Supplemental data

Figure S1.

prenylation and CaaX processing of proteins. I) Prenylation involves the attachment of farnesyldiphosphate (FPP) and geranylgeranyldiphosphate (GGPP) prenyl group substrate to the free thiol group of CaaX box proteins. **II**) STE24 or RCE1 cleave the aaX residues off the prenylated proteins. **III**) ICMT methylates the free carboxyl group of the isoprenyl cysteine.

Figure S2.

Processing of the a mating factor precursor and the pheromone diffusion halo assay. **A**) The **a**-factor precursor is farnesylated by PFT an in turn Ste24 (and Rce1) cleave the 3 C-terminal residues (aaX=VIA). In turn, Ste14 methylates the isoprenyl cysteine, and Ste24 and Ste23/Axl1 cleave the protein to yield the mature **a**-factor (**B**), which is then secreted out of the cell. **C**) The pheromone diffusion halo assay is based upon arrest of **α**-mating type cells by **a**-factor at the G1 stage of the cell cycle. *sst2α* are **a**-factor hypersensitive mutant cells used in the halo assays. The size of the growth inhibition halo corresponds to the amount of secreted **a**-factor. No growth inhibition halos are formed around *ste24Δ rce1Δ* cells, which do not process and secrete the **a**-factor.

Figure S3.

Co-localization of AtSTE24, AtICMTA, AtRCE1 and AtICMTB in the ER following transient expression in *N. benthamiana* leaf epidermal cells. **A**-**C**) Co-localization of YFP-AtSTE24 (**A**) and CFP-AtICMTA (**B**). **D**-**F**) Co-localization of YFP-AtICMTA (**D**) and CFP-RCE1 (**E**). **A** and **D** YFP channel. **B** and **E** CFP channel. **C** and **F** YFP/CFP overlay. **G**) GFP-AtICMTB localization in the reticulate ER.. Bars are 10 μm.

Figure S4.

Expression pattern of *AtICMTA* and *AtICMTB*. Microarray data from Genevestigator (Zimmermann et al., 2004; Laule et al., 2006) of expression levels of *AtICMTA* (red) and *AtICMTB* (blue) in different Arabidopsis tissues.

Figure S5

Identification and analysis of an *AtICMTA* T-DNA insertion mutant. A) Upper gel PCR on genomic DNA with T-DNA left border and *AtICMTA* gene-specific primer. Lower gel PCR on genomic DNA with *AtICMTA* gene specific primers. (+/+) WT plant, (-/-) T-DNA homozygote mutant plant and (+/-) heterozygote plant. **B**) RT-PCR on RNA isolated from *AticmtA*$^{-/-}$ mutant plants with *AtICMTA* or *AtICMTB* gene specific primers. Negative control – an RT-PCR reaction without RNA to verify lack of DNA contamination. The faint band in the faint band obtained with the *AtICMTA* primers is a result of low level amplification of *AtICMTB*.

Figure S6.

Nucleotide sequence of *AtICMTA* highlighting the insertion site of the T-DNA. Purple letters – the coding sequence of the AtICMTA, red and blue letters SYP300 and SYP301 primers used for screening. Underline arrow additional primer used and arrowhead the T-DNA insertion site as determined by sequencing.

Figure S7.

Nucleotide sequence alignment of *AtICMTA* and *AtICMTB*. Green and orange, Blue and red arrows correspond to the gene-specific primers used to amplify *AtICMTA* and *AtICMTB*, respectively in RT-PCR reactions.

Figure S8.

Amino acid sequence alignment of plant ICMT proteins. Shown are ICMT sequences from poplar (A9PB59_POPTR), Arabidopsis (AtICMTA, AtICMTB), rice (Os04g0602900, Q01SH5_ORYSA, OsI016666, OsJ_015369), *Physcomitrella patens* (A9SSC9_PHYPA), human (HsICMTA) and *Saccharomyces cerevisiae* (Ste14). Yellow highlights identical amino acids in all the proteins, blue highlights identical amino acids in some of the proteins and greens highlights conserved amino acids. Red rectangles denote the residues that are conserved between AtICMTB, yeast Ste14, human and moss ICMT proteins and differ in AtICMTA.

