

**Mitogen-activated protein kinase 4-like carrying a MEY motif instead of a TXY motif is involved in ozone tolerance and regulation of stomatal closure**

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**Supplementary Figure S1.** Comparison of the nucleotide sequences in open-reading frames among NtMPK4, NtMPK4L-1, and NtMPK4L-2. Bars indicate the regions used as IR constructs to generate NtMPK4L-silenced plants.

**Supplementary Figure S2.** Comparison of the amino acid sequences of NtMPK4L-1, NtMPK4L-2, and NtMPK4. A bar indicates the region used as an antigen in the production of the anti-NtMPK4L antibody. A square indicates the MEY and TXY motifs.

**Supplementary Figure S3.** Comparison of the amino acid sequences of SIMPK5 and SIMPK6. Bars indicate that sequence regions used as antigens against the anti-SIMPK5 and anti-SIMPK6 antibodies. A square indicates the MEY and TXY motifs.

**Supplementary Figure S4.** Quality test of the anti-NtMPK4L antibody. (A) SDS-PAGE analysis was performed using purified His-NtMPK4L-1 and His-NtMPK4 proteins. (B) Immunoblot analysis was performed using the anti-NtMPK4L antibody.

**Supplementary Figure S5.** Quality test of the anti-SIMPK5 and anti-SIMPK6 antibodies. (A) SDS-PAGE analysis was performed using purified His-SIMPK5 and His-SIMPK6 proteins. (B) Immunoblot analysis was performed using the anti-SIMPK5 or anti-SIMPK6 antibody.

**Supplementary Figure S6.** Subcellular localization of sGFP-fused NtMPK4 and NtMPK4L-1 in *N. tabacum*. Bar; 50  $\mu$ m.

**Supplementary Figure S7.** Crude extracts prepared from wounded leaves were separated by SDS-PAGE and stained with CBB. Tobacco leaves were wounded using a cork borer. After 0

and 10 min (A) or 0, 5, 10, 30, 60 and 120 min (B), the leaf discs were used for the preparation of crude extracts. (C) The expression level of *NtMPK4L* transcript was measured at 0, 5, 10, 30, 60 and 120 min after wounding. (D) Tobacco leaves expressing HA-SIMP6, or HA-SIMP6<sup>Y203F</sup> or HA-sGFP by agroinfiltration were wounded using a cork borer. After 0, 10 and 30 min, the leaf discs were used for the preparation of crude extracts. CBB staining or an immunoblot analysis using an anti-HA antibody was performed.

**Supplementary Figure S8.** SIMPK6 activity was upregulated by wounding. Phosphorylation of MBP as a substrate was detected by autoradiography. (A) Tobacco leaves were wounded using a cork borer. After 0 and 10 min, the leaf discs were used for the measurement of SIMPK5 or SIMPK6 activity. (B) *In vitro* activation of His-SIMP5 or His-SIMP6 by GST-SIMKK1 or GST-SIMKK1<sup>EE</sup> was examined.

**Supplementary Figure S9.** The relative expression levels of *NtMPK4L* and *NtMPK4* transcripts were measured in first-generation *NtMPK4L*-silenced plants. Five *NtMPK4L*-IR lines were compared with vector control plants.

**Supplementary Figure S10.** Leaves from wild-type (WT), *NtMPK4*-IR6 (4-IR6), *NtMPK4L*-IR6 (4L-IR6), and *NtMPK4L*-IR8 (4L-IR8) plants were wounded using a cork borer. After 0 or 10 min, the leaf discs were used for the preparation of crude extracts. CBB staining was performed.

**Supplementary Figure S11.** Relative expression levels of *NtMPK4* and *NtMPK4L* transcripts were measured. Error bars indicate standard deviations determined from three independent biological replicates. Asterisks indicate significant differences from analyses using Student's t-test compared with the relevant vector control at  $p < 0.05$  (\*).

**Supplementary Figure S12.** Wild-type tobacco leaves were exposed by ozone. (A) After 0 and 1 h, crude extracts were prepared. The crude extracts were separated by SDS-PAGE and stained

with CBB. (B) The expression levels of both *NtMPK4* and *NtMPK4L* transcripts were measured.

