***New Phytologist* Supporting Information**

Article title: **CIPK-B is essential for salt stress signalling in *Marchantia polymorpha***
Authors: Connor Tansley[1], James Houghton[1*], Althea M. E. Rose[1*], Bartosz Witek[1], Rocky D. Payet[1], Taoyang Wu[2], J. Benjamin Miller[1]
Article acceptance date: 13 November 2022

The following Supporting Information is available for this article:

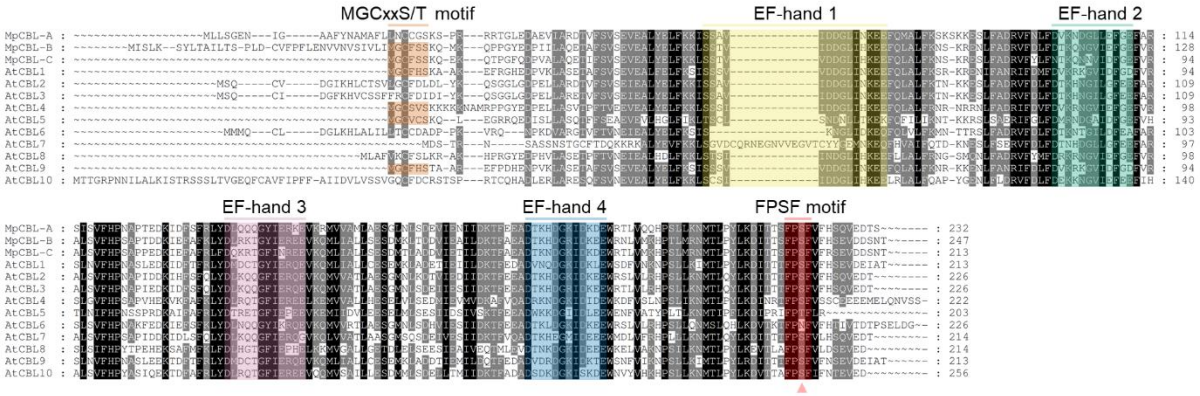

Fig. S1: Conservation of key domains in *Marchantia polymorpha* CBLs. Sequence alignment of CBL proteins from *A. thaliana* and *M. polymorpha*, with black/grey shading denoting the level of conservation. All three *M. polymorpha* CBLs have four conserved EF-hands (as indicated), including the first EF-hand containing a loop region of 14 amino acids. MpCBL-B/C (but MpCBL-A) contain the MGCxxS/T myristoylation and palmitoylation motif for localisation to the plasma membrane. All three *M. polymorpha* CBLs also have the conserved FPSF motif, including the conserved serine residue that can be regulated by phosphorylation (arrow head).

