Title: Suppressor of K^+ Transport Growth Defect 1 (SKD1) interacts with RING-type ubiquitin ligase and Sucrose Non-fermenting 1-related Protein Kinase (SnRK1) in halophyte ice plant

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Supplementary data: Two tables and 8 figures

Supplementary Table S1. Putative McSKD1-interacting proteins identified from salt-stressed ice plant cDNA library.

no	Prey genes (translated cDNA)	Homolog locus (CDS length)	Identity	Similarity	descriptions	β-galactosidase activity (units)	note
1	Vacuolar protein sorting13; VPS13 (263 aa)	*A. thaliana At4g17140* (4218 aa)	54%	72%	Contain vacuolar protein sorting-associated domain MRS6 at N- and C-terminus, involved in intracellular trafficking, secretion, and vesicular transport, located in vacuole	19.1±0.7	
2	Aspartic proteinase 1 (167 aa)	*Nepenthes alata AB045891* (514 aa)	76 %	86 %	A novel aspartic proteinase is targeted to the secretory pathway and to the vacuole, wound-induced	20.1±3.0	
3	Polygalacturonase/ pectinase, (103 aa)	*A. thaliana At1g60590* (540 aa)	69%	83%	Secretory proteins, hydrolytic enzymes involved in cell wall degradation, response to pathogen infection	24.2±2.0	
4	SNF1-related protein kinase 1; SNF1 (111 aa)	*Cucumis sativus Y10036* (504 aa)	87%	97%	Sucrose non-fermenting-related protein kinase 1 (SnRK1), N-terminal catalytic domain, C-terminal ubiquitin associated (UBA) and kinase associated 1 (KA1) domain, response to carbohydrate and stress	29.5±1.7	2 isolates
5	RING-type E3 ligase (243 aa)	*A. thaliana At5g1442*0 (468 aa)	78%	85%	RING domain Ligase2 (RGLG2), ubiquitin-protein ligase activity, N-terminal protein myristoylation, central Copine (vWA) domain, involved in auxin and cytokinin metabolic process, located in plasma membrane.	30.1±1.0	2 isolates
6	Epoxide hydrolase (104 aa)	*A. thaliana At3g51000* (323 aa)	49%	73%	Soluble epoxide hydrolase catalyzes the hydrolysis of aliphatic epoxy fatty acids, involved in lipid transport and metabolism, response to auxin and water deprivation	33.0±0.5	
7	catalase 3 (240 aa)	*A. thaliana At1g20620* (492 aa)	100%	100%	catalyzes the breakdown of H_2O_2 into water and oxygen, in response to nitrogen, phosphate, sulfate starvation, cold and oxidative stress, involved in defense response, located in apoplast/cell wall,	35.2±0.9	3 isolates

#7 candidate is identified by screening commercial Arabidopsis library (Arabidopsis MATCHMAKER cDNA Library, Clontech)

Supplementary Table S2. Primers used in 5'-RACE, 3'-RACE and RT-PCR of *McCPN1* and *McSnRK1*

Gene-specific primers of *McCPN1*

Primer Name	Sequence (5'→ 3')
5COP (5'RACE)	CCATCTCAATAACGGGTGCAAACGATGTAGGC
3COP (3'-RACE)	GGCGATGACAATTGTTGAGCAGAGCGGTGG
N5COP (5' Nest)	CTCCAAACCGTAACAAGGGATCAGATTATCATC
N3COP (3' Nest)	GGGCAGGTTACCAGGAGTGTTGATACAGAAC
COP coding-5 (RT-PCR)	TGCTGGCCTTGAATCATCTAATCT
COP coding-3 (RT-PCR)	CATCAACCGTTTTCTGCTCCTGT

RACE conditions were set up according to manufacturer's suggestion.
RT condition: RT was performed at 30°C for 10 min, 42℃ for 1 h and 99℃ for 5 min,
PCR condition: 30 cycles of 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec.