**Supplementary Table S1.** Primers used in this study.

# Supplementary Figure S1

NtMPK4	1	CTTCACATCTTTTTCTTGAAAGGCTACCGACACTAACAGGCCCATTAATAAACTGTCTTTG	60
NtMPK4L-1	0	-----	0
NtMPK4L-2	0	-----	0
NtMPK4	61	AACTGGGTTTGGCCAGATTTCAAFTTTTCGAGAAAAAARAAATTTTCTAAAT	119
NtMPK4L-1	1	-----CTGAAATGTCCAAAGGCTCTTCTTTCTTTTGGTCTAAATTTTCTGTTA	50
NtMPK4L-2	1	-----CTGAAATGTCCAAAGGCTCTTCTTTCTTTTGGTCTAAATTTTCTGTTA	52
NtMPK4	120	TTTTCTGTGTTTAAATTCCTATGCTGATGCTAAATTCATCTGATCAATTTTCAAAATTAAT	179
NtMPK4L-1	51	TTTTCTGTGTTTAAATTCCTATGCTGATGCTAAATTCATCTGATCAATTTTCAAAATTAAT	107
NtMPK4L-2	53	TTTTCTGTGTTTAAATTCCTATGCTGATGCTAAATTCATCTGATCAATTTTCAAAATTAAT	101
NtMPK4	180	TTTGGAGTTTCCAAACACATGGGGTCTGATGTCAGTATAATGTGATGGTATCTC	239
NtMPK4L-1	108	TTAAGAGGAGTTCCAAACACATGGGGTCTGATGTCAGTATAATGTGATGGTATCTC	167
NtMPK4L-2	102	TTAAGAGGAGTTCCAAACACATGGGGTCTGATGTCAGTATAATGTGATGGTATCTC	161
NtMPK4	240	TTTGAAGTTTCAAGAAAATATGTTCTCCCTATTAGACCTATTGGTCGTGGTCTAATGGC	296
NtMPK4L-1	168	TTTGAAGTTTCAAGAAAATATGTTCTCCCTATTAGACCTATTGGTCGTGGTCTAATGGC	227
NtMPK4L-2	162	TTTGAAGTTTCAAGAAAATATGTTCTCCCTATTAGACCTATTGGTCGTGGTCTAATGGC	221
NtMPK4	297	TTTTTTGCTGCTATGAATCTGAACTGAGGAGACCTGAGGAGTAGCAATTAAGAGATTGGC	356
NtMPK4L-1	228	TTGGTTTGTGCTGCTATGAATCTGAACTGAGGAGACCTGAGGAGTAGCAATTAAGAGATTGGC	287
NtMPK4L-2	222	TTGGTTTGTGCTGCTATGAATCTGAACTGAGGAGACCTGAGGAGTAGCAATTAAGAGATTGGC	281
NtMPK4	357	AATGCATTTGATAATGTAATAGATGCAAAAAGGACATTAAGAGAGATAAAGCTTCTGAT	416
NtMPK4L-1	288	AATGCATTTGATAATGTAATAGATGCAAAAAGGACATTAAGAGAGATAAAGCTTCTGAT	347
NtMPK4L-2	282	AATGCATTTGATAATGTAATAGATGCAAAAAGGACATTAAGAGAGATAAAGCTTCTGAT	341
NtMPK4	417	CACATGGATCATGAGAATGTTTTCGCAATTAAGATGTTTATAAGGCTCTCAAAATTAAG	476
NtMPK4L-1	348	CACATGGATCATGAGAATGTTTTCGCAATTAAGATGTTTATAAGGCTCTCAAAATTAAG	407
NtMPK4L-2	342	CACATGGATCATGAGAATGTTTTCGCAATTAAGATGTTTATAAGGCTCTCAAAATTAAG	401
NtMPK4	477	AAATTCATGATGTTTACATTTGTTTATGAACTGATGGACACTGATCTTCATCAGATTATT	536
NtMPK4L-1	408	AAATTCATGATGTTTACATTTGTTTATGAACTGATGGACACTGATCTTCATCAGATTATT	467
NtMPK4L-2	402	AAATTCATGATGTTTACATTTGTTTATGAACTGATGGACACTGATCTTCATCAGATTATT	461
NtMPK4	537	TTTCCAACCAACAGTTGACTGATGAACTGCGGATTTTCTTACCAATTTATTCGGA	596
NtMPK4L-1	468	TTTCCAACCAACAGTTGACTGATGAACTGCGGATTTTCTTACCAATTTATTCGGA	527
NtMPK4L-2	462	TTTCCAACCAACAGTTGACTGATGAACTGCGGATTTTCTTACCAATTTATTCGGA	521
NtMPK4	597	GGACTTAAGTACATTCACCTGCCCCATCTGTCATCGTGTATTAATAAAGCAGCAATTTG	656
NtMPK4L-1	528	GGACTTAAGTACATTCACCTGCCCCATCTGTCATCGTGTATTAATAAAGCAGCAATTTG	587
NtMPK4L-2	522	GGACTTAAGTACATTCACCTGCCCCATCTGTCATCGTGTATTAATAAAGCAGCAATTTG	581
NtMPK4	657	TTTCTCAATGCAAAAATGTCACCTGAAAGATTGGAGATTTTGGCTTGCAGAGCAACT	716
NtMPK4L-1	588	TTTCTCAATGCAAAAATGTCACCTGAAAGATTGGAGATTTTGGCTTGCAGAGCAACT	647
NtMPK4L-2	582	TTTCTCAATGCAAAAATGTCACCTGAAAGATTGGAGATTTTGGCTTGCAGAGCAACT	641