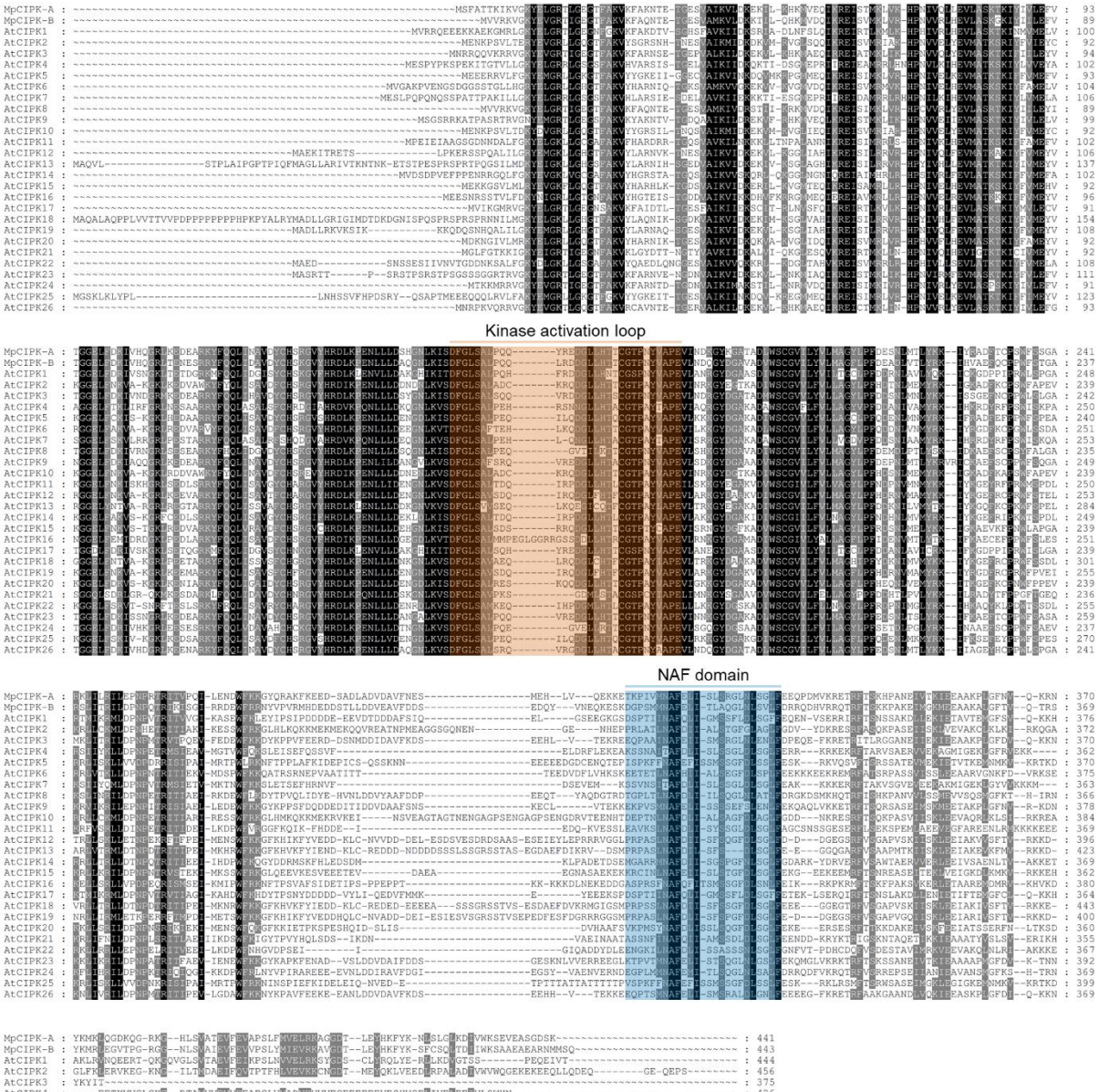

Fig. S2: Conservation of key domains in *Marchantia polymorpha* CIPKs. Sequence alignment of CIPK proteins from *A. thaliana* and *M. polymorpha*, with black/grey shading denoting the level of conservation. Both *M. polymorpha* CIPKs have conserved kinase domains, with an activation loop (defined between the DFG and APE residues), and conserved NAF domains (as indicated).

