Characterisation of a novel calcium sensor in *Arabidopsis thaliana*

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List of abbreviations

Abbreviation	Full term
3′	Three prime, of nucleic acid sequence
5'	Five prime, of nucleic acid sequence
~	Approximately
#	Number
%	Percent
±	Plus and minus
×	Times
β	Beta
ºC	Degree Celsius
µg	Microgram(s)
µM	Micromolar
µL	Microliter(s)
AGRF	Australian Genome Research Facility
Ala	Alanine
Asn	Asparagine
ATTED-II	*Arabidopsis thaliana* trans-factor and cis-element prediction database
BLAST	Basic Local Alignment Search Tool
bp	Base pairs, of nucleic acid
BSA	Bovine serum albumin
C-terminal	Carboxyl terminal
C-terminus	Carboxyl terminus
$Ca(NO_3)_2$	Calcium nitrate
$CaCl_2$	Calcium chloride
cAMP	Adenosine 3′,5′-cyclic monophosphate
Cd^{2+}	Cadmium ion
cDNA	Complementary deoxyribonucleic acid
cGMP	Guanosine 3′,5′-cyclic monophosphate
Cl^-	Chloride ion
cm	Centimetre(s)
$CuSO_4$	Cupric sulfate

Cys	Cysteine
d	Day(s)
Da	Dalton
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EGAT	Ethylene glycol-bis(2-aminoethylether) -N,N,N′,N′-tetraacetic acid
FW	Fresh weight
g	Gram(s)
GFP	Green fluorescent protein
Glu	Glutamic acid
Gly	Glycine
GSH	L-Glutathione
GSSG	L-Glutathione oxidized
H_3BO_3	Boric acid
His	Polyhistidine tag
hr	Hour(s)
K^+	Potassium ion
kb	Kilo base pairs, of nucleic acid
kcal	Kilocalorie
KCl	Potassium chloride
kDa	Kilo dalton
KH_2PO_4	Monopotassium phosphate
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
M	Molar
MAMP	Microbe-associated molecular patterns
MES	2- (N-Morpholino) ethanesulfonic acid, 4-morpholineethanesulfonic acid
mg	Milligram(s)
Mg^{2+}	Magnesium ion
$MgSO_4$	Magnesium sulfate
min	Minute(s)
mL	Millilitre(s)
mm	Millimetre(s)
mM	Millimolar

Mn^{2+}	Manganese ion
$MnCl_2$	Manganese chloride
mol	Mole
mRNA	Messenger RNA
N-terminal	Amine terminal
N-terminus	Amine terminus
Na^+	Sodium ion
Na_2HPO_4	Sodium phosphate dibasic
Na_2MoO_3	Sodium molybdate
NaCl	Sodium chloride
NaFe(III)EDTA	Sodium iron EDTA
NH_4NO_3	Ammonium nitrate
No.	Number
NO_3^-	Nitrate ion
ng	Nanogram(s)
nm	Nanometre(s)
nM	Nanomolar
RNA	Ribonucleic acid
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG 4000	Polyethylene glycol 4000
PO_4^{3-}	Phosphate ion
pv.	Pathovars
SD	Standard deviation
SE	Standard error
sec	Second(s)
Ser	Serine
SDS	Sodium dodecyl sulfate
T-DNA	Transfer deoxyribonucleic acid
Tm	Melting temperature, of primers
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
Triton X-100	Toctylphenoxypolyethoxyethanol
v/v	Volume per volume
w/v	Weight per volume

