The calcium sensor CBL10 mediates salt tolerance by regulating ion homeostasis in Arabidopsis

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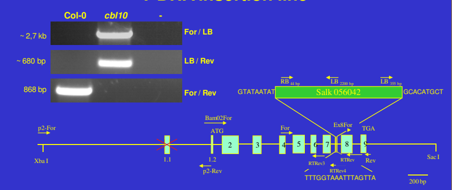
Abstract

Calcium signals regulate a multitude of biological responses and cellular processes in eukaryotic organisms. An important level of regulation in calcium signaling is decoded by calcium binding proteins functioning as calcium sensors.

The calcium sensor CBL10 belongs to the family of calcineurin-B-like-proteins (CBLs) which specifically interact with a family of serine-threonine protein kinases designated as CBL-interacting protein kinases (CIPKs). CBL10 is predominantly expressed in the shoot and likely to be involved in mediating salt stress tolerance, as revealed by the hypersensitivity of a T-DNA insertion mutant. Ion content determination of plants cultivated under salt stress conditions indicated a reduced Na^+ and an increased K^+ content in *cbl10* plants compared to wild type. This identified *cbl10* as the first plant salt sensitive mutant with an enhanced K^+/Na^+ ratio under salt stress conditions. Localization studies of GFP fusion proteins suggest that CBL10 is localized to moving punctate structures (endosomes or PVC) and at the tonoplast. Yeast-two-hybrid and BiFC analyses identified the salt tolerance factor CIPK24 (SOS2) as predominant interaction partner and revealed CBL10/CIPK24 complex formation at the tonoplast.

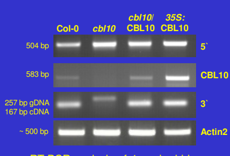
Our analyses suggest that CBL10 and CIPK24 constitute a novel Ca^{2+}-regulated salt tolerance pathway that regulates the sequestration/compartmentalization of Na^+ into vacuoles of green tissues.

Border- and transcript analysis of a CBL10 T-DNA insertion line



p2-For and p2-Rev primers were used to clone the promoter of CBL10.

For complementation a genomic fragment containing promoter, genomic DNA and 3`-UTR was subcloned with Xba I / Sac I from BAC F26P21 and transformed into *cbl10*.

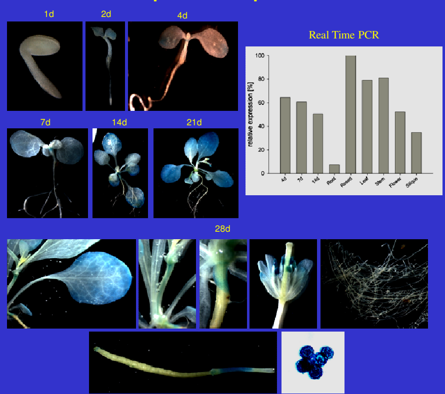


RT-PCR analysis of 4 week old leaves

Transcription of the CBL10 gene was detected upstream but not downstream of the T-DNA insertion.

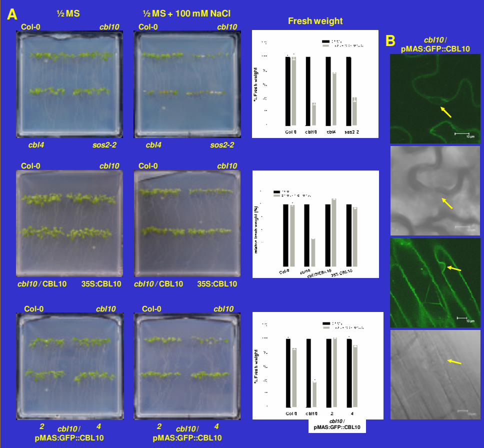
Theoretically, the T-DNA insertion could lead to a truncated protein containing only 191 out of 246 aa with loss of the 4th EF-hand.

CBL10 is predominantly expressed in the aerial parts of the plant



Promoter:GUS and real time PCR analyses revealed CBL10 expression throughout the development with a predominant expression in the aerial parts of the plant (hypocotyl, leaves, stem, antheres and pollen grains). In roots, CBL10 expression was not detectable in promoter:GUS transgenic plants and only weak expression was detected by real time PCR. In the real time PCR analyses Ubiquitin10 served as an internal reference.

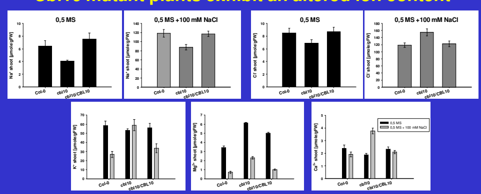
Mutation of *cbl10* renders plants salt sensitive



(A) Four day old seedlings grown on ½ MS media were transferred for additional seven days to vertical plates containing ½ MS ± 100 mM NaCl. Fresh weight is presented as a mean of 9 seedlings from six independent experiments.
(B) GFP and bright field images of two week old cbl10/pMAS:GFP::CBL10 transgenic plants (upper half, leaf; lower half, root hair zone).