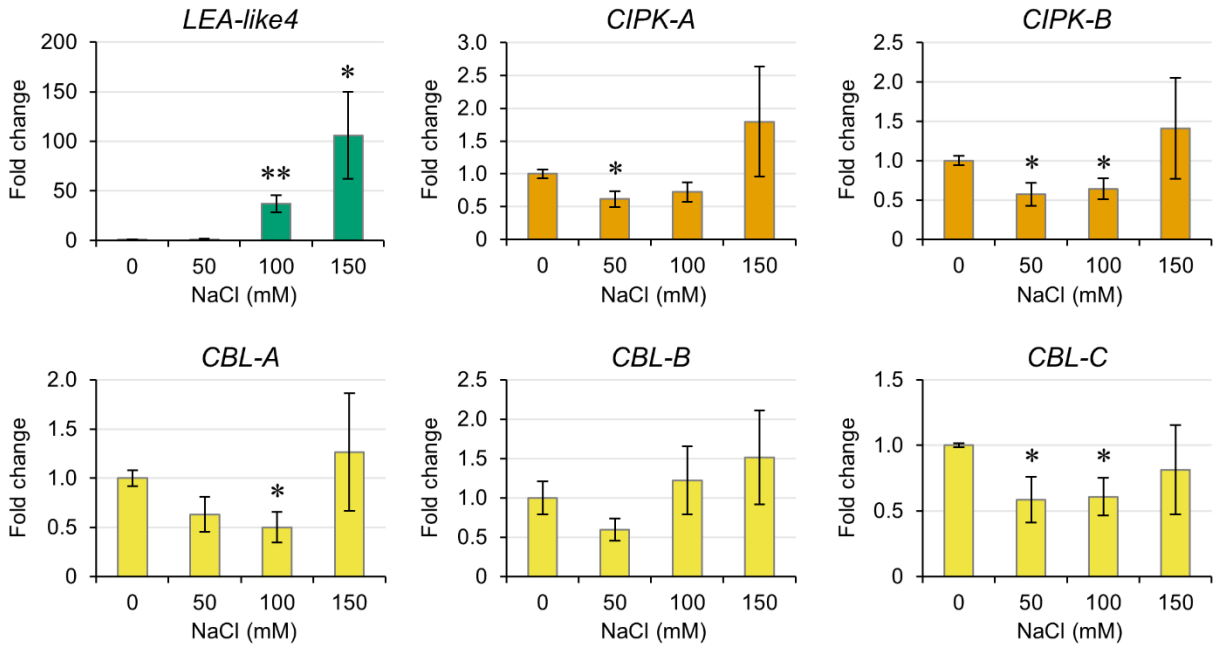

Fig. S3: Regulation of *CBL* and *CIPK* gene expression by salt stress in Tak-1 plants. Expression changes of the indicated genes were measured by RT-qPCR in thallus tissue of Tak-1 plants grown in the presence of the indicated concentrations of NaCl for 7 days. Data represent mean ± one standard error from three independent biological replicates. Significance in a pairwise two-tailed t-test relative to 0 mM treatment is indicated at $p<0.05$ (one star) or $p<0.01$ (two stars).