NtMPK4	717	BAGACAGATTTTCATGATGGAATATTGTTTACACGCTGGTATCGTGACCAAGATTGGTA	776
NtMPK4L-1	648	BAGACAGATTTTCATGATGGAATATTGTTTACACGCTGGTATCGTGACCAAGATTGGTA	707
NtMPK4L-2	642	BAGACAGATTTTCATGATGGAATATTGTTTACACGCTGGTATCGTGACCAAGATTGGTA	701
NtMPK4	777	TAAATTTGTTTACAGATGATACTGCAAAATGATGTTTGGTCAGTAGGTCGACTACTGGT	836
NtMPK4L-1	708	TAAATTTGTTTACAGATGATACTGCAAAATGATGTTTGGTCAGTAGGTCGACTACTGGT	767
NtMPK4L-2	702	TAAATTTGTTTACAGATGATACTGCAAAATGATGTTTGGTCAGTAGGTCGACTACTGGT	761
NtMPK4	837	BAAATATTGACAAGACAACCCCTTTTCCCGGCAGAGATTAATGACCAAGATTGAGACT	895
NtMPK4L-1	768	BAAATATTGACAAGACAACCCCTTTTCCCGGCAGAGATTAATGACCAAGATTGAGACT	826
NtMPK4L-2	762	BAAATATTGACAAGACAACCCCTTTTCCCGGCAGAGATTAATGACCAAGATTGAGACT	820
NtMPK4	896	TATCACTGAGCTCATAGGCTCACCTGATGATGCTGCTTTGGTTTCTCCGGAGTATAAA	955
NtMPK4L-1	827	TATCACTGAGCTCATAGGCTCACCTGATGATGCTGCTTTGGTTTCTCCGGAGTATAAA	886
NtMPK4L-2	821	TATCACTGAGCTCATAGGCTCACCTGATGATGCTGCTTTGGTTTCTCCGGAGTATAAA	880
NtMPK4	956	TGCTTCAAGATACTTACCTGAGCTCCCACTATGTTTCAACAAATTTTGGCTGAGATT	1015
NtMPK4L-1	887	TGCCCCAAGATACTTACCTGAGCTCCCACTATGTTTCAACAAATTTTGGCTGAGATT	946
NtMPK4L-2	881	TGCCCCAAGATACTTACCTGAGCTCCCACTATGTTTCAACAAATTTTGGCTGAGATT	940
NtMPK4	1016	CCCTAATTCATCTCCCTGAGCTGTGATTTGGTTGAAAAAATGCTCATCTTTGATCCAAG	1075
NtMPK4L-1	947	CCCTAATTCATCTCCCTGAGCTGTGATTTGGTTGAAAAAATGCTCATCTTTGATCCAAG	1006
NtMPK4L-2	941	CCCTAATTCATCTCCCTGAGCTGTGATTTGGTTGAAAAAATGCTCATCTTTGATCCAAG	1000
NtMPK4	1076	CAGGCCTTTACTTTGATGAGCTCTCTCCATCTTACCTTGGCCCTTTCATGATAT	1135
NtMPK4L-1	1007	CAGGCCTTTACTTTGATGAGCTCTCTCCATCTTACCTTGGCCCTTTCATGATAT	1066
NtMPK4L-2	1001	CAGGCCTTTACTTTGATGAGCTCTCTCCATCTTACCTTGGCCCTTTCATGATAT	1060
NtMPK4	1136	CAATGAGGAGCCCTTTTGTCTTAATCTTTTCAAGTTTGGCTTTGAGCAGCCATCTTTAC	1195
NtMPK4L-1	1067	CAACGAGGAGCCCTTTTGTCTTAATCTTTTCAAGTTTGGCTTTGAGCAGCCATCTTTAC	1126
NtMPK4L-2	1061	CAACGAGGAGCCCTTTTGTCTTAATCTTTTCAAGTTTGGCTTTGAGCAGCCATCTTTAC	1120
NtMPK4	1196	TGAAGATAAATCAAGGAGCTCATCTGGAGGGAACTCGTAAATTAATCTCCATCCAAC	1255
NtMPK4L-1	1127	TGAAGATAAATCAAGGAGCTCATCTGGAGGGAACTCGTAAATTAATCTCCATCCAAC	1186
NtMPK4L-2	1121	TGAAGATAAATCAAGGAGCTCATCTGGAGGGAACTCGTAAATTAATCTCCATCCAAC	1180
NtMPK4	1256	TCAATGAAAGAGATGTGGTTATATGATGATACAAATAGAAATTTGGTTATTTAACTTT	1315
NtMPK4L-1	1187	TCAATGAAAGAGATGTGGTTATATGATGATACAAATAGAAATTTGGTTATTTAACTTT	1244
NtMPK4L-2	1181	TCAATGAAAGAGATGTGGTTATATGATGATACAAATAGAAATTTGGTTATTTAACTTT	1238
NtMPK4	1316	TGATATACTGTTTCAATTTTGTAAATGTTCCCTGATGAAATTAATCTCCATCCAAC	1374
NtMPK4L-1	1245	TGATATACTGTTTCAATTTTGTAAATGTTCCCTGATGAAATTAATCTCCATCCAAC	1304
NtMPK4L-2	1239	TGATATACTGTTTCAATTTTGTAAATGTTCCCTGATGAAATTAATCTCCATCCAAC	1298
NtMPK4	1375	GG-----FGAGTTC	1383
NtMPK4L-1	1305	GGAGCTGTTGAGTTC	1319
NtMPK4L-2	1299	GGAGCTGTTGAGTTC	1313