Gene-specific primers of *McSnRK1*

Primer Name	Sequence (5' → 3')
5SNF (5'RACE)	GCTGAGCAAGGAAAGCTGCACAAAGATCAAGGAATA
3SNF (3'-RACE)	GGAAAAAGATTGGGCATTATAATATGAAGTGCAGGTGGATT
N5SNF (5' Nest)	CTCAAACTTCACTACATTGGGTGACCTCAACAC
N3SNF (3' Nest)	AATGATGGAGTGTTGAGGTCACCCAATGTAGT
SNF 3UTR-5 (RT-PCR)	GATCTACAGAGGGTTCAGG
SNF 3UTR-3 (RT-PCR)	AAGGCCACAAGTTTTCAATAG

RACE conditions were set up according to manufacturer's suggestion.
PCR condition: 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min.

RT-PCR primers of *FNR1*

Primer Name	Sequence (5' → 3')
FNR1-5	ATTGCCAGCAGGCCCTTG
FNR1-3	GAACCAGTCAATACCATCT

PCR condition: 30 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min.

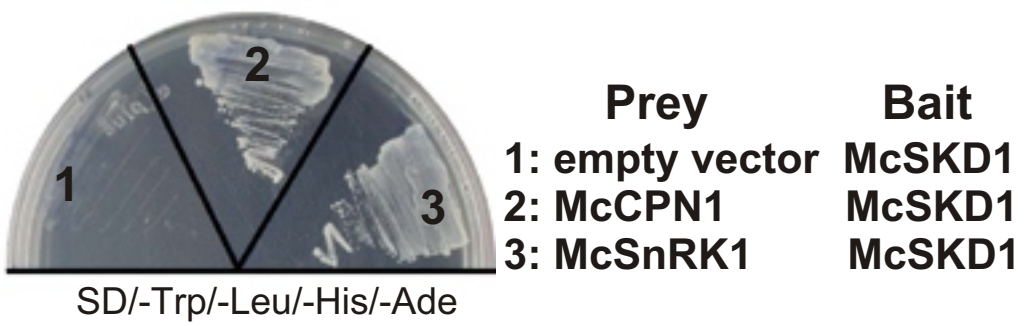

Fig. S1. *In vivo* interaction between McSKD1 and partial sequences of RING-type E3 ligase (McCPN1) and SNF1-related protein kinase (McSnRK1)

The growth of yeast in SD/-Trp/-Leu/-His/-Ade medium. The AH109 strain was co-transformed pGBKT7-McSKD1 plasmid with empty vector (pGADT7), pGADT7-McCPN1 or pGADT7-McSnRK1 plasmids and cultured on high stringent media. The prey constructs were original interacting clones identified by Y2H screen containing Copine domain of McCPN1 and KA1 domain of McSnRK1.