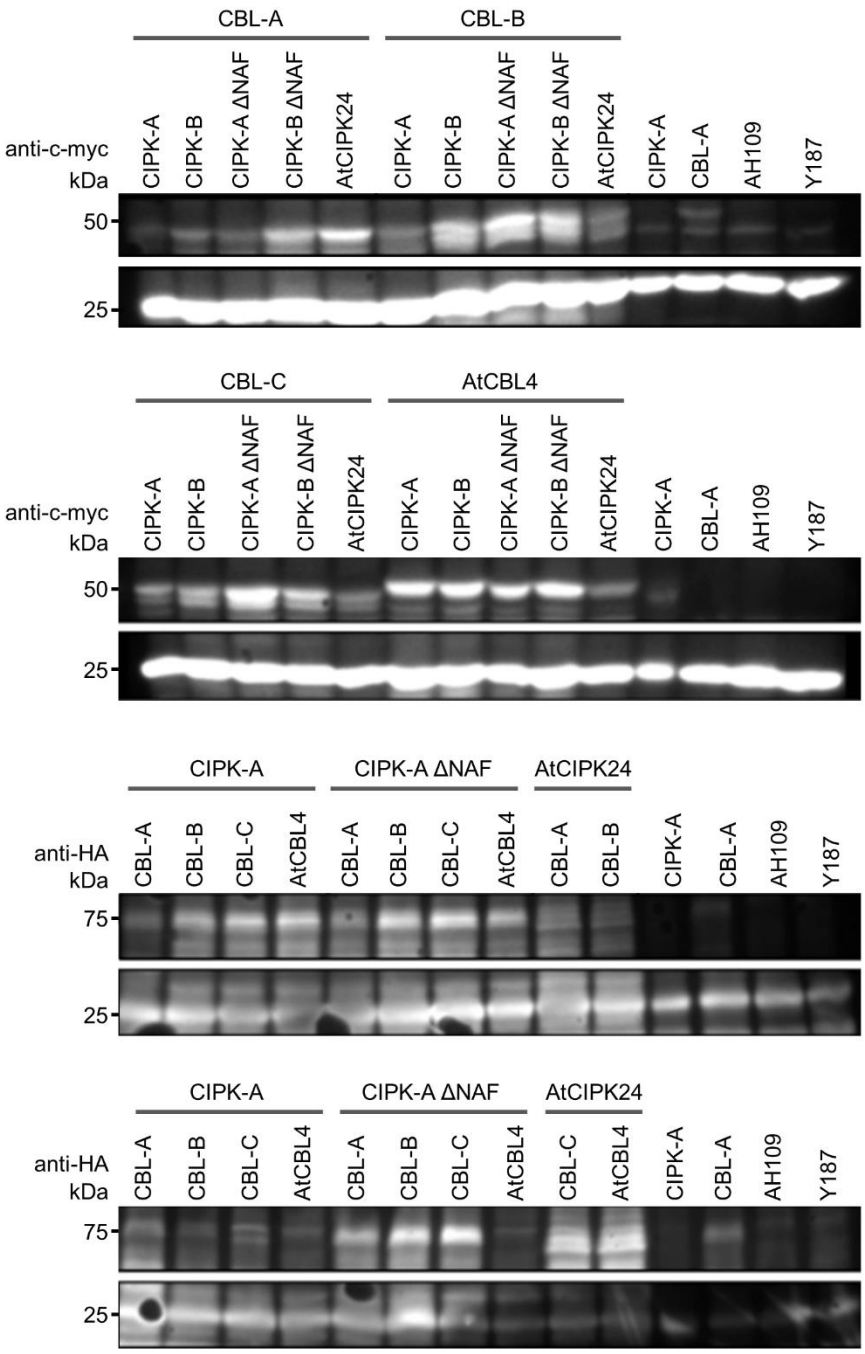

Fig. S4: Western blotting confirms expression of all *Marchantia polymorpha* CBLs and CIPKs in strains used for yeast two-hybrid assays. Western blotting using c-myc and HA antibodies identified proteins of the expected size for GAL4-BD-3xc-myc-CBLs and GAL4-AD-3xHA-CIPKs, respectively (upper panels). Expected sizes of the GAL4-BD-3xc-myc-CBLs are: 48.5 (MpCBL-A), 46.6 (MpCBL-B and MpCBL-C) and 47.8 kDa (AtCBL4). Expected sizes of the GAL4-AD-3xHA-CIPKs are: 69.7 (MpCIPK-A), 70.0 (MpCIPK-B), 67.1 (MpCIPK-A ΔNAF), 67.4 (MpCIPK-B ΔNAF) and 70.2 kDa (AtCIPK24). Lower panels correspond to loading control, using native yeast peroxidase (PRX1) activity as a proxy for total protein (expected size of 25 kDa).

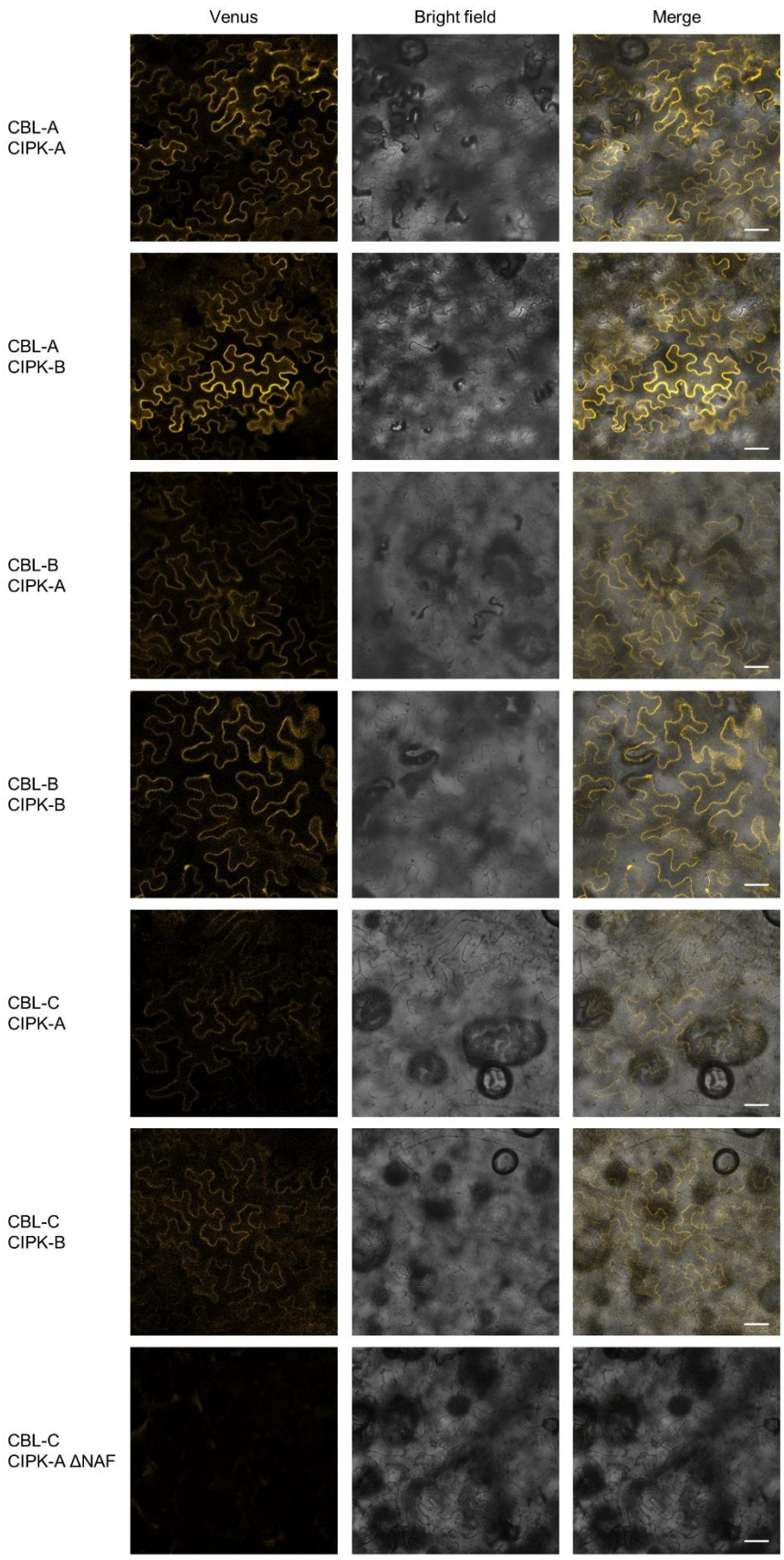
Venus
Bright field
Merge
CBL-A
CIPK-A
CBL-A
CIPK-B
CBL-B
CIPK-A
CBL-B
CIPK-B
CBL-C
CIPK-A
CBL-C
CIPK-B
CBL-C
CIPK-A ΔNAF