Table 1 – Oligonucleotide primers used in this study

Primer name	**Sequence** (5'-3')	**Restriction sites added**	**Used for**
SYP16	GATGCTGTCGACACAGAGATCTTCAGTGACACC	SalI	Cloning ICMTA (F)
SYP38	ACGGGATCCATGACAGAGATATTTAGTG	XbaI	Cloning ICMTB (F)
SYP39	CACGTCGACTCAGTTTACAAAAGGAAC	SalI	Cloning ICMTB (R)
SYP50	GTGTGGCGGTTTTTTGCTGAGAGAATACCGTACGAG		Point mutation ICMTA Gln^{165} to E (F)
SYP51	CTCGTACGGTATTCTCTCAGCAAAAAACCGCCACAC		Point mutation ICMTA Gln^{165} to E (R)
SYP52	CAGTATCTAGAGTATGCACAGAGAGTTGCCTCTGG		Point mutation ICMTA Glu^{187} and S^{188} to R and Q (F)
SYP53	CCAGAGGCAACTCTCTGTGCATACTCTAGATACTG		Point mutation ICMTA Glu^{187} and S^{188} to R and Q (R)
SYP54	GTTCACTCACCTCATAAAGATCCGCCGCGAAGAGCATCACGGGC		Point mutation ICMTA Asn^{111} and Tyr^{112} to R and R (F)
SYP55	GCCCGTGATGCTCTTCGCGGCGGATCTTTATGAGGTGAGTGAAC		Point mutation ICMTA Asn^{111} and Tyr^{112} to R and R (R)
SYP70	GCCGAGCTCATGGCCACCGATGGCGAG	SacI	Cloning RCE1 (F)
SYP71	GCCGAGCTCTCAATTCCACAAACAATAGC	SacI	Cloning RCE1 (R)
SYP807	GTGAGAGCTCATGACAGAGATCTTC	SacI	Cloning ICMTA with ATG (F)
SYP808	CACTGAGCTCTCAGTTCACAAATGG	SacI	Cloning ICMTA with stop (R)
SYP300	GGTTGGTGGGTTTTCTGTAAAATCTCAAT		ICMTA F', used for screening T-DNA insertion mutants
SYP301	CATATCCACGTCAAGACCTTCTCTTTCAG		ICMTA R', used for screening T-DNA insertion mutants
SYP304	ACTCTCGAGGACAGAGATCTTCAGTGACAC	XhoI	ICMTA sense F' 380 bp for RNAi
SYP305	ACTCCATGGAGTCTATACACACCATGAGTCA	NcoI	ICMTA sense R' 380 bp for RNAi
SYP306	ACTTCTAGAGACAGAGATCTTCAGTGACAC	SacI	ICMTA antisense F' 380 bp for RNAi
SYP307	ACTGAGCTCAGTCTATACACACCATGAGTCA	XbaI	ICMTA antisense R' 380 bp for RNAi
SYP321	ATATGTTCAAAAATAATTCAAGTGTC		Sequencing the T-DNA insertion site in *ICMTA*
JL202	CATTTTATAATAACGCTGCGGACATCTAC		T-DNA-specific

SYP322	AGAGATCTTCAGTGACACCAGC	ICMTA-F' specific
SYP323	GTGTTTACCTTGTGGTCTCCG	ICMTA-R' specific
SYP324	ATGACAGAGATATTTAGTGACACCGG	ICMTB-F' specific
SYP325	AGTCAAATGTTTTCCTTGTGGTCTTCC	ICMTB-R' specific
SYP1008	AGCATCTCTCGTCTTCACAGC	RCE1-F' - Q-PCR
SYP1009	CCACTCCTTGCCACAGATG	RCE1-R' – Q-PCR
SYP1006	AATGATCATCGTTGGGGAAA	ICMTA-F' – Q-PCR
SYP1007	AACGATCTTCCCGCTGTTATT	ICMTA-R' – Q-PCR
SYP1004	AAAGTCCACGCGATTCTCAC	ICMTB-F' – Q-PCR
SYP1005	TCTGTCATTGAGTCTTCACAAGATTA	ICMTB-R' – Q-PCR
SYP446	CACTTTCCAGCAGATGTGGATC	ACTIN8-F' – Q-PCR
SYP447	AATGCCTGGACCTGCTTCAT	ACTIN8 – Q-PCR