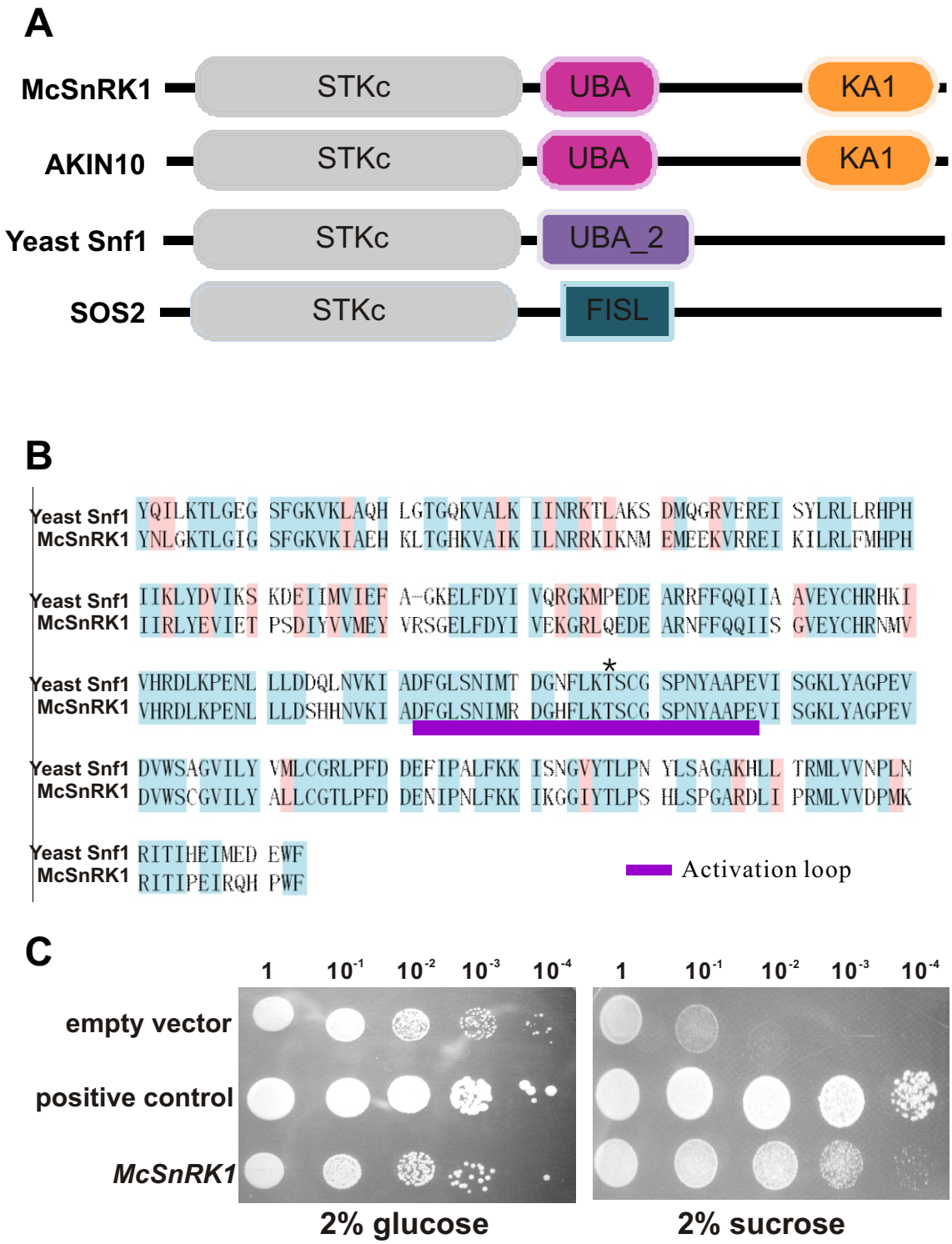

Fig. S2. Domain and sequence alignments of McSnRK1 and related protein kinases
(A) Conserved domains of McSnRK1, Arabidopsis KIN10, yeast Snf1, and Arabidopsis SOS2. STKc: ser/thr kinase catalytic domain; UBA: UBA/TS-N domain; KA1: kinase assocaited domain1; UBA_2: ubiquitin assocaited domain; FISL: FISL motif.
(B) Sequence comparison of the STKc domain between McSnRK1 and yeast Snf1. The purple underline indicates the region of the activation loop, the blue regions indicate identical amino acids, and the pink regions indicate similar amino acids.
(C) Complementation of yeast *snf1* mutant with *McSnRK1*. The yeast *snf1* mutant was transformed with either an empty vector or with full-length McSnRK1 and grown on SD medium supplemented with 2% glucose or 2% sucrose. The wild-type yeast grew equally well in both glucose and sucrose media while the *snf1* mutant transformed with empty vector grew poorly in the sucrose medium. The transformants expressing *McSnRK1* exhibited better growth in sucrose medium than the transformants carrying an empty vector. Empty vector: transformants carrying an empty expression vector; *McSnRK1*: transformants carrying full-length McSnRK1; positive control: wild-type yeast carrying a functional yeast *Snf1* gene.