Fig. S5: Bimolecular fluorescence complementation confirms all *Marchantia polymorpha* CBLs and CIPKS interact in *N. benthamiana*. Constructs containing the C-terminal half of the Venus fluorescent protein fused to each CIPK (Venus^{C}-CIPK) and the N-terminal half of Venus fused to each CBL (CBL-Venus^{N}) were transiently expressed in *N. benthamiana* leaf epidermal cells. Venus fluorescence, bright field and merged images are shown for each *M. polymorpha* CBL-CIPK interaction. Scale bar in all panels = 100 µm.

Fig. S6: Both *cipk-b* knockout mutant lines encode proteins with premature stop codons. Two 20-nucleotide sgRNAs (indicated) were designed to target exon 1 of *CIPK-B* to generate two independent *cipk-b* mutant lines via CRISPR/Cas9 gene editing in the Cam-2 background. Nucleotide deletions and substitutions in the resulting two *cipk-b* mutant lines are indicated in red. The protein sequence encoded by each mutant line is also shown, with amino acid differences compared with the wildtype CIPK-B sequence indicated in red. The *cipk-b-1* mutant contains a 35 bp deletion and the *cipk-b-2* mutant contains two small deletions and a single nucleotide substitution (Fig. 5), both leading to missense mutations and introduction of a premature stop codon (resulting in predicted proteins containing 29 and 39 amino acids, respectively).

Table S1: Gene ID numbers for sequences used in bioinformatics in this study

Gene name	Species	Gene ID
MpCBL-A	*M. polymorpha*	Mp2g07750 (Mapoly0015s0061)
MpCBL-B	*M. polymorpha*	Mp4g00900 (Mapoly0066s0053)
MpCBL-C	*M. polymorpha*	Mp5g19810 (Mapoly0134s0040)
MpCIPK-A	*M. polymorpha*	Mp1g05680 (Mapoly0005s0039)
MpCIPK-B	*M. polymorpha*	Mp2g26670 (Mapoly0025s0017)
KnCBL1	*K. nitens*	GAQ81593.1
KnCBL2	*K. nitens*	GAQ84394.1
KnCBL3	*K. nitens*	GAQ80563.1
KnCIPK1	*K. nitens*	GAQ84395.1
PpCBL1	*P. patens*	Pp3c1_36780V3.1.p
PpCBL2	*P. patens*	Pp3c16_24350V3.1.p
PpCBL3	*P. patens*	Pp3c13_3530V3.1.p
PpCBL4	*P. patens*	Pp3c5_9970V3.1.p
PpCIPK1	*P. patens*	Pp3c16_15230V3.1.p
PpCIPK2	*P. patens*	Pp3c2_13790V3.1.p
PpCIPK3	*P. patens*	Pp3c10_7160V3.1.p
PpCIPK4	*P. patens*	Pp3c14_7960V3.1.p
PpCIPK5	*P. patens*	Pp3c5_7750V3.1.p
PpCIPK6	*P. patens*	Pp3c12_210V3.1.p
PpCIPK7	*P. patens*	Pp3c15_1210V3.1.p