Table 2. Plasmids used in this study

Plasmid Name	Description	Source or Reference
pJR1131	Yeast low copy *CEN* shuttle vector containing a *URA3* marker, a glyceraldehydes-3-phosphate dehydrogenase gene (GPD) promoter and phosphoglycerate kinase gene (PGK) terminator, separated by restriction sites, Amp^R gene and bacterial origin of replication.	(Yalovsky et al., 1997).
pJR1133	Yeast high copy 2μ shuttle vector otherwise, identical to pJR1131.	(Yalovsky et al., 1997)
pJR1138	Yeast high copy 2μ shuttle vector containing a *LEU2* (instead of *URA3*) marker otherwise, identical to pJR113	(Yalovsky et al., 1997)
pGFP-MRC	35S::GFP-NOS 3' end, Amp^R	(Rodriguez-Concepcion et al., 1999)
pCAMBIA 2300	Plant TDNA-based binary vector, 35S promoter and NOS 3' end, Kan^R	CAMBIA
pGEM	PCR product TA cloning vector, Amp^R	Promega
pSY24	pJR1133-AtSTE24	(Bracha et al., 2002)
pSY25	pJR1131-AtSTE24	(Bracha et al., 2002)
pSY27	pCAMBIA 2300-GFP-AtSTE24	Present study
pSY28	pCAMBIA 2300-GFP-AtICMTA	Present study
pSY31	pJR1138 GFP-Atrop9/rac7mS	Present study
pSY32	pJR1138 GFP-AtROP9/RAC7	Present study
pSY39	pGFP-AtICMTA	Present study
pSY60	pJR1133-AtICMTA	Present study
pSY64	pCAMBIA2300-YFP- $Atste24mA^{284}$	Present study
pSY65	pCAMBIA2300-GFP- $Atste24mA^{284}$	Present study
pSY66	pCAMBIA2300-YFP-AtICMTA	Present study
pSY67	pCAMBIA2300-CFP-AtICMTA	Present study
pSY68	pCFP-AtICMTA	Present study
pSY69	pYFP-AtICMTA	Present study
PSY87	pPCRII-AtPCM/ICMTA	(Rodriguez-Concepcion et al., 2000)
pSY96	pJR1133-AtICMTB	Present study
pSY98	pJR1133-AtRCE1	Present study
pSY125	pGEM AtRAC7	Present study
pSY900	pJR1131-AtICMTB	Present Study
pSY901	pJR1131-$AticmtAmR^{111}R^{112}E^{165}$	Present study
pSY902	pJR1133-$AticmtAmR^{111}R^{112}E^{165}$	Present study
pSY903	pJR1131-$AticmtAmR^{154}R^{155}E^{165}Q^{187}R^{188}$	Present study
pSY904	pJR1133-$AticmtAmR^{154}R^{155}E^{165}Q^{187}R^{188}$	Present study
pSY905	pCAMBIA2300-GFP-AtICMTB	Present study
pSY906	pCAMBIA2300-CFP-AtICMTB	Present study
pSY907	pCAMBIA2300-CFP- AtRCE1	Present study
pSY908	pCAMBIA2300-GFP-AtRCE1	Present study
pSY915	pJR1133-$AticmtAmE^{165}$	Present study
pSY302	pGFP-MRC-AtICMTA-380 bp sense	Present study
pSY303	pGFP-MRC-dsAtICMTA-380 bp RNAi	Present study
pSY304	pCAMBIA2300-dsICMTA-RNAi-GFP	Present study
pmCherry-ER	mCherry-ER an ER marker plant binary vector	(Nelson et al., 2007)

