

Figure S1. Fluorescence analysis of (A) Tobacco protoplasts prepared from leaf mesophyll cells expressing ARA6-mCherry and Golgi-mCherry; (B) Arabidopsis protoplasts transformed with AtCML5-YFP and AtCML4-YFP expressed under their respective endogenous promoters or (C) tobacco protoplasts prepared from leaf mesophyll cells co-expressing OEP7-saGFP₁₁+Cyt-saGFP₁₋₁₀.

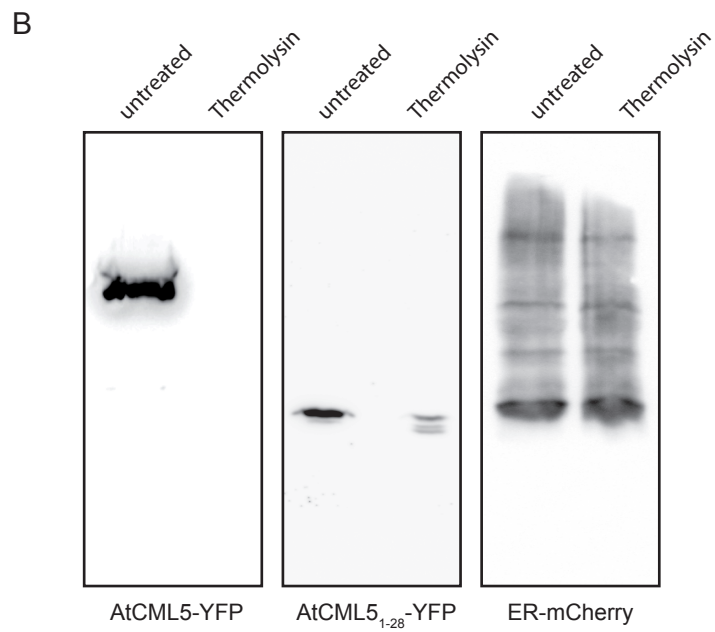
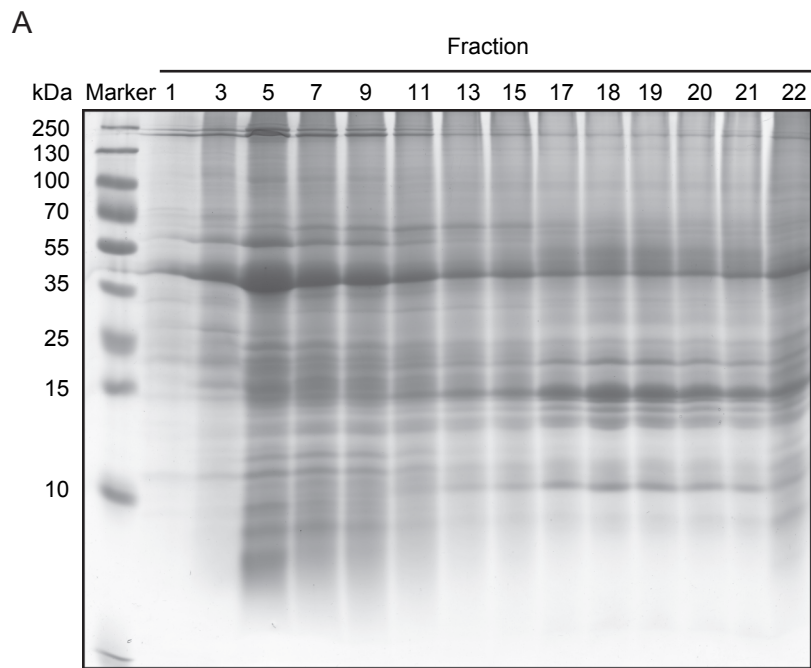


Figure S2. (A) SDS-PAGE separation and Coomassie staining of fractions from the sucrose density gradient of isolated microsomes. (B) Full-size western blot of the thermolysin treatment shown in Fig. 3C.

