Appendix

The Arabidopsis CERK1-associated kinase PBL27 connects chitin perception to MAPK activation

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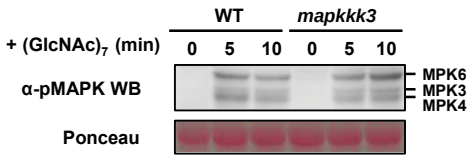
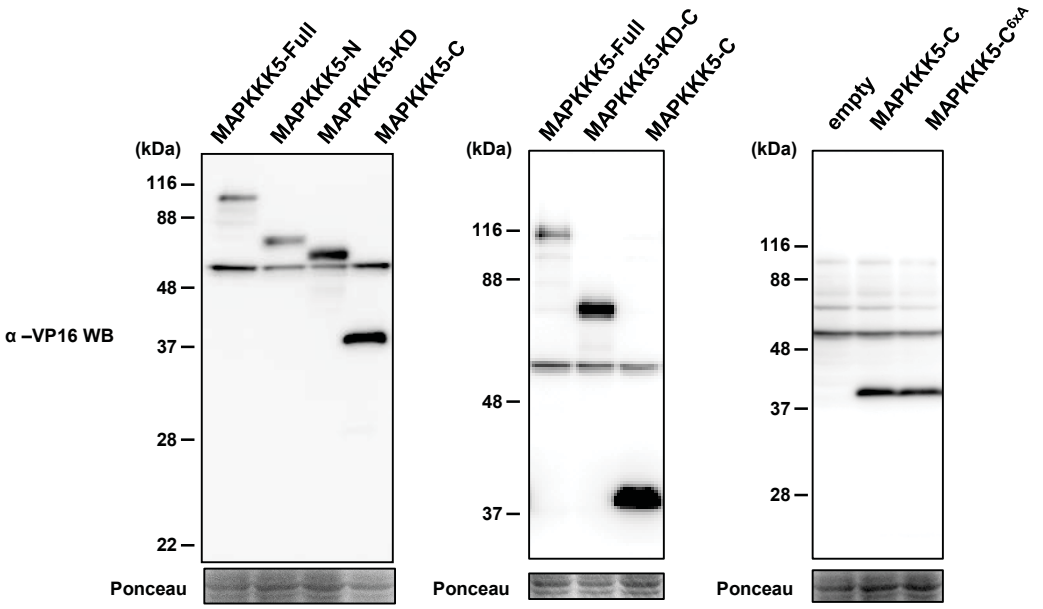