# Supplementary Figure S2

NtMPK4L-1	1	MSVDSSSGDHSSNIRGVPTHGGRYVQYNVHGSLFEVSRKYVPPPIRPIGRGANGMVCAAM	59
NtMPK4L-2	1	MSVDSSSGDHSSNIRGVPTHGGRYVQYNVHGSLFEVSRKYVPPPIRPIGRGANGMVCAAM	59
NtMPK4	1	MEATISGDQGVQS-NFKGVPTHGGRYVQYNVHGSLFEVSRKYVPL-RPWGRGAIISIVCAAM	58
NtMPK4L-1	60	NSETREEVAIKKIGNAFDNVIDAKRTLREIKLLSHMDHENVIAIKDVIRPPQKKNFNDVY	119
NtMPK4L-2	60	NSETREEVAIKKIGNAFDNVIDAKRTLREIKLLSHMDHENVIAIKDVIRPPQKKNFNDVY	119
NtMPK4	59	NSETREEVAIKKIGNAFDNVIDAKRTLREIKLLSHMDHENVIAIKDVIRPPQKKNFNDVY	118
NtMPK4L-1	120	IVYELMDTDLHQIIHSNQQLTDEHCRHFLYQVLRGLKYYIHSANILHRDLKPSNLLVNAKC	179
NtMPK4L-2	120	IVYELMDTDLHQIIHSNQQLTDEHCRHFLYQVLRGLKYYIHSANILHRDLKPSNLLVNAKC	179
NtMPK4	119	IVYELMDTDLHQIISNQQLTDEHCRHFLYQVLRGLKYYIHSANILHRDLKPSNLLVNAKC	178
NtMPK4L-1	180	DLKIGDFGLARTTTTETDFMMEYCVTRWYRAPELLNCSEYTSVIDVWSVGCILGEILTRQ	239
NtMPK4L-2	180	DLKIGDFGLARTTTTETDFMMEYCVTRWYRAPELLNCSEYTSVIDVWSVGCILGEILTRQ	239
NtMPK4	179	DLKIGDFGLARTTTTETDFMMEYCVTRWYRAPELLNCSEYTSVIDVWSVGCILGEILTRQ	238
NtMPK4L-1	240	PLFPPGRDYVHQLRLITELIGSPDDASLGFLRSNNARRYVRQLPRYPQQFSARFPNSSPR	299
NtMPK4L-2	240	PLFPPGRDYVHQLRLITELIGSPDDASLGFLRSNNARRYVRQLPRYPQQFSARFPNSSPR	299
NtMPK4	239	PLFPPGRDYVHQLRLITELIGSPDDASLGFLRSNNARRYVRQLPRYPQQFSARFPNSSPR	298
NtMPK4L-1	300	AVDLEKMLIFDPSRRITVDEALSHPYLAPLHDINEEPCPRPFLDFEQPSFTEDNIKE	359
NtMPK4L-2	300	AVDLEKMLIFDPSRRITVDEALSHPYLAPLHDINEEPCPRPFLDFEQPSFTEDNIKE	359
NtMPK4	299	AVDLEKMLIFDPSRRITVDEALSHPYLAPLHDINEEPCPRPFLDFEQPSFTEDNIKE	358
NtMPK4L-1	360	LIWREAVKFNPDPTH	374
NtMPK4L-2	360	LIWREAVKFNPDPTH	374
NtMPK4	359	LIWREAVKFNPDPTH	373

# Supplementary Figure S3

S1MPK5 1 M--EASITGDHGVQSNFRGVPTHGGRYVQYNVYGNLFEVSKKYVPL-RPVGRGAYGIVCAA 57  
 S1MPK6 1 MSLDSSADHGGHSNIRGVPTHGGRYVQYNVHGS LFEVSKKYVVPPIRPIGRGANGMVCAA 60

S1MPK5 58 LNSETREEVAIKKIGNAFDNRIDAKRTLREIKLLRHMDHENVIAIRDIIRPPQTEINFNDV 117  
 S1MPK6 61 VNSETREEVAIKKIGNAFDNVIDAKRTLREIKLLSHMDHENVIAIKDVIIRPPQKKNFNDV 120