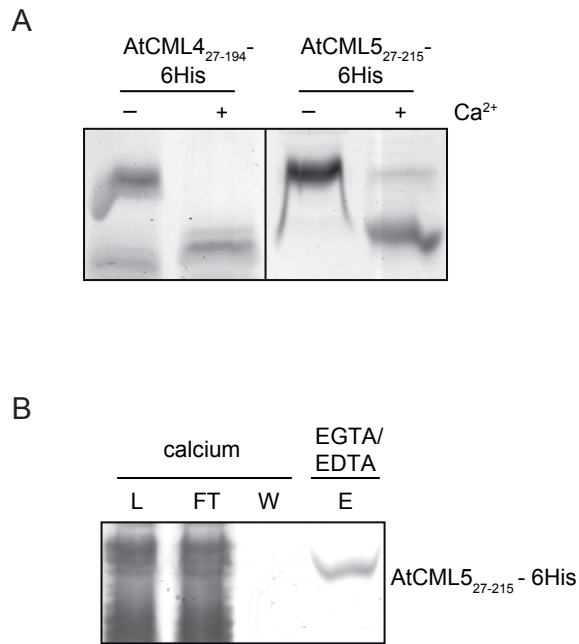


Figure S3. (A) Purified recombinant AtCML4₂₇₋₁₉₄-6His and AtCML5₂₇₋₂₁₅-6His were separated by SDS-PAGE in the presence or absence of calcium. Calcium-dependent shifts in electrophoretic mobility were analysed by SDS-PAGE and Coomassie staining. (B) Soluble extracts from *E. coli* cells expressing AtCML5₂₇₋₂₁₅-6His were loaded on phenyl-sepharose in the presence of calcium. Elution was performed by substituting EDTA for calcium and fractions were analysed by SDS-PAGE and Coomassie staining. L: load; FT: flow-through; W: wash; E: elution.