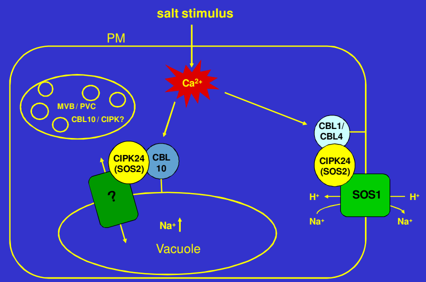
Salt sensitivity of the *cbl10* mutant specifically affects the shoot tissue. *Cbl10* mutants exhibit a reduced fresh weight but not a reduced root growth upon NaCl exposure. In comparison, *cbl4* (GABI_015F02) and *sos2-2* exhibited a strong growth reduction of shoots and roots. Fresh weight measurements indicated only a slight reduction in *cbl4* (20 %) whereas *cbl10* and *sos2-2* exhibited a comparable reduction of fresh weight of about 60 %. A complementation line (cbl10/CBL10) and two transgenic *cbl10* mutant lines expressing GFP::CBL10 were able to restore the CBL10 function. Overexpression of CBL10 (35S:CBL10) did not visibly affect plant growth.

Cbl10 mutant plants exhibit an altered ion content



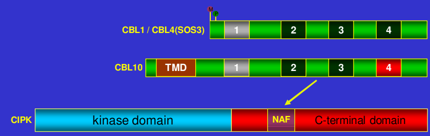
Ion content of *cbl10*, wild type and complemented plants as determined by ICP. Plants were cultivated for four days on ½ MS media. Subsequently, the plants were transferred for seven days on the indicated media and shoots were harvested for ion content determination by ICP. Compared to wild type and complemented plants, the *cbl10* mutant contains less Na^+ under control and salt stress conditions. The Cl^- content is reduced under control conditions and elevated under salt stress conditions. Furthermore, under salt stress conditions, the contents of K^+, Mg^{2+} and Ca^{2+} are increased in the *cbl10* mutant.

Model for CBL/CIPK function in salt stress responses



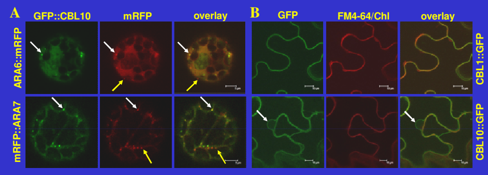
Na^+ stress triggers cytosolic Ca^{2+} release that targets CBL1, CBL4 and CBL10. All three calcium sensors can interact with CIPK24(SOS2) forming alternative calcium sensor-kinase complexes. The CBL1/CIPK24(SOS2) and CBL4/CIPK24(SOS2) complexes target the plasma membrane localized Na^+/H^+ exchanger SOS1 whereas the CBL10/CIPK24(SOS2) complex regulates ion transport processes at the vacuolar membrane and potentially also in prevacuolar compartments (PVC).

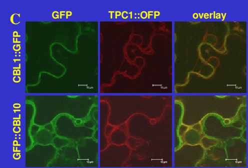
Structural model of CBLs and CIPKs



The Arabidopsis genome contains 10 CBLs and 25 CIPKs. CBLs contain 4 EF-hand motifs with different Ca^{2+} binding specificities. Four of these CBLs e.g. 1, 4, 5 and 9 can be myristoylated at their N-terminus and are therefore predicted to be localized at the plasma membrane. Distinct from the other CBLs is CBL10 as it contains an elongated N-terminus with a predicted transmembrane domain (TMD).
CIPKs contain an N-terminal kinase domain and a C-terminal regulatory domain. This regulatory domain includes the NAF-domain which is necessary and sufficient for CBL interaction.

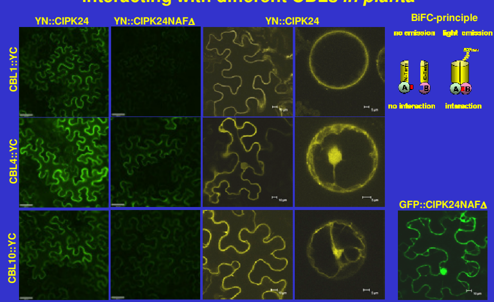
CBL10 co-localizes with the tonoplast marker TPC1 and partially also with endosomal markers





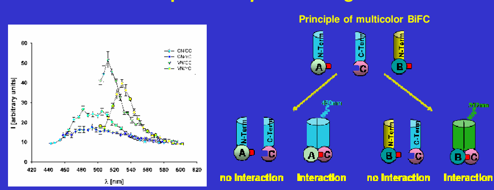
(A) Localization of GFP::CBL10 partially overlaps with the endosomal markers ARA6::mRFP and mRFP::ARA7 (white arrows, co-localization; yellow arrows, no overlap).
(B) CBL1::GFP co-localizes with the fluorescent marker dye FM4-64 which labels the plasma membrane. Localization of CBL10::GFP is distinct from this pattern as indicated by white arrows.
(C) GFP::CBL10 but not CBL1::GFP co-localizes with the tonoplast marker TPC1::OFP as indicated by the yellow fluorescence in the overlay picture.
Shown are protoplasts and epidermal cells from transiently transformed tobacco.