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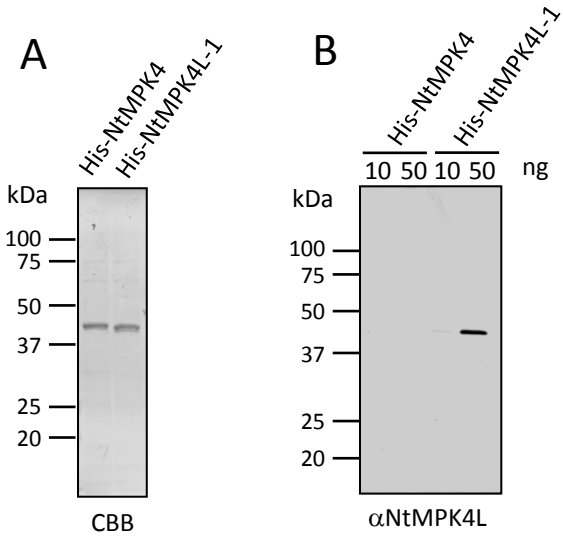
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S1MPK5 238 QPLFPGRDYVHQLKRLITELIGSPDDASLGFLRSNNARRVYRQLPQYPRQQFAAKFPNASP 297  
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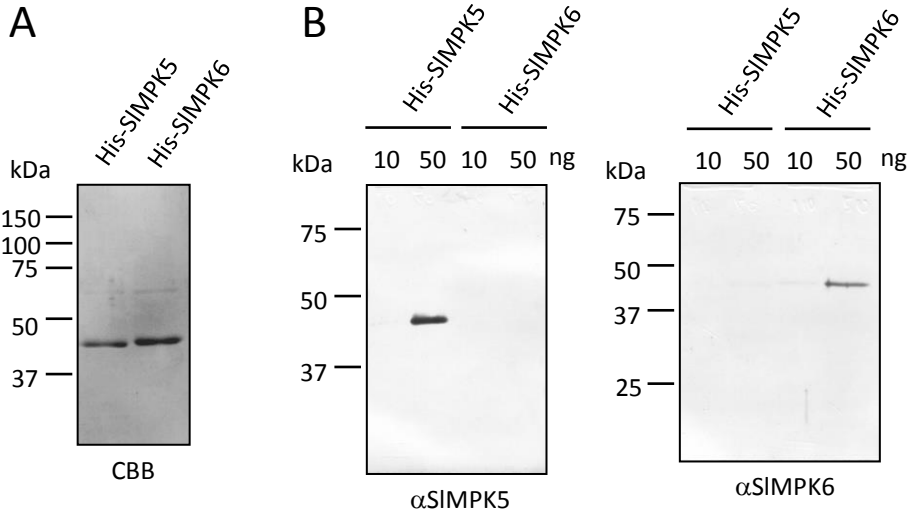
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# Supplementary Figure S4