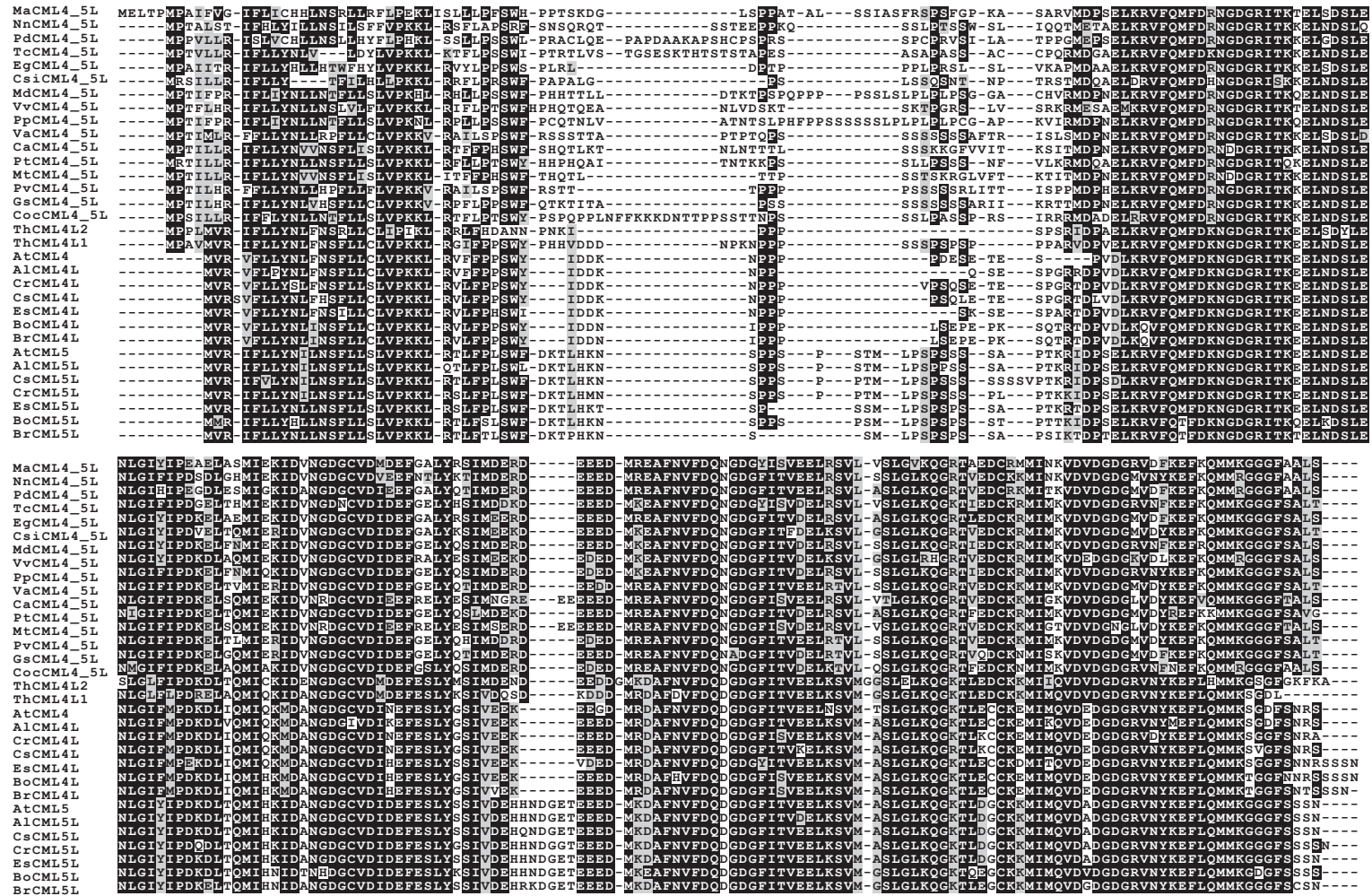


Figure S4. Amino acid sequence alignment of CML4_5 proteins from species representing different orders of flowering plants. Black boxes indicate identical amino acid residues while grey boxes indicate a conserved amino acid substitution.