CIPK24 is recruited to different compartments by interacting with different CBLs *in planta*



In planta interaction, using the BiFC-system, revealed an interaction of the kinase CIPK24(SOS2) with the calcium sensor CBL1 at the plasma membrane, with CBL4(SOS3) in the cytoplasm and the nucleus and with CBL10 at the vacuolar membrane. Deletion of the interaction domain (NAFΔ) results only in back ground fluorescence.

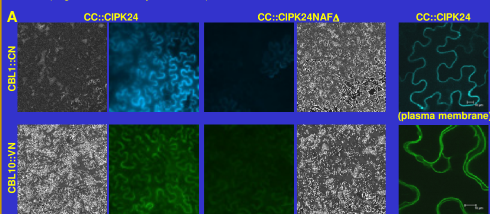
Simultaneous visualization of CBL1/CIPK24 and CBL10/CIPK24 complexes *in planta* using multicolor BiFC

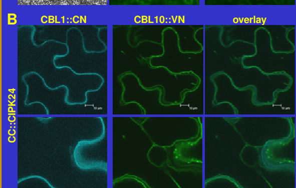


	mean Amp max. [nm]	SE
CN/CC	483,05 (cyan)	2,19
CN/YC	499,50 (blue)	4,95
VN/CC	511,70 (green)	1,45
VN/YC	528,24 (yellow)	0,99

In the multicolor BiFC system N- and C- terminal fragments of CFP can be combined with the N- and C-termini of YFP, resulting in different spectral properties with emission maxima from cyan over blue and green to yellow.

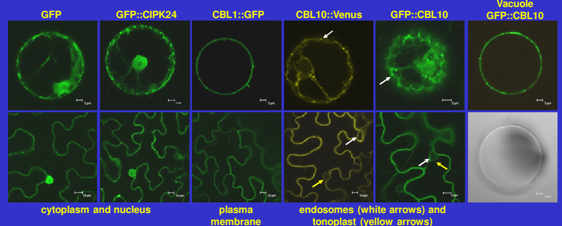
The best combinations for multicolor BiFC are CFP-C with CFP-N and YFP-N because these combinations exhibit the highest emission rate and the most distant emission maxima (Shyu *et al.* 2006). Here we used the Venus-N (Nagai *et al.* 2002; Shyu *et al.* 2006) instead of YFP-N.



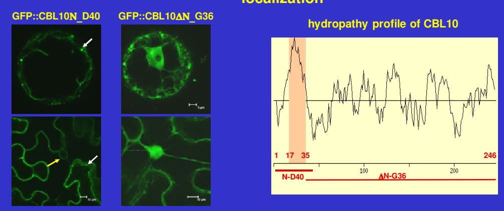


A) In double infiltrated *N. benthamiana* leaves CBL1/CIPK24 complexes are localized at the plasma membrane (cyan) whereas CBL10/CIPK24 complexes are localized at the vacuolar membrane (green). Deletion of the CIPK24 interaction domain (NAFΔ) resulted only in back ground fluorescence.
B) Tripple infiltrated *N. benthamiana* leaves exhibit the same localization pattern of CBL1/CIPK24 and CBL10/CIPK24 complexes as in A) but with reduced fluorescence emission.

CBL10 is localized to moving punctate structures and to the vacuolar membrane

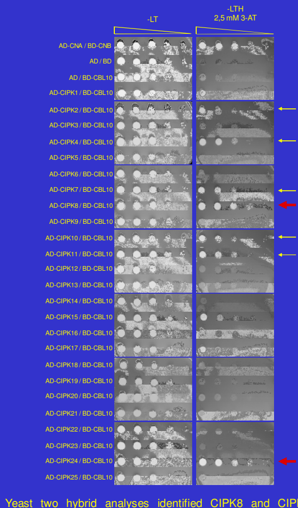


The N-terminus of CBL10 is necessary and sufficient to determine the localization



CBL10 contains a predicted N-terminal transmembrane domain from aa17 to aa35. Subcellular localization studies of GFP fusion proteins using protoplasts and epidermal cells of *Agrobacterium* infiltrated *N. benthamiana* leaves indicated that the 1st 40 aa of CBL10 are sufficient and necessary for its localization to endosomes (white arrows) and to the tonoplast as indicated by forming the nuclear pocket (yellow arrows). Deletion of the N-Terminus shifts the GFP localization to the cytoplasm.

CBL10 interacts with CIPK24 and CIPK8 in yeast two hybrid assays



Yeast two hybrid analyses identified CIPK8 and CIPK24 as predominant CBL10 interactors. In addition, we also observed a weak interaction with CIPK2, 4, 7, 10 and 11.