Table 3. Yeast strains used in this study

Strain	Genotype	Source or Reference
JRY 6958	*MATa his3 leu2 met15 ura3 pep4Δ*::KanMX	(Trueblood et al., 2000)
JRY 6959	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3*	(Trueblood et al., 2000)
JRY 3443	*MATα sst2 trp1 his3 ura3 can1*	(Trueblood et al., 2000)
4246 *ste14Δ*	*MATa ste14Δ his3 leu2 lys2 trp1 ura3*	Research Genetics
SYY 500	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pSY24	Present study
SYY 501	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pJR1133	Present study
SYY 502	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pSY25	Present study
SYY 503	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pJR1131	Present study
SYY 525	*MATa ste14Δ his3 leu2 lys2 trp1 ura3* + pSY96	Present study
SYY 526	*MATa ste14Δ his3 leu2 lys2 trp1 ura3* + pSY60	Present study
SYY 535	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pSY 32 + pSY24	Present study
SYY 538	*MATa his3 leu2 met15 ura3 pep4Δ*::KanMX + pSY32	Present study
SYY 539	*MATa his3 leu2 met15 ura3 pep4Δ*::KanMX + pSY31	Present study
SYY 542	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pSY32	Present study
SYY 563	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pSY98	Present study
SYY 564	*MATa ste14Δ his3 leu2 lys2 trp1 ura3* + pSY96	Present study
SYY 565	*MATa ste14Δ his3 leu2 lys2 trp1 ura3* + pSY900	Present study
SYY 569	*MATa ste24Δ::HIS rce1Δ::TRP his3 leu2 lys2 met15 ura3* + pSY98 + pSY32	Present study
SYY 578	*MATa ste14Δ his3 leu2 lys2 trp1 ura3* + pSY903	Present study
SYY 579	*MATa ste14Δ his3 leu2 lys2 trp1 ura3* + pSY904	Present study

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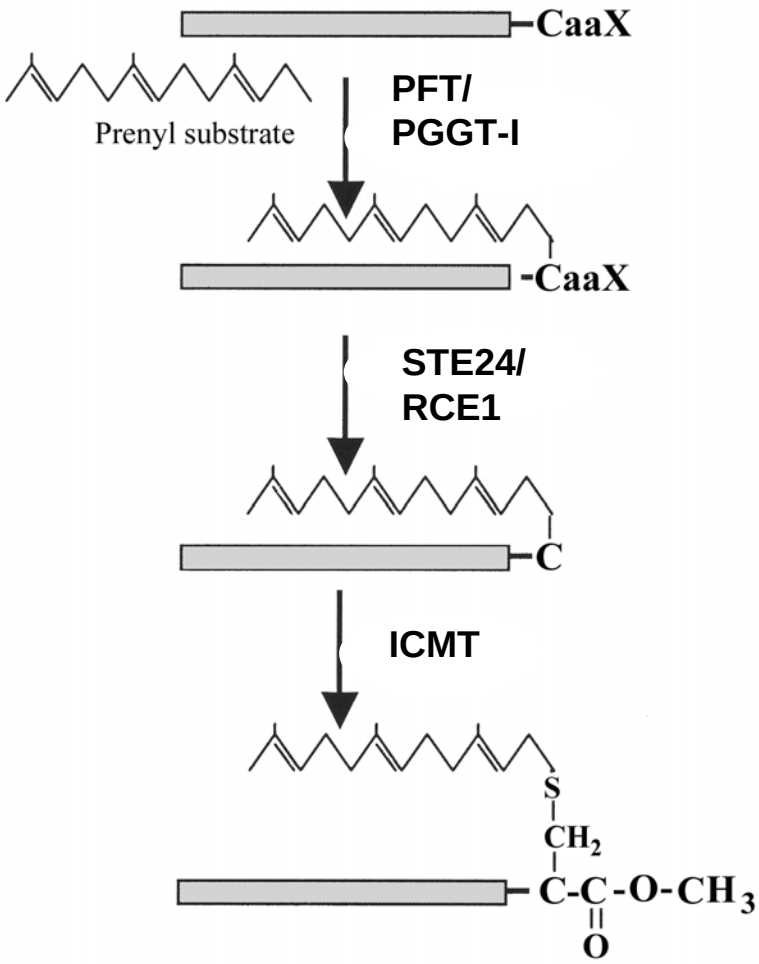


Figure S1. prenylation and CaaX processing of proteins. I) Prenylation involves the attachment of farnesyldiphosphate (FPP) and geranylgeranyldiphosphate (GGPP) prenyl group substrate to the free thiol group of CaaX box proteins. **II**) STE24 or RCE1 cleave the aaX residues off the prenylated proteins. **III**) ICMT methylates the free carboxyl group of the isoprenyl cysteine.