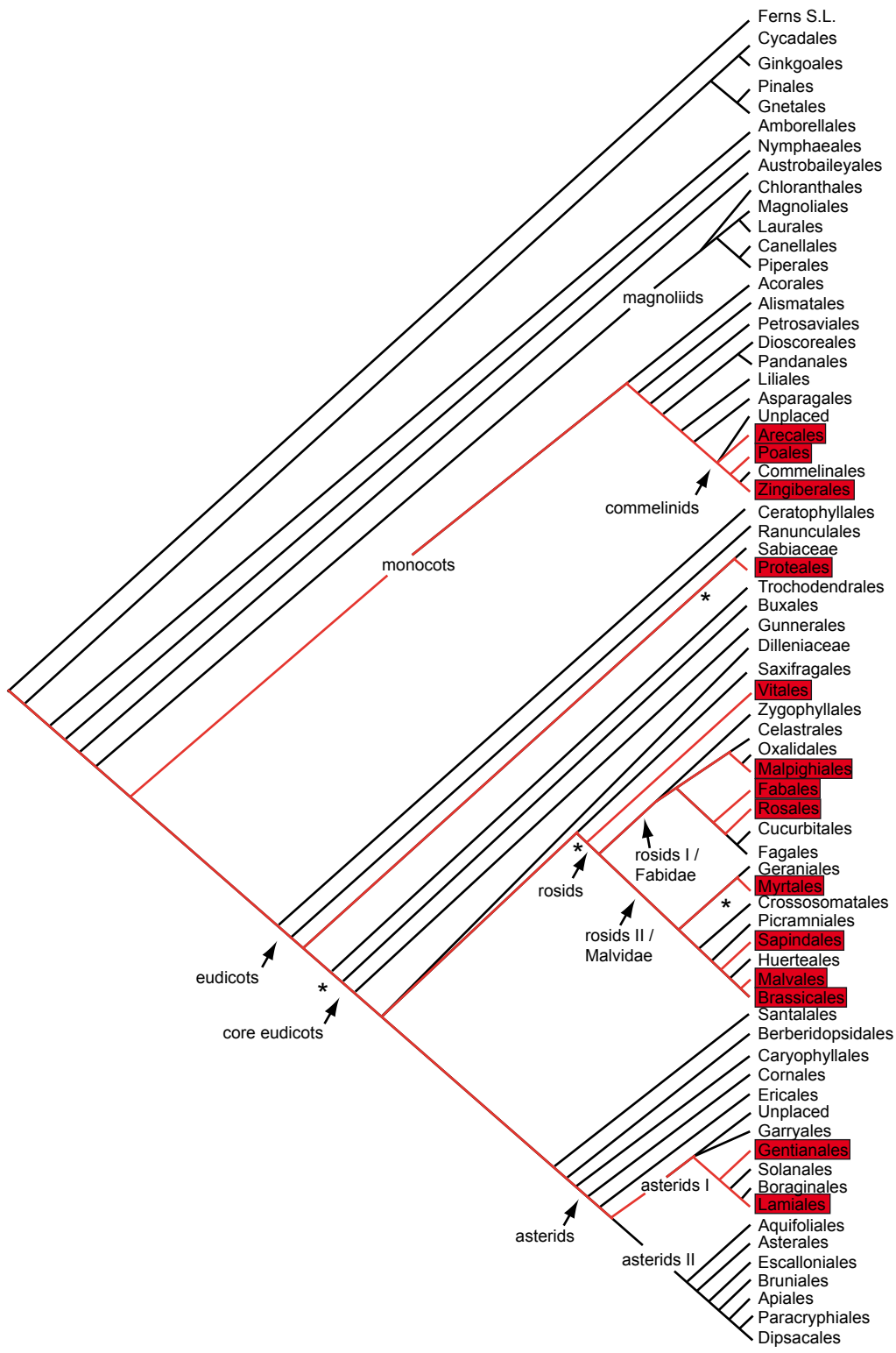


Figure S5. Distribution CML4_5-like proteins within the different orders of flowering plants. Orders in which CML4_5-like proteins were identified are marked by red branches and red boxes. Due to lack of full genome sequences covering all orders of plants, the absence of a marking does not indicate absence of CML4_5-like protein. The tree was constructed by the Angiosperm Phylogeny Website and used with the kind permission of the author (<http://www.mobot.org/MOBOT/research/APweb/welcome.html>).

Table S1. List of primer sequences

Primer	Sequence (5'→3')
AtCML5 ₁₋₂₈ NotI rv	AATGCGGCCGCCAAGAGGGAAAAGAGTTCG
AtCML5 ₂₇₋₂₁₅ ApaI fw	ATGGGCCCATGCCTCTTTCTTGGTTCGAC
AtCML5 ApaI fw	CAGGGCCCATGGTGAGAATATTCCTTCTC
AtCML5 NotI rv	TTAGCGGCCGCCATTACTGCTGCTAAAG
AtCML5 ₂₇₋₂₁₅ NdeI fw	CATCATATGCCTCTTTCTTGGTTCGACAAAAC
AtCML5 XhoI rv	TGTGCTCGAGATTACTGCTGCTAAAGCCACC
AtCML4 ₂₇₋₁₉₄ NdeI fw	CATCATATGCCTCCTTCTTGGTACATCGACG
AtCML4 XhoI rv	TGTGCTCGAGTGATCTATTGCTAAAGTCACCAC
ARA6ApaI fw	TGGGCCCATGGGATGTGCTTCTTCTTTC
ARA6NotI rv	AATGCGGCCGCCTGACGAAGGAGCAGGACG
AtCML4-BINfw	GAGGGCCCATGGTGAGAGTCTTTC
AtCML4-BINrv	TTAGCGGCCGCCTGATCTATTGCTAAAG
mCher_ApaI_fw	TCCGGGCCCATGGTGAGCAAGGGCG
mCherSKLnotIrv2	CGTTAGCGGCCGCTTACAATTTTGACTTGTACAGCTCGTC
1g66410 pBINfw	CCGGGCCCATGGCGGATCAG
1g66410 pBINrv	TTAGCGGCCGCCCTTAGCCATCATAATC
Fw_C4_PrC5_EcoRI	CTTGAATCTTTTCTGTCTGAATCTCTG
Rv_C4_PrC5_XhoI	AAGCTCGAGAACTCTTGGCTTTG
Fw_CML4_XhoI	AAGCTCGAGATGGTGAGAGTCTTTC
Rv_CML4_NcoI	TCGCCCTTGCTCACCATGGCTGATCTATTGCTAAAGTC
Fw_YFP_C4_NcoI	GACTTTAGCAATAGATCAGCCATGGTGAGCAAGGGCGA
Rv_YFP_SpeI	AATCCTCGGACTAGTCTAGCGCCCGCTCTTGTAC
FwC5specPrC5EcoRI	GCAACTTGAATTCACATTTTTCAGTTATTTTGTG
Rv_C5_PrC5_XhoI	AAGCTCGAGAACTGTTGAATCACAATC
Fw_CML5_XhoI	GCCAAGCTCGAGATGGTGAGAATATTCCTTCTC
Rv_CML5_NcoI	TCGCCCTTGCTCACCATGGCATTACTGCTGCTAAAG
Fw_YFP_C5_NcoI	CTTTAGCAGCAGTAATGCCATGGTGAGCAAGGGCGA
Rv_YFP_SpeI	AATCCTCGGACTAGTCTAGCGCCCGCTCTTGTAC

