydrophilic residues below the mid-line. Asterisks (*) mark the positions of the AtICMTB Ste14, HsICMT, PhyPaICMT conserved and the AtICMTA unique residues.

Figure 5.

Pheromone diffusion halo assay identify amino acids responsible for reduced efficiency of α-factor processing by AtICMTA.  Growth inhibition halos produced by
a-factor secretion on a mat of sst2α cells.  A) Wild type Ste14 cells.  B-G) ste14Δ mutants cells expressing: AtICMTA (B), AtICMTB (C), AticmtAmR154R155E165Q187R188 (D), AtICMTB (E), AticmtAmR154R155E165Q187R188 (F) and vector control (G). B, C, D and G cells were transformed with 2µ high copy number plasmids.  E and F cells were transformed with CEN single copy plasmids.

Figure 6
Expression levels of AtICMTA and AtICMTB and AtRCE1.  A) Q-PCR determined, relative and normalized expression levels of AtRCE1, AtICMTA and AtICMTB.  ACTIN was use as internal control in the Q-PCR. Average was calculated using three replicates. The error bars represent SD.  B) Microarray data taken from Genevestigator (Zimmermann et al., 2004; Laule et al., 2006) show the expression levels of AtICMTA (red) and AtICMTB (blue) during development of Arabidopsis.

Figure 7.
Phenotype of ICMT RNAi plants.  A-C) Altered phylotaxis of flower arrangement on inflorescence stems.  D) Development of multiple axillary buds.  E) A fasciated inflorescence stem.  F) Adjacent siliques on a stem tip results from development of multiple buds.  G) WT Col-0 inflorescence stem.  H) era1-2 inflorescence stem. Note the altered phylotaxis (arrow heads).
Figure 1

Arabidopsis AtSTE24 and AtRCE1 facilitate yeast α factor processing. Growth inhibition halos of α factor hypersensitive sst2α cells formed around wild type Ste24 Rce1 cells (A), ste24Δ rce1Δ expressing AtSTE24 (B) or ste24Δ rce1Δ cells expressing AtRCE1 (C) but not around ste24Δ rce1Δ cells transformed with empty vector control (D).
AtRCE1 promote plasma membrane localization of GFP:AtROP9 in ste24D rce1D double mutant yeast cells.  

A) GFP:AtROP9 detected in both plasma and endo-membranes following expression in wild type Ste24 Rce1 cells.  

B) GFP:AtROP9 detected only in the endo membranes in ste24D rce1D double mutant cells.  

C) AtRCE1 restored plasma membrane localization of GFP-AtROP9 in ste24D rce1D cells.  

D) non-prenylated GFP:Atrop9mS mutant protein dispersed in the cytoplasm following expression in wild type Ste24 Rce1 cells.  

Bars are 5 mm.
Figure 4.
Sequence alignment of AtICMTA, AtICMTB and *S. cerevisiae* Ste14p human and *Physcomitrella patens* ICMT. A) Identical residues in all five proteins are highlighted in yellow. Identical residues between two of the proteins are highlighted in blue. Similar residues are highlighted in green. Red rectangles denote five residues that are structurally conserved between AtICMTB and Ste14p human and moss ICMTs and differ in AtICMTA. B) Kyte and Doolittle hydropathy plot of ICMTB with a 9-amino acid window size. Hydrophobic residues appear above the mid-line (labeled 0) and hydrophilic residues below the mid-line line. Asterisks (*) mark the positions of the AtICMTB Ste14, HsICMT, PhyPaICMT conserved and the AtICMTA unique residues.
Figure 5.
Pheromone diffusion halo assay identify amino acids responsible for reduced efficiency of a factor processing by AtICMTA. Growth inhibition halos produced by a factor secretion on a mat of sst2α cells. A) Wild type Ste14 cells. B-G) ste14Δ mutants cells expressing: AtICMTA (B), AtICMTB (C), AticmtAmR111R112E165Q187R188 (D), AtICMTB (E), AticmtAmR111R112E165Q187R188 (F) and vector control (G). B, C, D and G cells were transformed with 2m high copy number plasmids. E and F cells were transformed with CEN single copy plasmids.
Figure 6
Expression levels of AtICMTA and AtICMTB and AtRCE1. A) Q-PCR determined relative and normalized levels of AtRCE1, AtICMTA and AtICMTB. Actin was use as internal control in the Q-PCR. Average was calculated using five replicates. The error bars represent SE. B) Microarray data taken from Genevestigator (Zimmermann et al., 2004; Laule et al., 2006) show the expression levels of AtICMTA (red) and AtICMTB (blue) during development of Arabidopsis.
Figure 7.
Phenotype of ICMT RNAi plants. A-C) Altered phylotaxis of flower arrangement on inflorescence stems. D) Development of multiple axillary buds. E) A fasciated bifurcated inflorescence stem. F) Adjacent siliques on a stem tip. G) WT Col-0 inflorescence stem. H) era1-2 inflorescence stem. Note the altered phylotaxis (arrow heads).