YFP	YFP fluorescent protein
Zn^{2+}	Zinc ion
$ZnSO_4$	Zinc sulfate

Abstract

In dicotyledonous plants calcium is predominantly stored in the vacuoles of leaf mesophyll cells, a process in which the *Arabidopsis thaliana* tonoplast-localised Ca^{2+}/H^{+} antiporter 1 (AtCAX1) was previously identified as having an essential role. Simultaneous loss-of-function of *AtCAX1,* and its close homolog *AtCAX3,* or an overexpression of a constitutively active form (*sCAX1*) can cause a number of physiological perturbations. The transcriptional profiles concurrent with these perturbations were examined in a set of Arabidopsis *cax* mutants (*cax1*, *cax3*, *cax1/cax3* and *cax1/sCAX1,* and parental wildtype Col-0) as means to uncover novel Ca^{2+}-signalling elements. A core set of misexpressed genes was examined, in a preliminary screen using putative loss-of-function Arabidopsis mutants, but no calcium-related phenotypes were identified. Instead, the most highly misexpressed gene in *cax1* and *cax1/cax3* lines was selected for further functional characterisation. Calmodulin-like 41 (*CML41*) was negatively correlated with *CAX1* expression so it was hypothesised that it might behave as a transcriptional regulator of *CAX1* or as a Ca^{2+} signalling element downstream of CAX1 function.

During cloning it was discovered that *CML41* was likely transcribed into two transcripts – a full-length *CML41* (*CML41FL*), which is annotated in the NCBI database, and a novel shorter-splicing transcript named *CML41* Short (*CML41S*). The proteins encoded by *CML41FL* and *CML41S* were predicted to have 4 and 3 putative EF-hand calcium binding domains respectively, and both were demonstrated to have calcium-binding capacity *in vitro,* indicating that CML41FL and CML41S may act as Ca^{2+} sensors *in planta*. Both proteins have the same targeting signal peptide and share a similar subcellular localisation pattern being predominantly localised in the cytoplasm of young developing leaves, and roots under standard growth conditions, but are translocated to plasmodesmata (PD) in mature and old vegetative leaves. Furthermore, a TEOSINTE BRANCHED 1, cycloidea and proliferating cell factor (TCP) transcription factor 14 (TCP14) was demonstrated to interact with both CML41FL and CML41S, but the function of these interactions remains obscure.

Misexpression (35S CMV driven amiRNA knockdown or overexpression) of either *CML41FL* or *CML41S* had no effect on *CAX1* transcript abundance, so it is more likely that CML41 acts as a downstream Ca^{2+} signal element rather than in controlling *CAX1* expression. *In silico* analysis of gene expression indicates that *CML41* is highly up-regulated during biotic stress, senescence, in response to changes in photoperiod and calcium treatments, so the phenotypes of *CML41* misexpressing plants were examined under these and related conditions.

Both *CML41FL* and *CML41S* expression was induced in leaves infiltrated with flg22 – an elicitor of *P. syringae* inducing pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) signalling in plants. Knocking-down *CML41FL* expression significantly reduced the callose

deposition at PD in leaves in response to flg22, whereas in normal conditions a constitutive overexpression of *CML41FL* failed to increased callose deposition. Together this implies that CML41FL (and/or CML41S) may function as a Ca^{2+} sensor downstream of the flg22-triggered immune response to modulate callose accumulation, and its activation may require an elevation of cytosolic Ca^{2+}. The overexpression of *CML41S* and silencing of *CML41FL* both accelerated chlorophyll breakdown and senescence of individual leaves induced by dark, although their expression was not altered during the conditions imposed here. High calcium supplementation (50 mM) inhibited primary root growth of wild-type and *CML41* overexpression lines whereas it was not affected in *CML41*-knocked-down amiRNA lines. At 12.5 mM calcium, as compared to 0.3 mM, primary root growth of wild-type and *CML41*-knocked-down amiRNA plants was stimulated but this was not observed in *CML41FL*- or *CML41S*-overexpression plants. In plants expressing *CML41-GFP* translational fusions, both CML41FL and CML41S were translocated from the cytoplasm to the PD at the root tip under high calcium conditions. These results suggest that a root-growth responses to high external calcium might involve the translocation of CML41 from the cytoplasm to the PD.

Here, I demonstrate that a previously uncharacterised member of the CML family is likely to have key roles in biotic stress responses, in regulation of dark-induced leaf senescence and regulation of root sensitivity to environmental calcium levels. A number of experimental avenues are opened up by this work, especially in respect to the relative contributions of CML41FL and CML41S to the above phenotypes.