Table S2. List of constructs used in this work

Construct	Vector	Reference
AtCML5-YFP	pBIN19	this publication
AtCML5 ₁₋₂₈ -YFP	pBIN19	this publication
AtCML5 ₂₇₋₂₁₅ -YFP	pBIN19	this publication
AtCML5 ₂₇₋₂₁₅ -His	pET21b	this publication
AtCML4 ₂₇₋₁₉₄ -His	pET21b	this publication
Ara6-mCherry	pBIN19	this publication
Golgi-mCherry	pBIN	Nelson et al. 2007
ER-mCherry	pBIN	Nelson et al. 2007
mCherry-SKL	pBIN	this publication
Cyt-YFP	pBIN19	this publication
OEP7-YFP-AEQ	pBIN19	Mehlmer et al. 2012
OEP7-saGFP _{1-10C}	pBIN19	this publication
Cyt- saGFP _{1-10C}	pBIN19	this publication
Cyt- saGFP _{11C}	pBIN19	this publication
AtCML5-saGFP _{1-10C}	pBIN19	this publication
AtCML5-saGFP _{11C}	pBIN19	this publication
AtCML4-saGFP _{1-10C}	pBIN19	this publication
AtCML4-saGFP _{11C}	pBIN19	this publication
<i>cml5</i> ::AtCML5-YFP	pGREENII	this publication
<i>cml4</i> ::AtCML4-YFP	pGREENII	this publication

Table S3. List of accession numbers and the corresponding species for sequences used in alignments and phylogenetic tree analysis