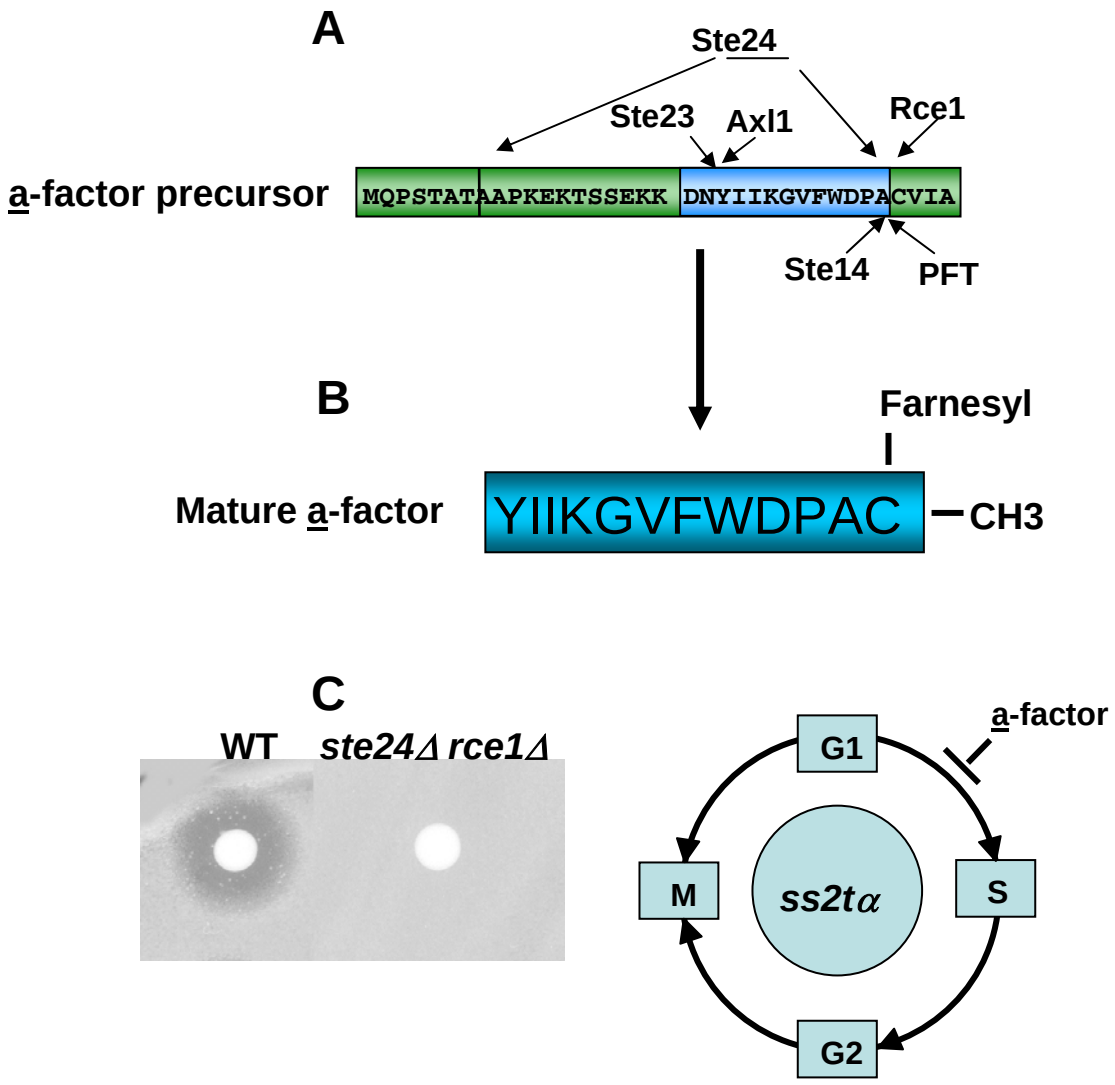


Figure S2. Processing of the a mating factor precursor and the pheromone diffusion halo assay. **A**) The **a**-factor precursor is farnesylated by PFT an in turn Ste24 (and Rce1) cleave the 3 C-terminal residues (aaX=VIA). In turn, Ste14 methylates the isoprenyl cysteine, and Ste24 and Ste23/Axl1 cleave the protein to yield the mature **a**-factor (**B**), which is then secreted out of the cell. **C**) The pheromone diffusion halo assay is based upon arrest of α-mating type cells by **a**-factor at the G1 stage of the cell cycle. *sst2α* are **a**-factor hypersensitive mutant cells used in the halo assays. The size of the growth inhibition halo corresponds to the amount of secreted **a**-factor. No growth inhibition halos are formed around *ste24Δ rce1Δ* cells, which do not process and secrete the **a**-factor.