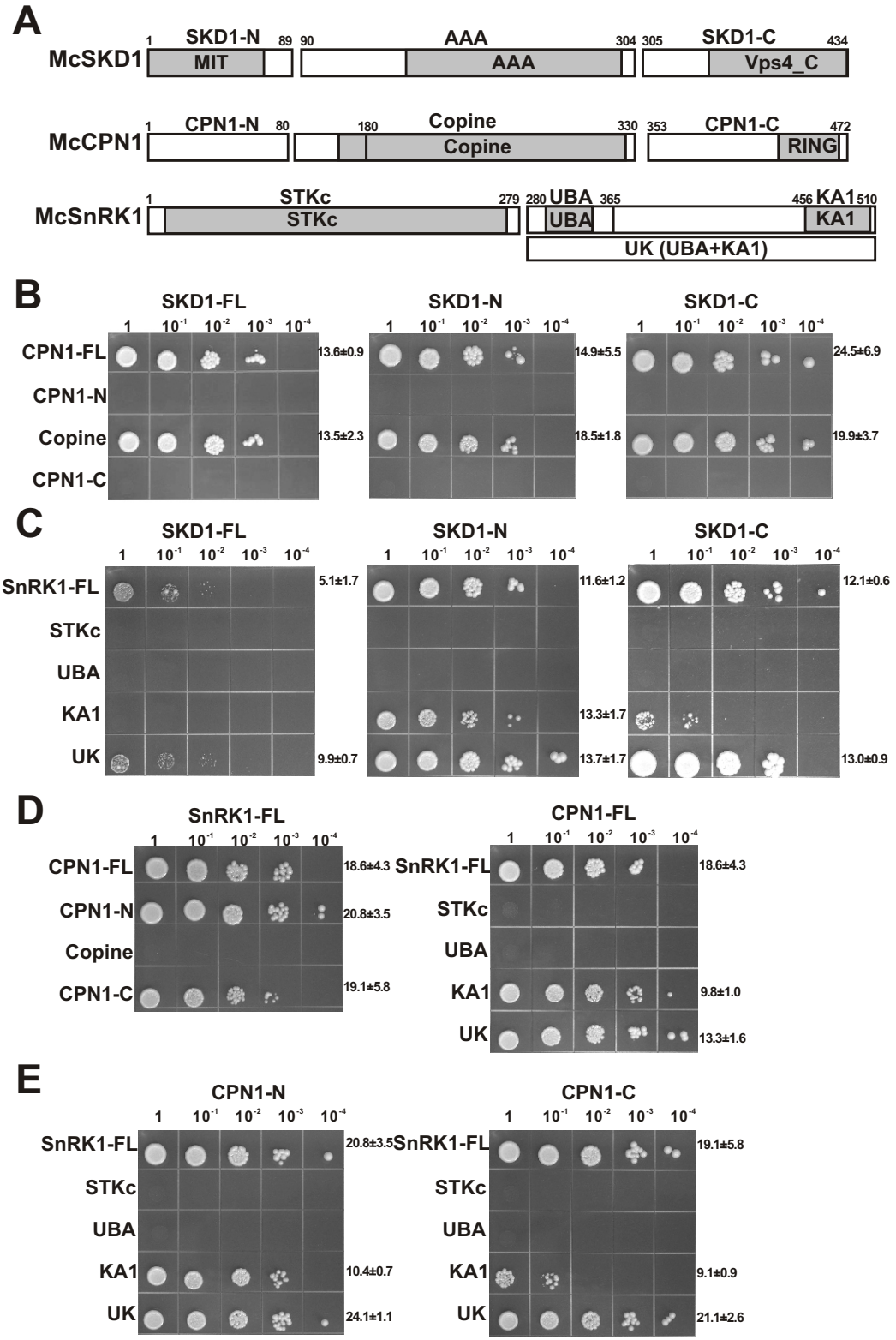

Fig. S3. Pair-wise two-hybrid analysis of domain-domain interaction between McSKD1, McCPN1 and McSnRK1
(A) Schematic representation of domain structures of McSKD1, McCPN1, and McSnRK1 and abbreviations used in (B) through (E). The known domains of each protein are shown in gray boxes. Numbers on the top indicate the positions of the construct used in Y2H interaction assay. (B) Interaction of McCPN1 with full-length McSKD1 (SKD1-FL), the N-terminal region (SKD1-N) and C-terminal region (SKD1-C). Growth of yeast AH109 transformants carrying pGBKT7-based bait plasmids and pGADT7-based prey plasmids (CPN1-FL, CPN1-N, Copine, or CPN1-C). (C) Interaction of McSnRK1 with full-length McSKD1 (SKD1-FL), the N-terminal region (SKD1-N) and C-terminal region (SKD1-C). (D) Interaction between full-length McCPN1 and full-length McSNF1. On the left, full-length McSNF1 cloned into pGADT7 plasmid was co-transformed with pGBKT7-based plasmid carrying CPN1-FL, CPN1-N, Copine, or CPN1-C. On the right, full-length McCPN1 cloned into pGBKT7 plasmid was co-transformed with pGADT7-based plasmid carrying SnRK1-FL, STKc, UBA, KA1, or UK. (E) N- and C-terminus of McCPN1 interact with the UK region of McSnRK1.

All transformants were serial diluted, spotted on SD/-Trp/-Leu/-His/-Ade selection plates, and incubated at 30°C for 3 to 5 days. At least three independent experiments provided similar results. Numbers to the right of each photograph are beta-galactosidase activity expressed in Miller units (mean±SD, n=5). The beta-galactosidase activity of transformants carrying pGBKT7-p53 and pGADT7-T (positive control) was about 60 Miller units.