Table S2: Primers used for RT-qPCR in this study

Gene name	Gene ID	Forward primer	Reverse primer
CBL-A	Mp2g07750	AGCGGAAAGAGGTGAAACGG	GAGAGGGATGCTGCTGAACC
CBL-B	Mp4g00900	GGGCTGCTTCAGCTCAAAAC	CGCAAGCTGGAACTCTTCCT
CBL-C	Mp5g19810	CAAGTGCTCCACCAGAGGAC	GCCTCCGCAAATGTCTTGTC
CIPK-A	Mp1g05680	AAACACCCTGCGAACGAGAT	ACCTCAAACACCTCTGTGGC
CIPK-B	Mp2g26670	CCTGTACGGATGCACGATGA	AGAACGGAAAGGTTGAGCCC
LEA-like4	Mp1g23200	GCTAACAGACCCAGGTGAC	TGTTTCCAACGGCAGAGTG
ACT1	Mp6g10990	GAGCGCGGTTACTCTTTCAC	GACCGTCAGGAAGCTCGTAG
APT	Mp3g35140	CGAAAGCCCAAGAAGCTACC	GTACCCCCGGTTGCAATAAG

Table S3: Final constructs created and used in this study

ID	Construct details	Experiment
01044	pTRP1-LEU2-tADH1 pADH1-GAL4-AD-3xHA-MpCIPK-A-tADH1	Yeast two-hybrid GAL4-AD-MpCIPK-A
01045	pTRP1-LEU2-tADH1 pADH1-GAL4-AD-3xHA-MpCIPK-B-tADH1	Yeast two-hybrid GAL4-AD-MpCIPK-B
01046	pTRP1-TRP1-tADH1 pADH1-GAL4-BD-3xc-myc-MpCBL-A-tADH1	Yeast two-hybrid GAL4-BD-MpCBL-A
01147	pTRP1-TRP1-tADH1 pADH1-GAL4-BD-3xc-myc-MpCBL-B-tADH1	Yeast two-hybrid GAL4-BD-MpCBL-B
01048	pTRP1-TRP1-tADH1 pADH1-GAL4-BD-3xc-myc-MpCBL-C-tADH1	Yeast two-hybrid GAL4-BD-MpCBL-C
01133	pTRP1-LEU2-tADH1 pADH1-GAL4-AD-3xHA-MpCIPK-A-ΔNAF-tADH1	Yeast two-hybrid GAL4-AD-MpCIPK-A ΔNAF
01134	pTRP1-LEU2-tADH1 pADH1-GAL4-AD-3xHA-MpCIPK-B-ΔNAF-tADH1	Yeast two-hybrid GAL4-AD-MpCIPK-B ΔNAF
01145	pTRP1-TRP1-tADH1 pADH1-GAL4-BD-3xc-myc-AtCBL4-tADH1	Yeast two-hybrid GAL4-BD-AtCBL4
01146	pTRP1-LEU2-tADH1 pADH1-GAL4-AD-3xHA-AtCIPK24-tADH1	Yeast two-hybrid GAL4-AD-AtCIPK24
01268	p35S-MpCBL-A-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-A-LgBiT SmBiT-MpCIPK-A
01269	p35S-MpCBL-B-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-B-LgBiT SmBiT-MpCIPK-A
01270	p35S-MpCBL-C-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-C-LgBiT SmBiT-MpCIPK-A
01271	p35S-AtCBL4-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-tNOS pAtUBI10-GUS-tNOS	Split luciferase AtCBL4-LgBiT SmBiT-MpCIPK-A
01272	p35S-MpCBL-A-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-A-LgBiT SmBiT-MpCIPK-B
01273	p35S-MpCBL-B-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-B-LgBiT SmBiT-MpCIPK-B
01274	p35S-MpCBL-C-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-C-LgBiT SmBiT-MpCIPK-B
01275	p35S-AtCBL4-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-tNOS	Split luciferase AtCBL4-LgBiT