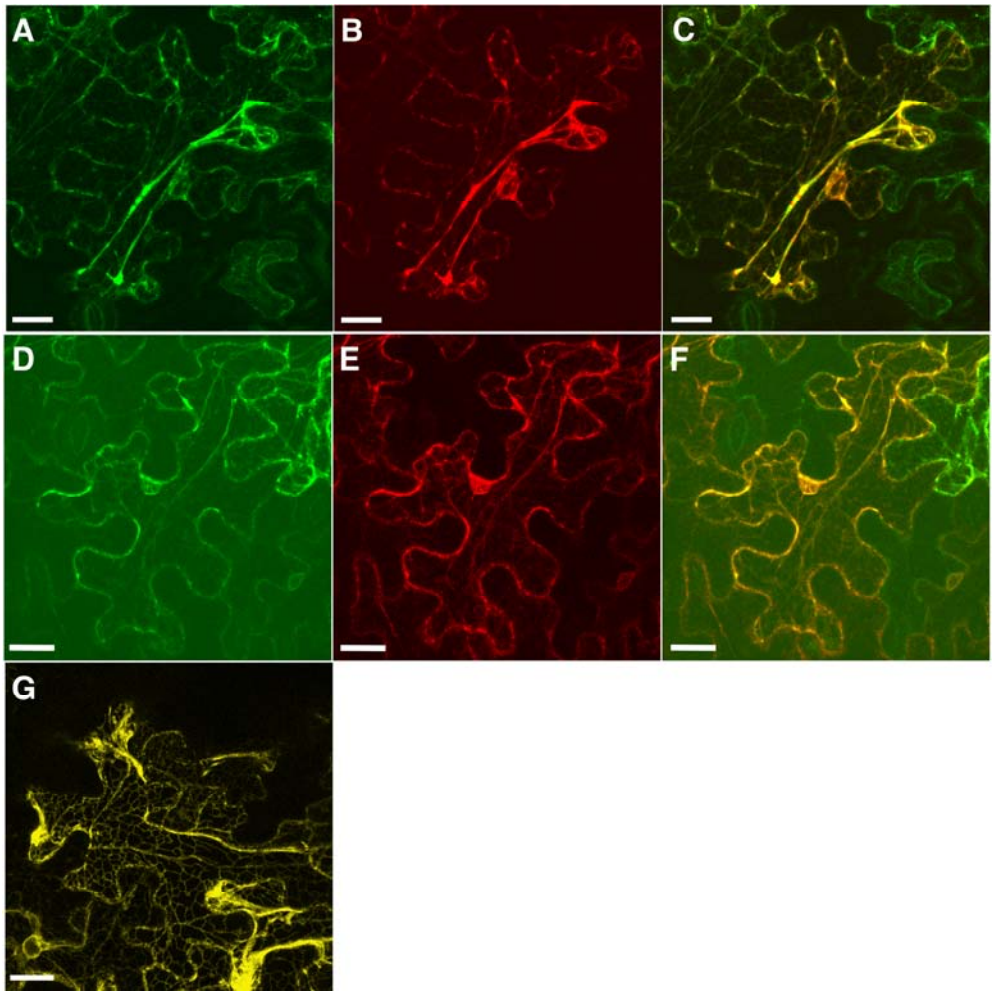
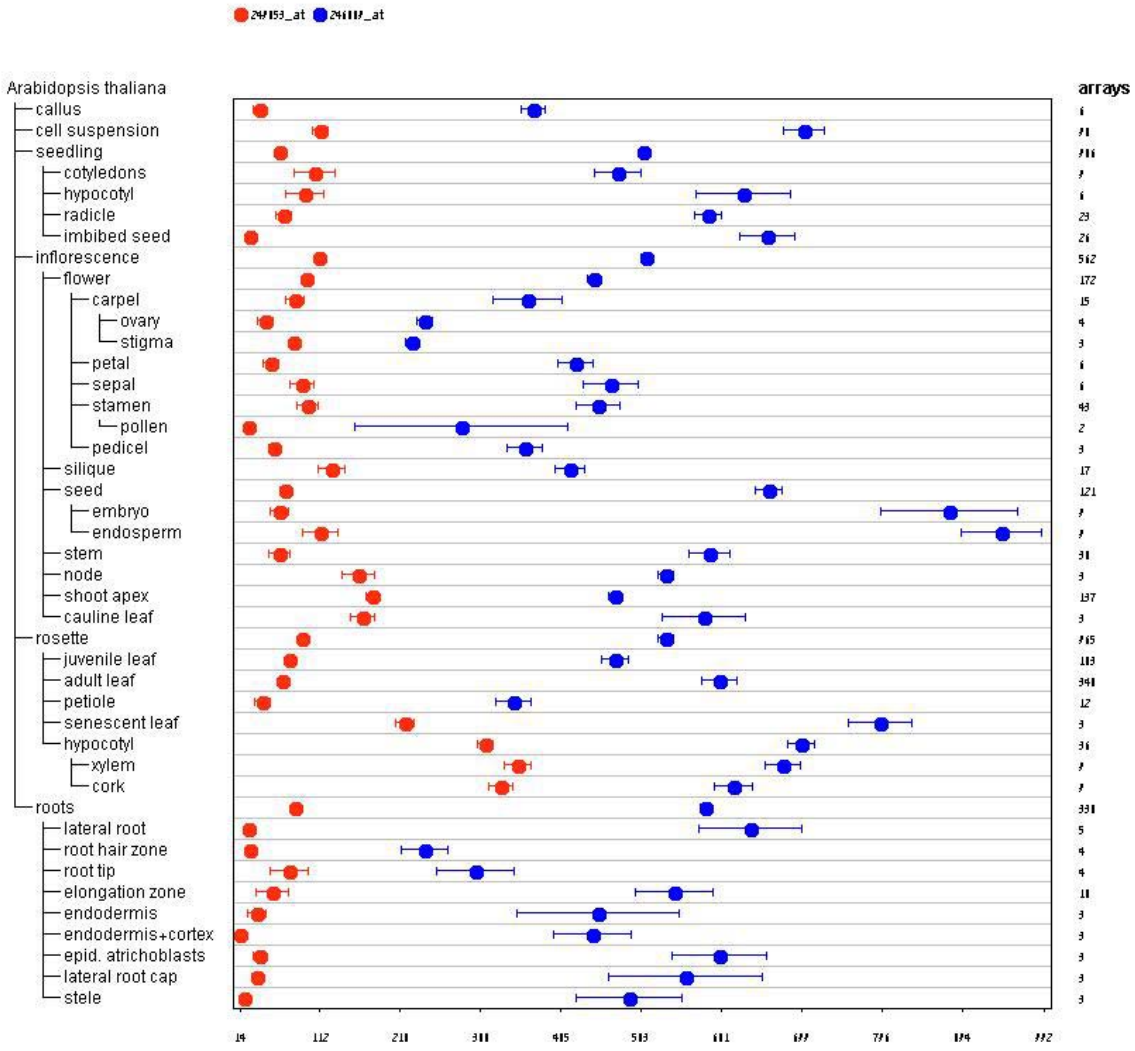