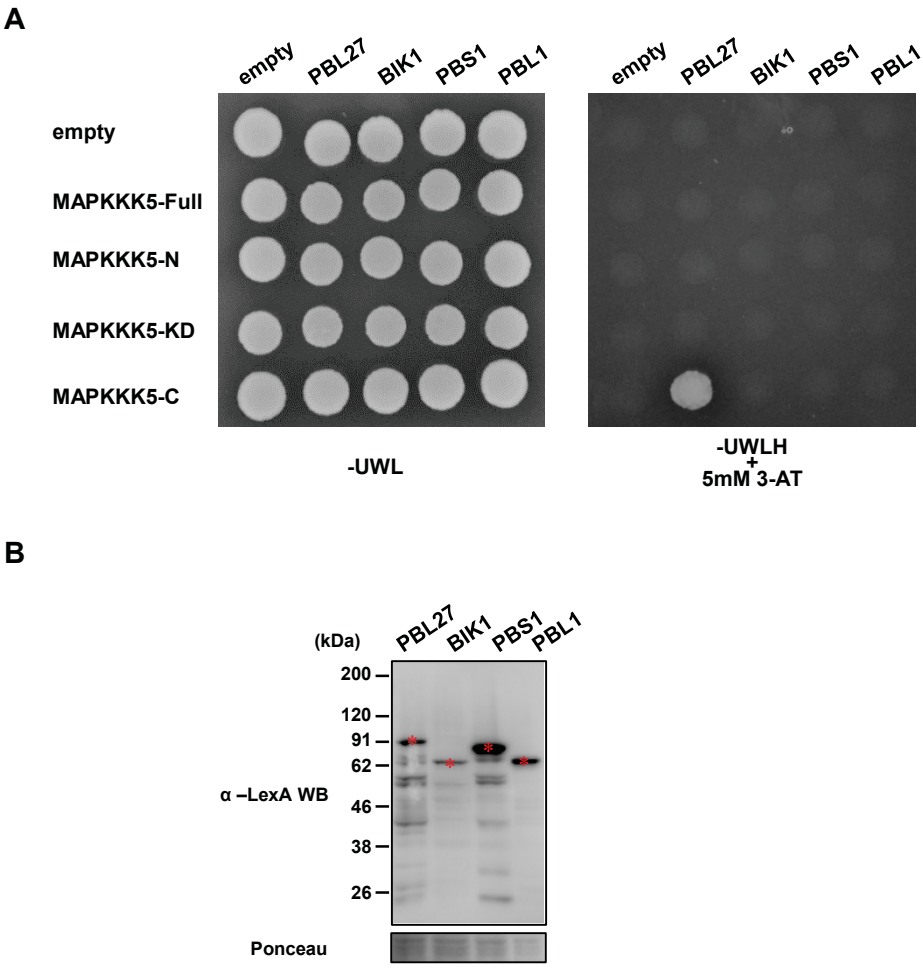
Appendix Figure S1. Chitin-Induced Activation of MAPKs in the *mapkkk3* Mutant (SALK_203147C).

The MAPK activation was analyzed by immunoblot with α-pMAPK. The protein loading control was shown by Ponceau staining. Experiments were repeated at least three times with similar results.



Appendix Figure S2. The Levels of the VP16-fused Proteins in Yeast.

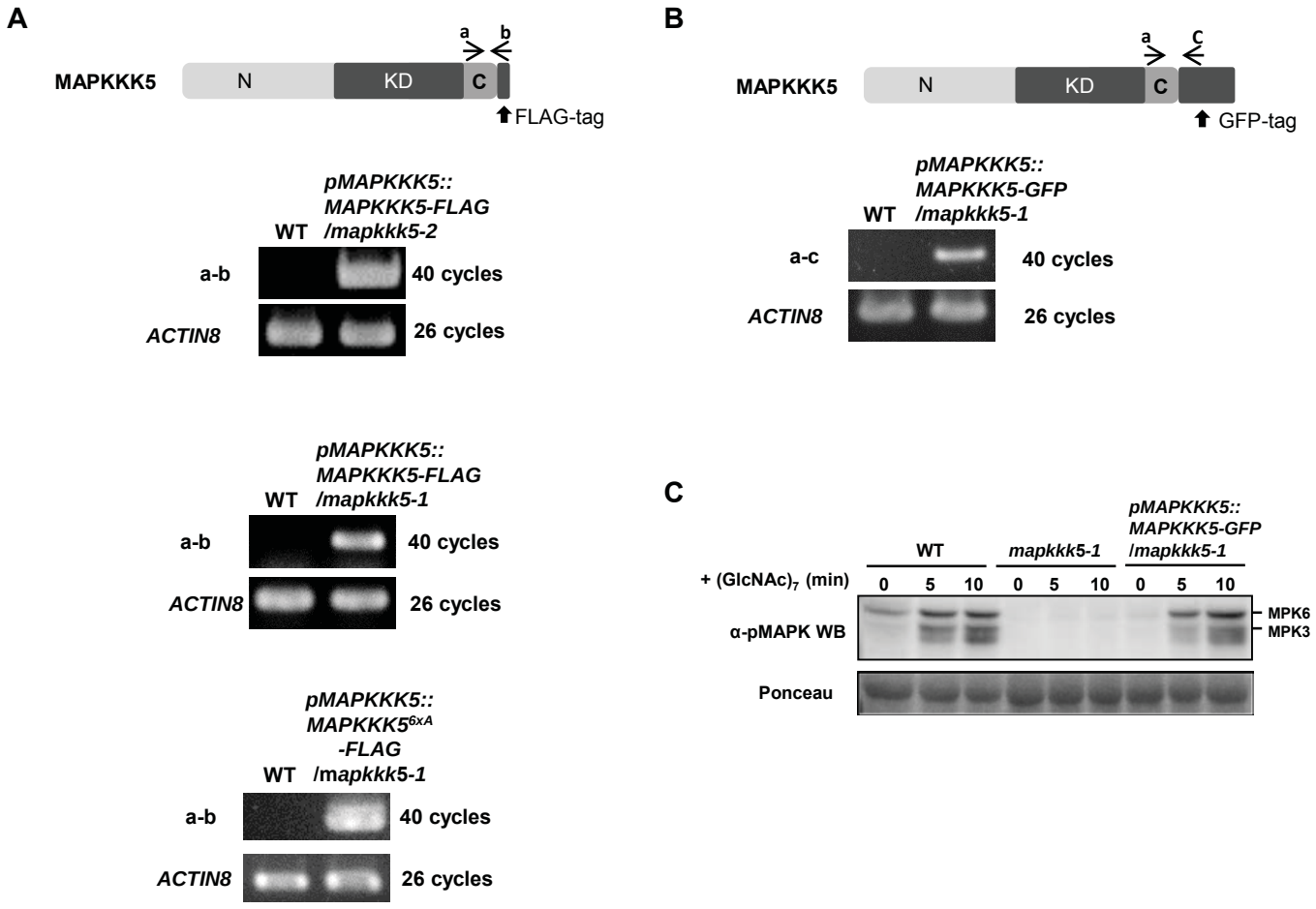
Immunoblot analysis of yeast extracts containing the VP16 fusion proteins. Total proteins were prepared from the yeast cells used for the two-hybrid assays in Figure1B, 6E and 8A, and subjected to immunoblots with α-VP16. The protein loading control was shown by Ponceau staining.