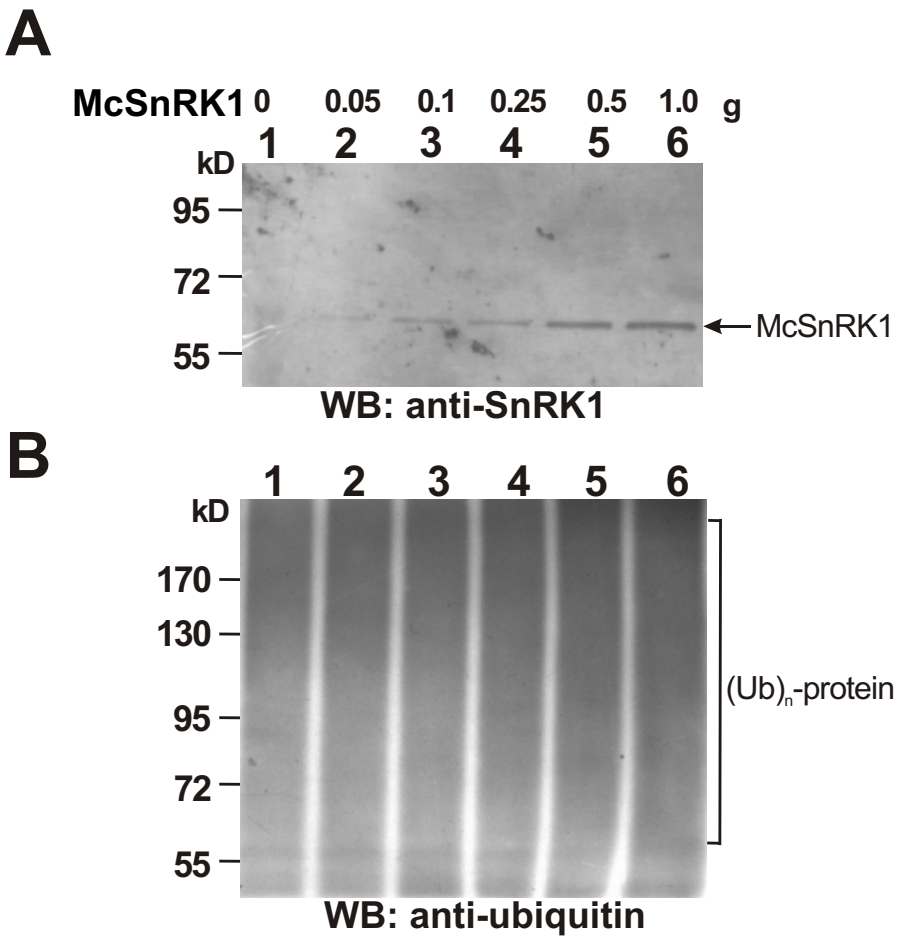

Fig. S4. *In vitro* ubiquitination reaction by McCPN1 using McSnRK1 as a substrate
Various concentrations of purified McSnRK1-$(His)_6$ were added to the complete *in vitro* ubiquitination mixture using McCPN1 as E3. After separation by 8% SDS-PAGE, anti-SnRK1 antiserum was used to detect McSnRK1 (A) and anti-ubiquitin antibody was used to detect protein-ubiquitin conjugates (B). The position of McSnRK1 is indicated by an arrow and no laddered bands appeared in the position higher than McSnRK1.

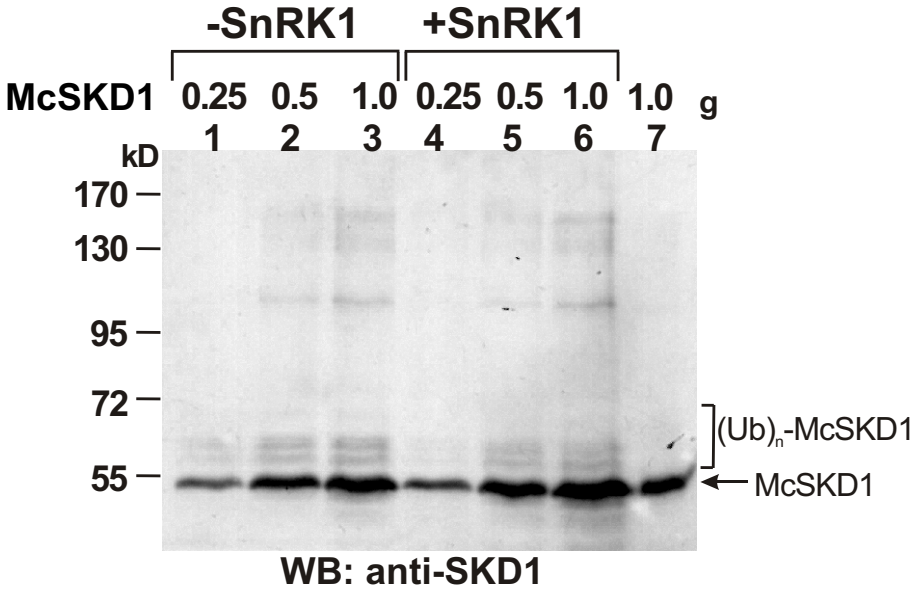

Fig. S5. The effect of McSnRK1 on McSKD1 ubiquitination
In vitro ubiquitination reactions containing McCPN1 as E3 and McSKD1 as substrate were performed in the absence or in the presence of purified McSnRK1-$(His)_6$. The reaction mixture contains 1 mM ATP that can serve as substrate for McSnRK1. Anti-SKD1antiserum was used to detect McSKD1 and its ubiquitin-conjugated forms $(UB)_n$-McSKD1. The arrow indicates the position of McSKD1 (51 kD), no significant changes of the $(UB)_n$-McSKD1 pattern in the presence of McSnRK1. Lane 7 is a reaction without E3 as a negative control.