	pAtUBI10-GUS-tNOS	SmBiT-MpCIPK-B
01276	p35S-MpCBL-A-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-A-LgBiT SmBiT-MpCIPK-A ΔNAF
01277	p35S-MpCBL-B-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-B-LgBiT SmBiT-MpCIPK-A ΔNAF
01278	p35S-MpCBL-C-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-C-LgBiT SmBiT-MpCIPK-A ΔNAF
01279	p35S-AtCBL4-LgBiT-t35S pNOS-SmBiT-MpCIPK-A-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase AtCBL4-LgBiT SmBiT-MpCIPK-A ΔNAF
01280	p35S-MpCBL-A-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-A-LgBiT SmBiT-MpCIPK-B ΔNAF
01281	p35S-MpCBL-B-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-B-LgBiT SmBiT-MpCIPK-B ΔNAF
01282	p35S-MpCBL-C-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-C-LgBiT SmBiT-MpCIPK-B ΔNAF
01283	p35S-AtCBL4-LgBiT-t35S pNOS-SmBiT-MpCIPK-B-ΔNAF-tNOS pAtUBI10-GUS-tNOS	Split luciferase AtCBL4-LgBiT SmBiT-MpCIPK-B ΔNAF
01284	p35S-MpCBL-A-LgBiT-t35S pNOS-SmBiT-AtCIPK24-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-A-LgBiT SmBiT-AtCIPK24
01285	p35S-MpCBL-B-LgBiT-t35S pNOS-SmBiT-AtCIPK24-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-B-LgBiT SmBiT-AtCIPK24
01286	p35S-MpCBL-C-LgBiT-t35S pNOS-SmBiT-AtCIPK24-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-C-LgBiT SmBiT-AtCIPK24
01287	p35S-AtCBL4-LgBiT-t35S pNOS-SmBiT-AtCIPK24-A-tNOS pAtUBI10-GUS-tNOS	Split luciferase AtCBL4-LgBiT SmBiT-AtCIPK24
01288	p35S-MpCBL-A-LgBiT-t35S	Split luciferase

	pNOS-LgBiT-MpCIPK-A-tNOS pAtUBI10-GUS-tNOS	MpCBL-A-LgBiT LgBiT-MpCIPK-A
01289	p35S-MpCBL-A-SmBiT-t35S pNOS-SmBiT-MpCIPK-A-tNOS pAtUBI10-GUS-tNOS	Split luciferase MpCBL-A-SmBiT SmBiT-MpCIPK-A
01247	p35S-HYG-tNOS pMpEF1α-NLS-pcoCas9-t35S pMpU6-1-sgRNA-MpCIPK-B1-tRNAP pMpU6-1-sgRNA-MpCIPK-B2-tRNAP	CRISPR/Cas9 MpCIPK-B knockout
01135	pNOS-KAN-tNOS p35S-MpCBL-A-VYNE-t35S pAtUBI10-dsRED-tNOS	BiFC MpCBL-A-Venus^{N}
01188	pNOS-KAN-tNOS p35S-MpCBL-B-VYNE-t35S pAtUBI10-dsRED-tNOS	BiFC MpCBL-B-Venus^{N}
01137	pNOS-KAN-tNOS p35S-MpCBL-C-VYNE-t35S pAtUBI10-dsRED-tNOS	BiFC MpCBL-C-Venus^{N}
01138	pNOS-KAN-tNOS p35S-VYCE(R)-MpCIPK-A-t35S pAtUBI10-dsRED-tNOS	BiFC Venus^{C}-MpCIPK-A
01139	pNOS-KAN-tNOS p35S-VYCE(R)-MpCIPK-B-t35S pAtUBI10-dsRED-tNOS	BiFC Venus^{C}-MpCIPK-B
01140	pNOS-KAN-tNOS p35S-VYCE(R)-MpCIPK-A-ΔNAF-t35S pAtUBI10-dsRED-tNOS	BiFC Venus^{C}-MpCIPK-A ΔNAF