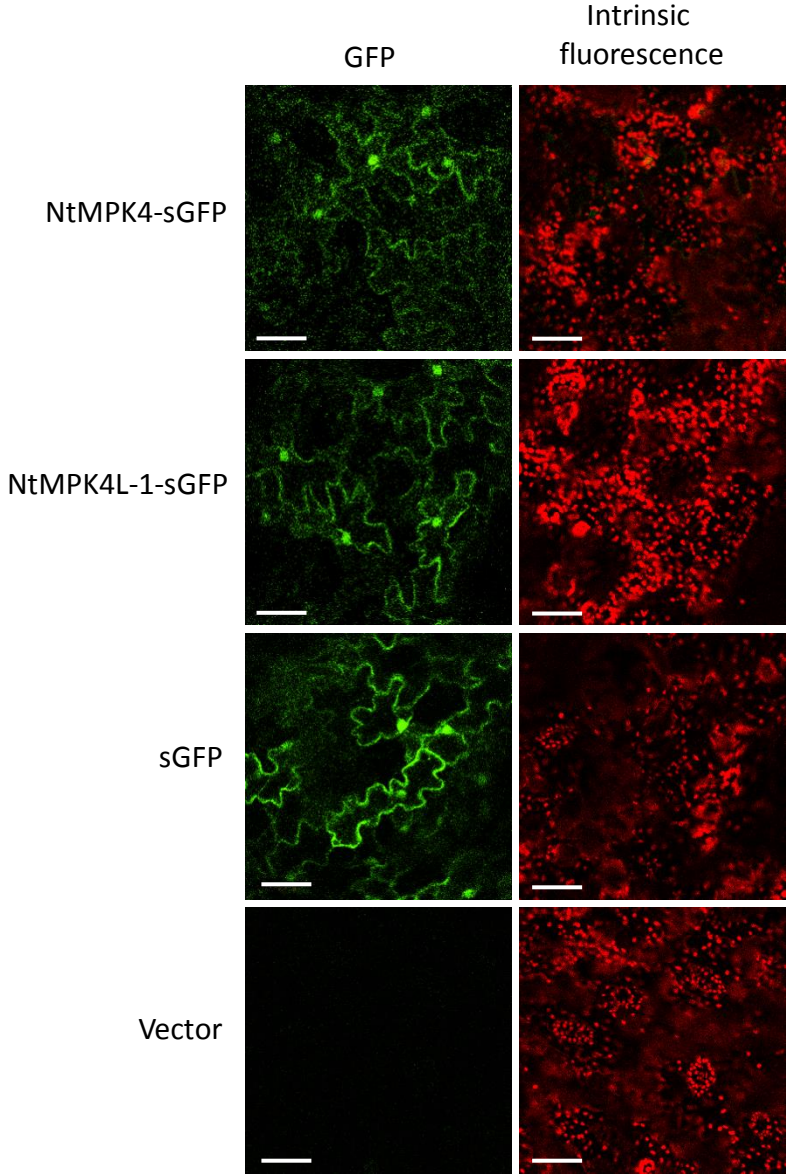


# Supplementary Figure S5

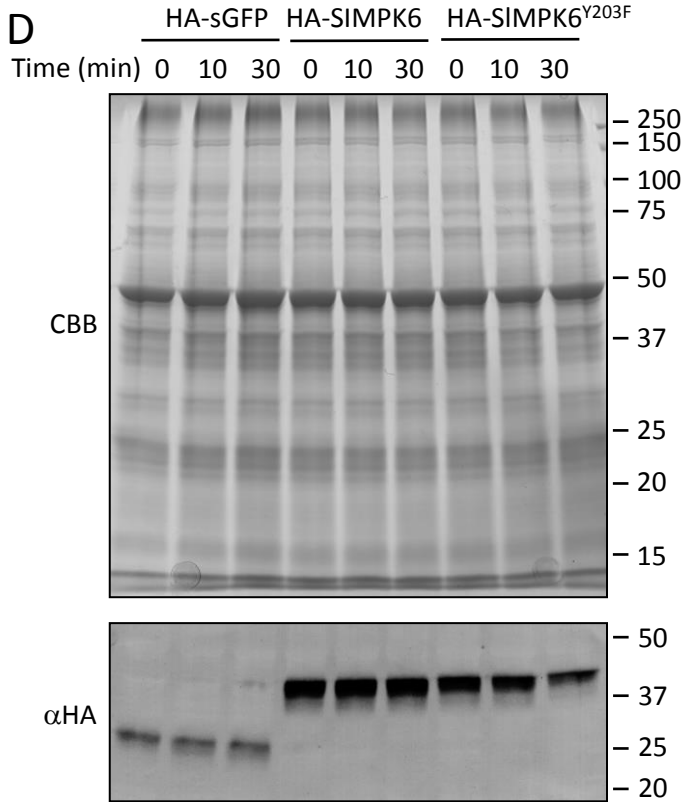
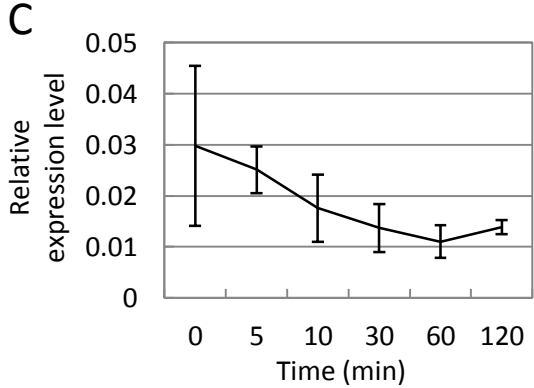
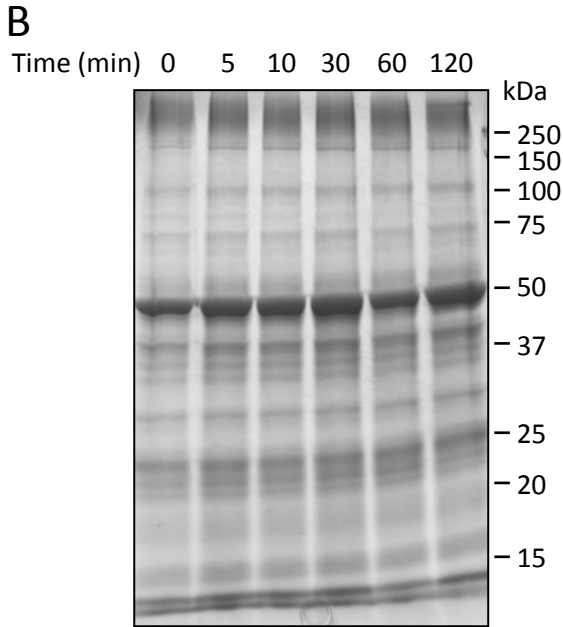
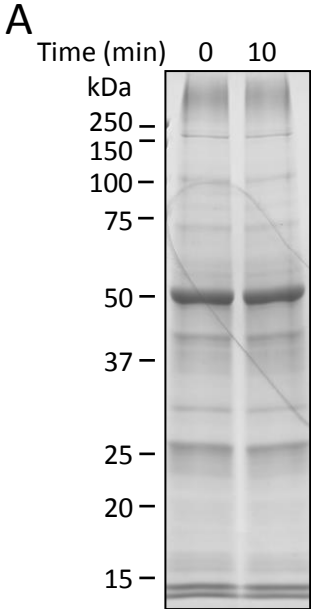




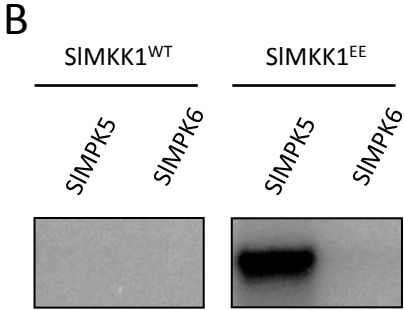
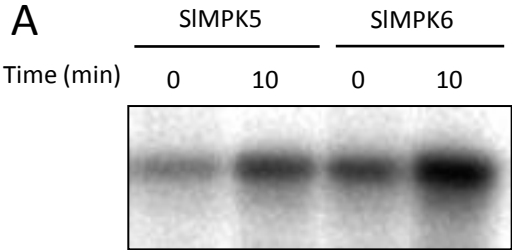
# Supplementary Figure S6