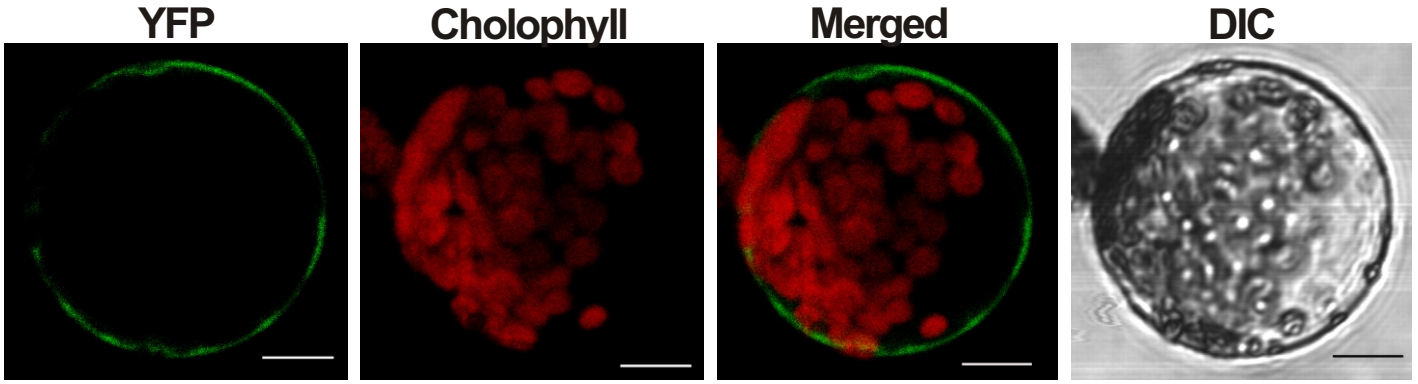

Fig. S6. Localization of McCPN1 fusion YFP on the plasma membrane of Arabidopsis protoplasts. Constructs carrying McCPN1-YFP were transfected into protoplasts isolated from mesophyll cells. Green: YFP signal; red: chlorophyll autofluorescence. DIC: differential interference contrast showing the protoplast image. All bars = 10 μm.

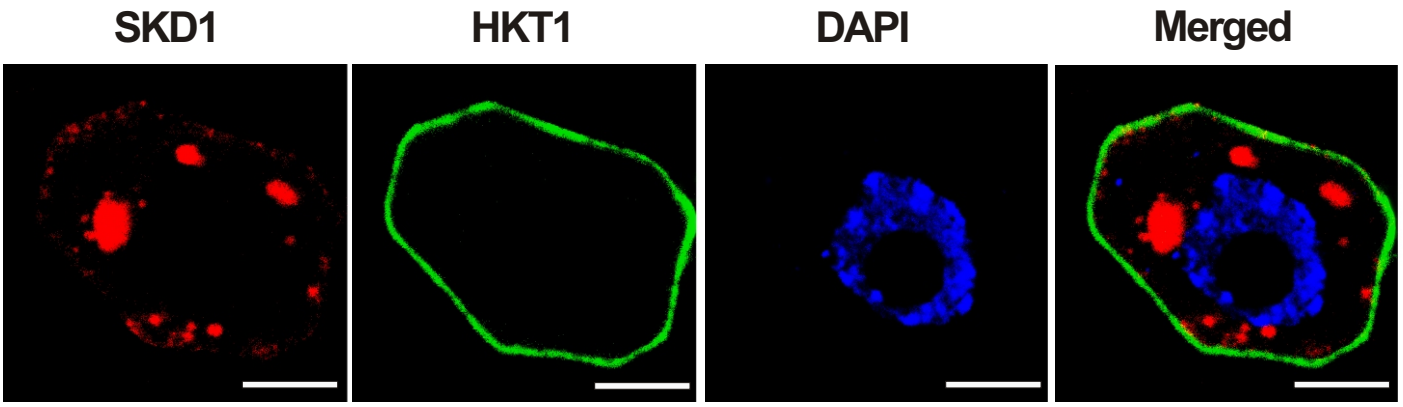

Fig. S7. Immunofluorescence detecting cellular localization of McSKD1 in root tip cells. Root cells isolated from 3-d-old seedlings were dual labeled by anti-HKT1 and anti-SKD1. HKT1 is a high-affinity K^+ transporter that serves as a plasma membrane marker. Confocal images represent single images of Cy3/Alexa 488 dual labeling of a representative cell. Blue color is DAPI staining that indicates the position of nucleus. Red fluorescence indicates McSKD1 image while HKT1 is shown by green fluorescence. Bars=5 μm