Figure S3.
Co-localization of AtSTE24, AtICMTA, AtRCE1 and AtICMTB in the ER following transient expression in *N. benthamiana* leaf epidermal cells. A-C) Co-localization of YFP-AtSTE24 (**A**) and CFP-AtICMTA (**B**). **D-F**) Co-localization of YFP-AtICMTA (**D**) and CFP-RCE1 (**E**). **A** and **D** YFP channel. **B** and **E** CFP channel. **C** and **F** YFP/CFP overlay. **G**) GFP-AtICMTB localization in the reticulate ER. Bars are 10 μm.



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Expression pattern of *AtICMTA* and *AtICMTB*. Microarray data from Genevestigator (Zimmermann et al., 2004; Laule et al., 2006) of expression levels of *AtICMTA* (red) and *AtICMTB* (blue) in different Arabidopsis tissues.

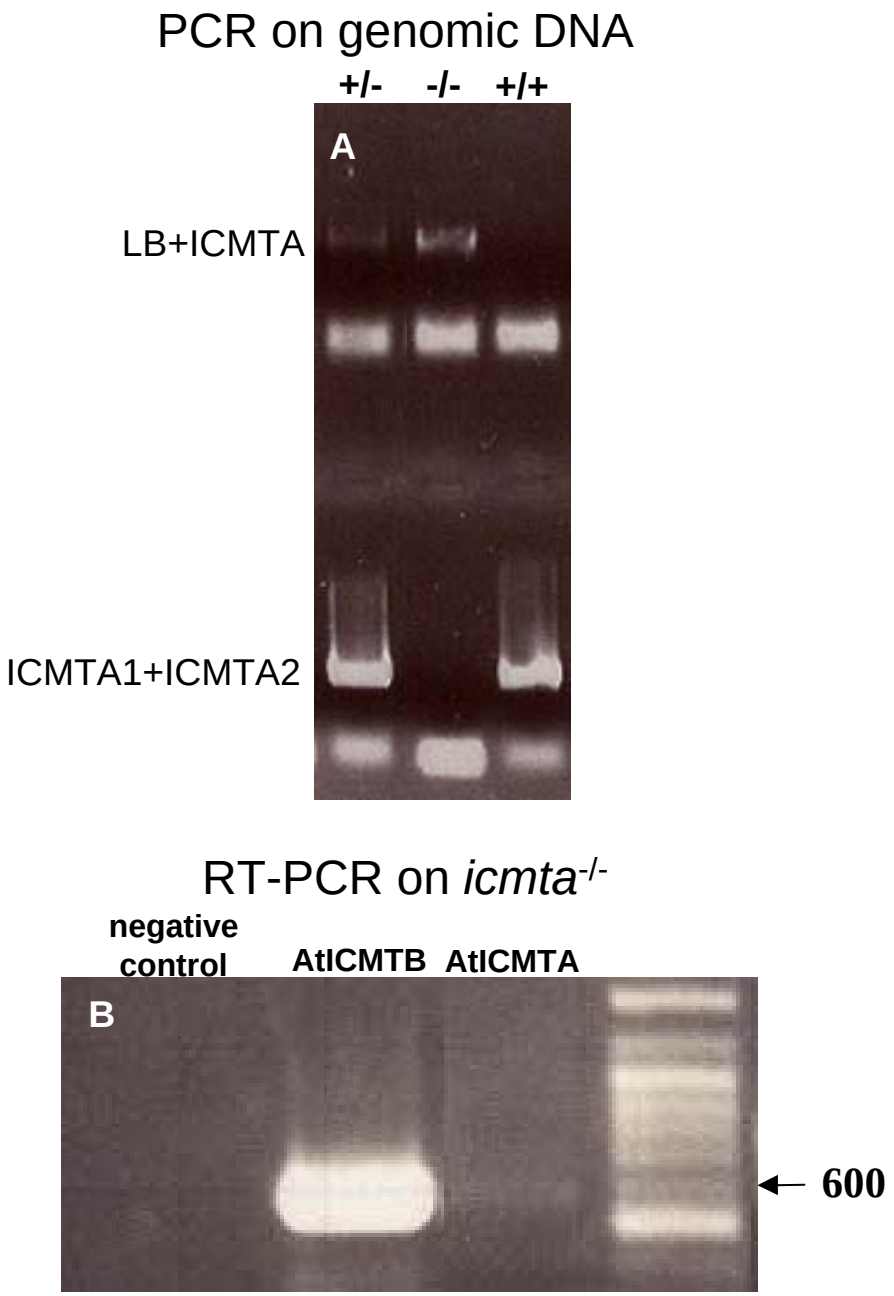

Figure S5.
Identification and analysis of an *AtICMTA* T-DNA insertion mutant. **A**) Upper gel PCR on genomic DNA with T-DNA left border and AtICMTA gene-specific primer. Lower gel PCR on genomic DNA with AtICMTA gene specific primers. (**+/+**) Wild type plant, (**-/-**) T-DNA homozygote mutant plant and (+/-) heterozygote plant. **B**) RT-PCR on RNA isolated from *AticmtA*$^{-/-}$ mutant plants with *AtICMTA* or *AtICMTB* gene specific primers. Negative control – an RT-PCR reaction without RNA to verify lack of DNA contamination. The faint band in the faint band obtained with the *AtICMTA* primers is a result of low level amplification of *AtICMTB*.

64021 aaacttgcaa tgcgattgtt gtgttg**gttg gtgggttttc tgtaaaatct caa**tgttcat

64081 gtaggacctg ggttttggca gattcagtgc cataaagact gtagtgttag tcctcatatt

64141 acatttgtat ggttgctaaa tggtcaaact gatactagta taactcagag attacaagat

64201 ttatttggat caagatcata tgtttccttg atgccaaaac taattcattg ttaagattca

64261 taaaatcgag tatatagcga gttaacgttt acttgaattt cctcgttctt tatcctttgt

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64441 taagctttga tcaaacagga aagaaaacct cttataatct ttcttgattg ataaaa**atga**

64501 cagagatctt cagtgacacc agcatcagac agttatctca aatgctacta tcactaatct

64561 tcttccacat atccgaatac attctagcca tcaccattca cggagcatca aacgtaactc

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65161 aagaccctct **ggctgaaaga gaaggtcttg acgtggatat g**aaatttacc acagcgttaa

65221 ttgtttatgt gacacctgtc accggtgatg catagctccg gcgaagagtt tcggagaaca

Figure S6.
Nucleotide sequence of AtICMTA highlighting the insertion site of the T-DNA. Purple letters – the coding sequence of the AtICMTA, red and blue letters SYP300 and SYP301 primers used for screening. Underline arrow additional primer used and arrowhead the T-DNA insertion site as determined by sequence.