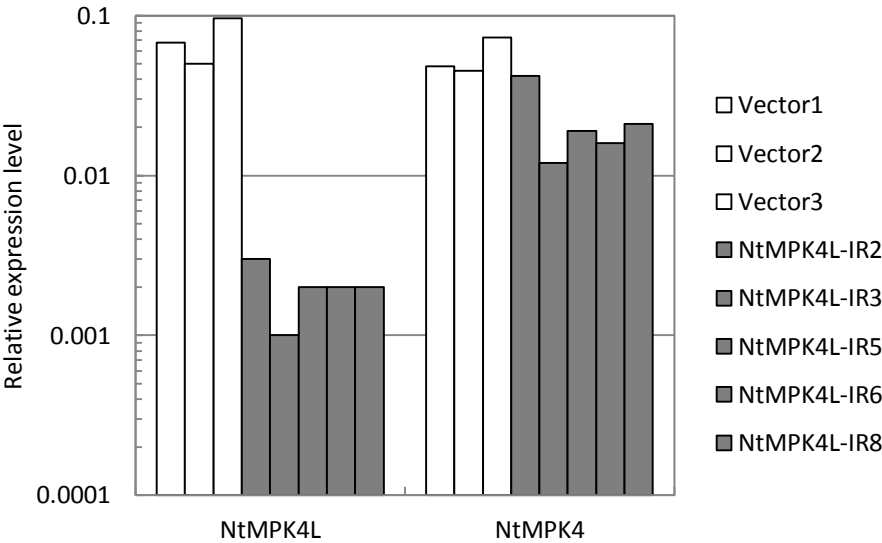
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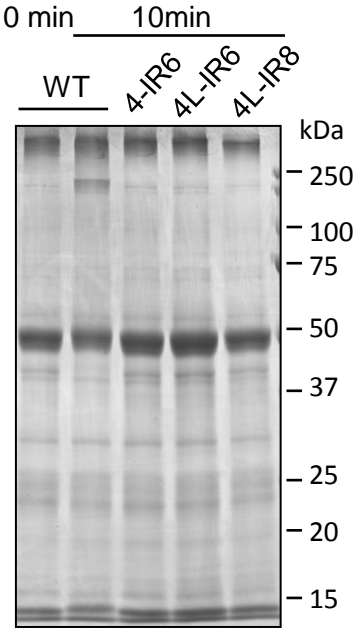
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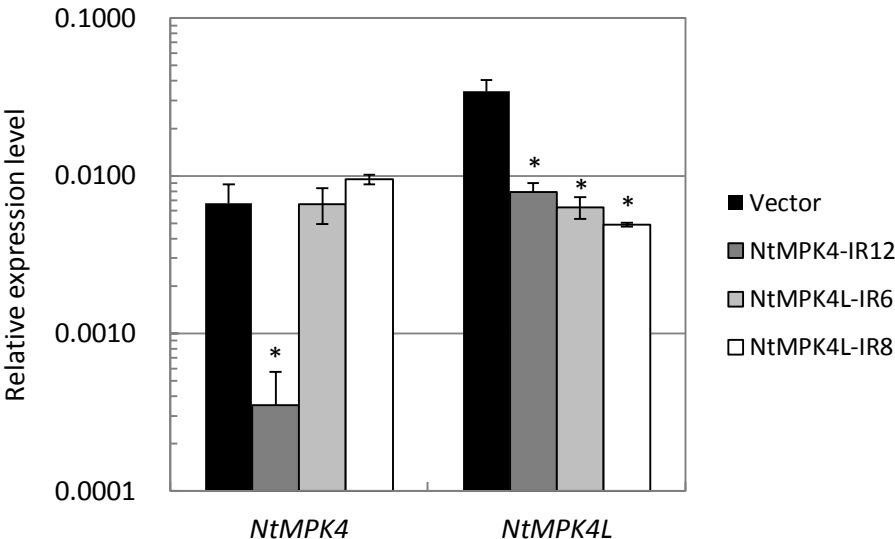
# Supplementary Figure S9



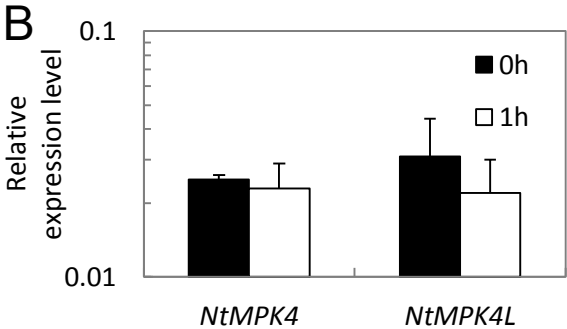
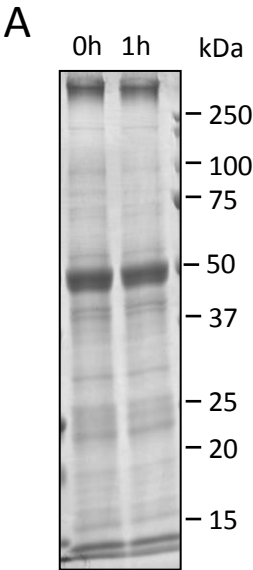
Supplementary Figure S10