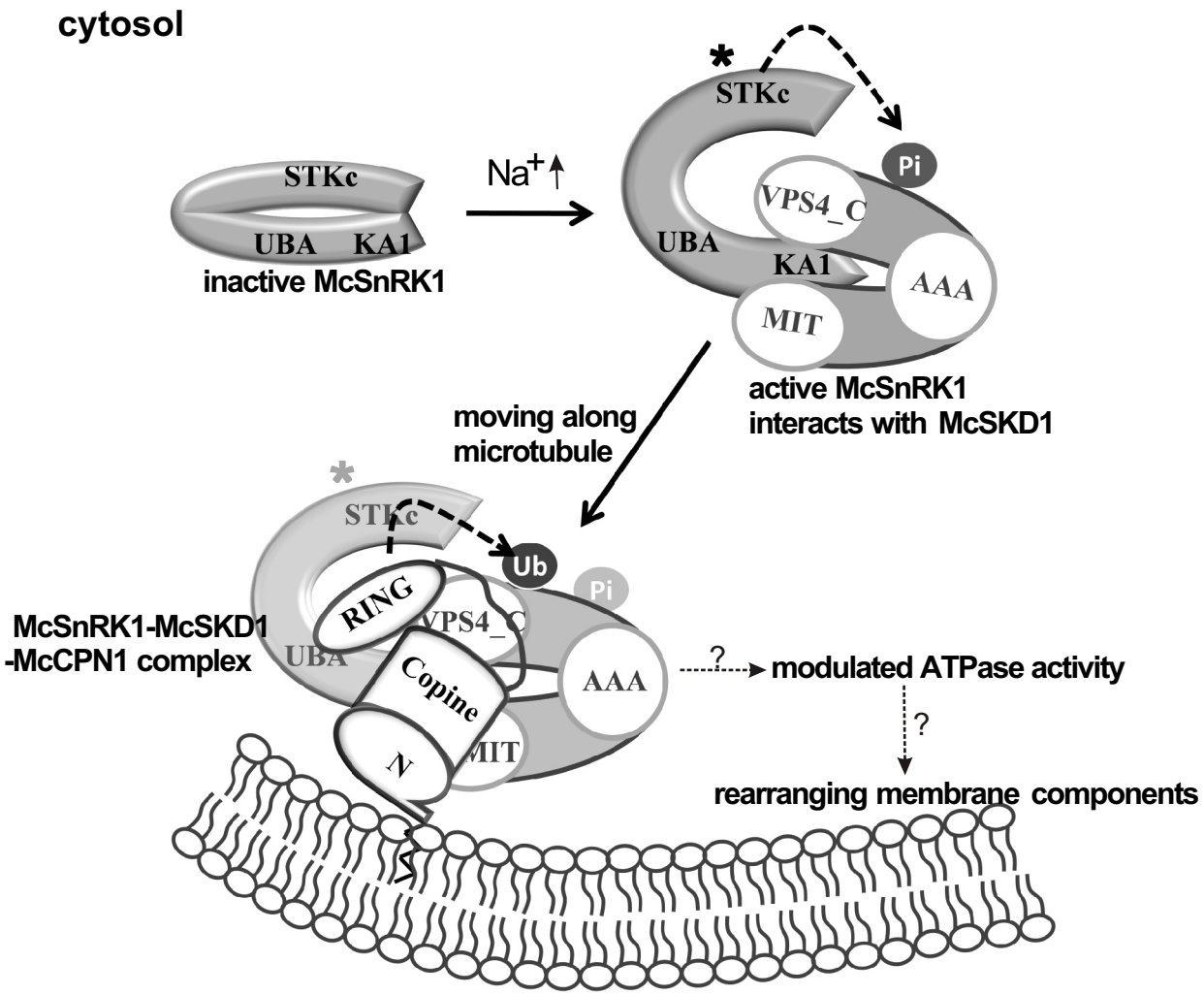

Fig. S8. A proposed model for the formation of McSKD1-McSnRK1-McCPN1 ternary complex under high salinity. Pi: addition of phosphate groups; Ub: addition of ubiquitin molecules; N: CPN1-N containing a potential site of myristoylation. The asterisk indicates the position of phosphorylated Thr-172 in the activation loop of the STKc domain.