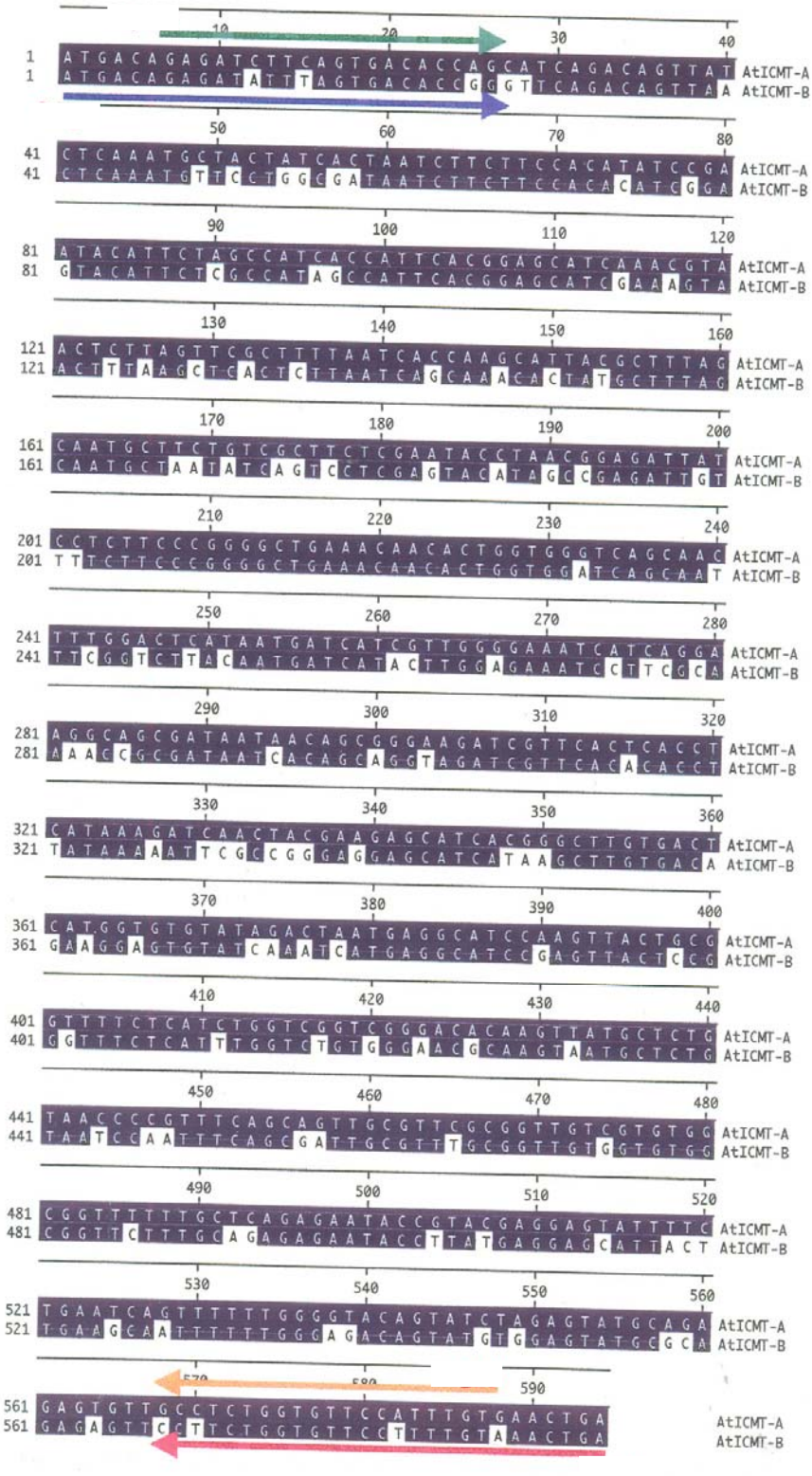

Figure S7.
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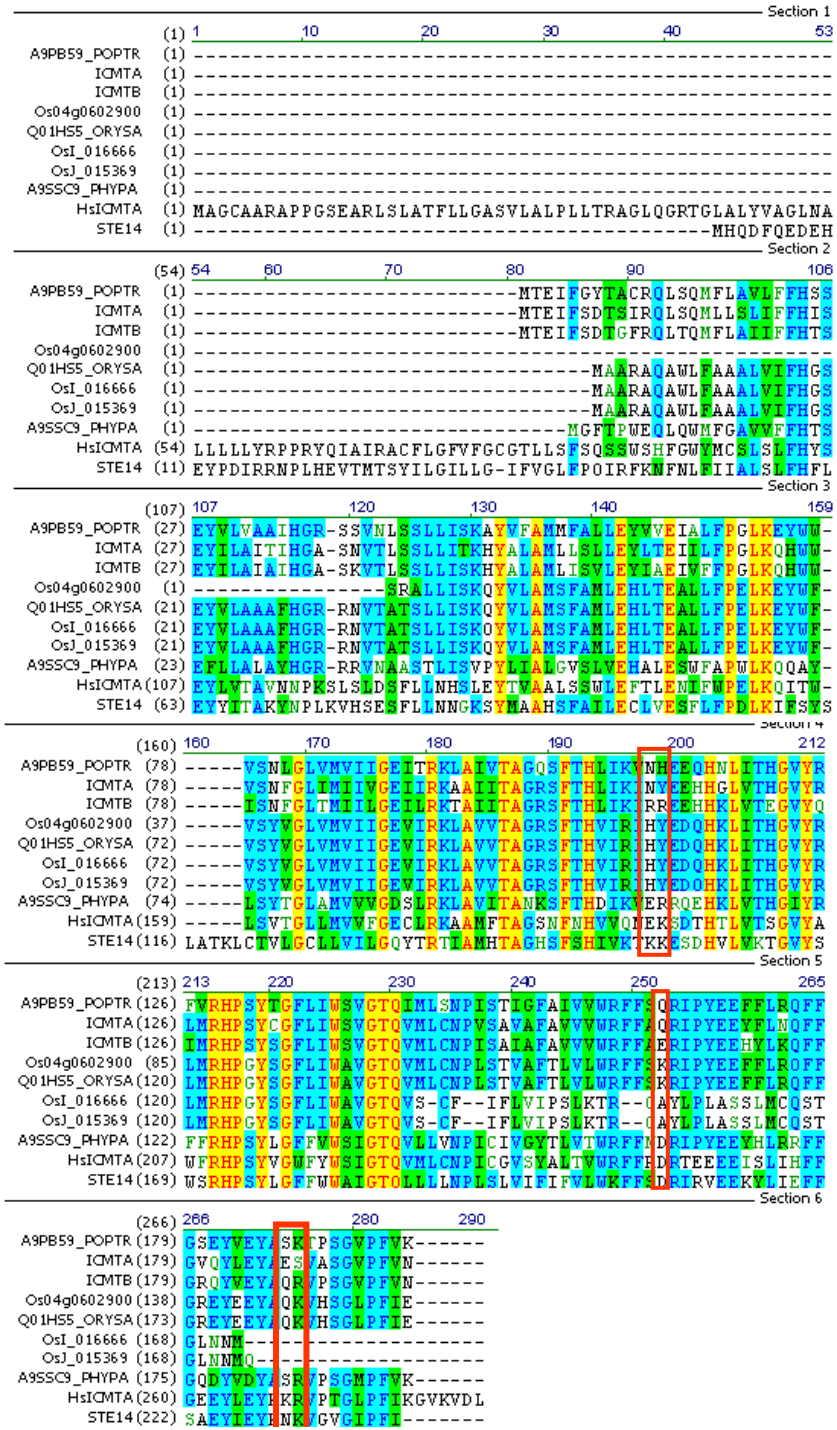
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Section 2
Section 3
Section 4
Section 5
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