Name	Accession number	Species	Order
AICML4L	XP_002876503.1	<i>Arabidopsis lyrata subsp. lyrata</i>	Brassicales
AICML5L	XP_002881895.1	<i>Arabidopsis lyrata subsp. lyrata</i>	Brassicales
AtCaM4	NP_001185330.1	<i>Arabidopsis thaliana</i>	Brassicales
AtCML2	NP_193022.1	<i>Arabidopsis thaliana</i>	Brassicales
AtCML3	NP_187405.1	<i>Arabidopsis thaliana</i>	Brassicales
AtCML4	NP_191503.1	<i>Arabidopsis thaliana</i>	Brassicales
AtCML5	NP_565996.1	<i>Arabidopsis thaliana</i>	Brassicales
AtCML6	NP_192238.1	<i>Arabidopsis thaliana</i>	Brassicales
AtCML7	NP_172089.1	<i>Arabidopsis thaliana</i>	Brassicales
CrCML5L	XP_006296176.1	<i>Capsella rubella</i>	Brassicales
CsCML4L	XP_010469361.1	<i>Camelina sativa</i>	Brassicales
CsCML5L	XP_010508448.1	<i>Camelina sativa</i>	Brassicales
CsiCML4_5L	KDO46255.1	<i>Citrus sinensis</i>	Sapindales
EgCML4_5L	XP_010069294.1	<i>Eucalyptus grandis</i>	Myrtales
EsCML4L	XP_006402687.1	<i>Eutrema salsugineum</i>	Brassicales
EsCML5L	XP_006397493.1	<i>Eutrema salsugineum</i>	Brassicales
GsCML4_5L	KHN33309.1	<i>Glycine soja</i>	Fabales
MaCML4_5L	XP_009417008.1	<i>Musa acuminata subsp. malaccensis</i>	Zingiberales
MdCML4_5L	XP_008369144.1	<i>Malus domestica</i>	Rosales
MtCML4_5L	XP_003597517.1	<i>Medicago truncatula</i>	Fabales
NnCML4_5L	XP_010268140.1	<i>Nelumbo pentapetala</i>	Proteales
PdCML4_5L	XP_008791779.1	<i>Phoenix dactylifera</i>	Arecales
PpCML4_5L	XP_007227575.1	<i>Prunus persica</i>	Rosales
PtCML4_5L	XP_002310432.2	<i>Populus trichocarpa</i>	Malpighiales
PvCML4_5L	XP_007150430.1	<i>Phaseolus vulgaris</i>	Fabales
TcCML4_5L	XP_007033950.1	<i>Theobroma cacao</i>	Malvales
ThCML4L1	XP_010526084.1	<i>Tarenaya hassleriana</i>	Brassicales
ThCML4L2	XP_010525867.1	<i>Tarenaya hassleriana</i>	Brassicales
VaCML4_5L	KOM44447.1	<i>Vigna angularis</i>	Fabales
VvCML4_5L	XP_002266359.1	<i>Vitis vinifera</i>	Vitales

Table S4. Targeting prediction analysis for AtCML5 and AtCML4

The full length coding sequences of AtCML5 and AtCML4 were analysed on the web interphase of a number of different targeting prediction programs. The results of those programs predicting subcellular targeting for at least one of the two proteins are shown.

Prediction tool	AtCML5 (At2g43290)	AtCML4 (At3g59440)
TargetP 1.1 (a)	Secretory protein	Secretory protein
Psort (b)	ER membrane	Plasma membrane
BaCelLo (c)	Secretory protein	Secretory protein
Predotar (d)	ER	ER
Yloc (e)	Vacuole (38.9%), Cytoplasm (22.8%), Mitochondrion (17.3%)	Extracellular space (79.45 %)
Golgi Predictor (f)	Golgi (score 26.13; threshold 20)	Golgi (score 29.56; threshold 20)
MemPype (g)	Signal peptide (1-21aa) 93 % internal membrane score; no TMD	Signal peptide (1-19aa) 66% Internal membrane score; no TMD

(a) <http://www.cbs.dtu.dk/services/TargetP/>

(b) <http://psort.hgc.jp/form.html>

(c) <http://gpcr.biocomp.unibo.it/bacello/>

(d) <https://urgi.versailles.inra.fr/predotar/predotar.html>

(e) <http://abi.inf.uni-tuebingen.de/Services/YLoc/webloc.cgi>

(f) http://ccb.imb.uq.edu.au/golgi/golgi_predictor.shtml

(g) <http://mu2py.biocomp.unibo.it/mempype/default/predict>

Table S5. TM-domain predictions for AtCML5 and AtCML4

The full length coding sequences of AtCML5 and AtCML4 were analysed on the web interphase of different programs for TM-domain analysis.

Prediction tool	AtCML5 (At2g43290)	AtCML4 (At3g59440)
DAS-TMfilter (a)	Potential signal peptide, no TMD	Potential signal peptide, no TMD
TMpred (b)	TMD (1-19 aa) N-terminus lumen (strong preference)	TMD (1-19 aa) N-terminus lumen (strong preference)
TopPred 1.10 (c)	TMD predicted (1-21 aa score 0.854, 39-59 aa score 0.617)	TMD predicted (1-21 aa, score 0.944)

(a) <http://www.enzim.hu/DAS/DAS.html>

(b) http://www.ch.embnet.org/software/TMPRED_form.html

(c) <http://mobylye.pasteur.fr/cgi-bin/portal.py#forms::toppred>