Supplementary Figure S11



# Supplementary Figure S12



Supplementary Table S1 Primers used in this study

Primer	Sequence (5' - 3')	Application
NtMMPK4L Dege 5'	GGIGTICCIACICAYGGIGG	Cloning
NtMMPK4L Dege 3'	TGIGTIGGRTCIGGRTTTRAAAYTTIACIGC	Cloning
NtMPK4L 5'	CTGAAATGTCCAAAGGCTCTTC	Cloning
NtMPK4L 3'	GAACTCAACAGCTCCACCATA	Cloning
5'+3'UTR BamHI 5'	TTAGGATCCCTGAAACCAAAGGCTC	Silencing vector
5'UTR+3'UTR TR Rv	CAGGATTAATTTTACAGCCCATGTGTTGGTACTCCTC	Silencing vector
3'UTR Fw	GCTGTAAAATTTAATCCTGATCC	Silencing vector
5'+3'UTR XhoI 3'	TTACTCGAGGAACTCAACAGCTCCACC	Silencing vector
5'+3'UTR SacI 5'	TTAGAGCTCCTGAAATGTCCAAAGGCTC	Silencing vector
5'+3'UTR KpnI 3'	TTAGGTACCGAACTCAACAGCTCCACC	Silencing vector
NtActin RT 51	GGGTTTGCTGGAGATGATGCT	qPCR
NtActin RT 31	GCTTCGTCACCAACATATGCAT	qPCR
NtMPK4L-Fw-2	TCAGAGTATACATCGGCAATTGATG	qPCR
NtMPK4L-Rv-2	ATAAGTCTCAACTGGTGTACATAATCTC	qPCR
NtMPK4-5'-1	GTTGGGTTGATTTCTATGGAAG	qPCR
NtMPK4-rv-1	CATAACAATGTAGACATCATTGAAATTCTC	qPCR
NtMPK4 NdeI 5'	TTCCATATGATGGAAGCAATTTTCAGGTGATC	Recombinant protein
NtMPK4 BamHI 3'	TTCGGATCCTCAGTGAGTTGGATCTGG	Recombinant protein
NtMPK4L NdeI 5'	TTCCATATGATGTCTGTTGATTTCGAGTTC	Recombinant protein
NtMPK4L BamHI 3'	TTCGGATCCTCAATGAGTTGGATCAGG	Recombinant protein
5' NdeI-SIMPK5	CTACATATGGATCATGACAATGTGATT	Recombinant protein
3' BamHI-SIMPK5	ATCGGATCCTCAGTGAGTTGAATCTGGA	Recombinant protein
5' NdeI-SIMPK6	CATATGTCTCTTGATTCAAGTTCAGCT	Recombinant protein
3' BamHI-SIMPK6	GGATCCTCAATGAGTTGGATCA	Recombinant protein
5' EcoRI-SIMKK1	GAATTCATGAAGAAAGGATCTTTTGCA	Recombinant protein
3' SalI-SIMKK1	GTCGACTTATAGCTCAGTAAGTGT	Recombinant protein
SIMKK1 <sup>EE</sup> Fw	GCAAGCGAATCTGGACTGGCCAATGAATTTGTC	Recombinant protein
SIMKK1 <sup>EE</sup> Rv	GACAAATTCATTGGCCAGTCCAGATTCGCTTGC	Recombinant protein
SIMPK6YF Fw	TTCATGATGGAATTTTGTGTGACACGCTGGTACCGT	Immuno complex kinase assay
SIMPK6YF Rv	ACGGTACCAGCGTGTACACAAAATTCCATCATGAA	Immuno complex kinase assay
SIMPK6-BamHI Fw	CTAGGATCCATGTCTCTTGATTCAAGTTCA	Immuno complex kinase assay
SIMPK6-XhoI Rv	ATTCTCGAGTCAATGAGTTGGATCAGGATT	Immuno complex kinase assay
EcoRI-sGFP	TACGAATTCATGGTGAGCAAGGGCGAGG	Immuno complex kinase assay
sGFP-XhoI	AGTCTCGAGTTACTTGTACAGCTCGTCCATGC	Immuno complex kinase assay
SpeI-sGPF-F	TACTAGTATGGTGAGCAAGGGCGAGGAG	Subcellular localization
SacI-sGFP-R	TGAGCTCTTACTTGTACAGCTCGTCCATG	Subcellular localization
XbaI-NtMPK4-F	TATCTAGAATGGAAGCAATTTTCAGGTGATCAAG	Subcellular localization
EcoRI-NtMPK4-R	AGAATTCGTGAGTTGGATCTGGATTAATTTTAC	Subcellular localization
XbaI-NtMPK4L-1-F	TATCTAGAATGTCTGTTGATTTCGAGTTTCAGG	Subcellular localization
EcoRI-NtMPK4L-1-R	AGAATTCATGAGTTGGATCAGGATTAATTTTACAGC	Subcellular localization