Appendix Figure S3. MAPKKK5-C Interacts with PBL27 but not Three Related RLCKs, BIK1, PBS1 and PBL1 in Yeast Two-Hybrid Experiments.

A The growth of yeast colonies on plates (-ULWH) lacking uracil (U), leucine (L), tryptophan (W), and histidine (H) with 5 mM 3-aminotriazole (3-AT) indicates a positive interaction.

B Immunoblot analysis of yeast extracts containing the LexA fusion proteins. Protein extracts from the yeast cells were separated on a 10% SDS-PAGE gel, transferred to PVDF membrane and incubated with α-LexA. The bands marked with an asterisk correspond to the products of RLCKs.



Appendix Figure S4. Expression of the Transgenes in the *MAPKKK5-FLAG* and *MAPKKK5-GFP* Plants.

A (Upper panel) Structure of transgene. Exons are indicated by black boxes. Arrows indicate specific primers. (Bottom panel) Expression of *MAPKKK5-FLAG* was analyzed by semi-quantitative RT-PCR using specific primers for the MAPKKK5 and FLAG sequences. *ACTIN8* (At1g49240) was used as a control. The PCR-amplifications for transgene and *ACTIN8* were carried out using 40 and 26 cycles, respectively.

B (Upper panel) Structure of transgene. Exons are indicated by black boxes. Arrows indicate specific primer. (Bottom panel) Expression of *MAPKKK5-GFP* was analyzed by semi-quantitative RT-PCR using specific primers for the MAPKKK5 and GFP sequences. *ACTIN8* was used as a control. The PCR-amplifications for transgene and ACTIN8 were carried out using 40 and 26 cycles, respectively.

C Complementation of chitin-induced MAPK activation in *mapkkk5* mutants by expression of *MAPKKK5-GFP*. MAPK activation was analyzed by immunoblots with α-pMAPK.

Logo	Database	Name	*p*-value	Adjusted *p*-value
	ArabidopsisPBM 20140210	KAN1	2.11e-9	2.38e-7
	ArabidopsisPBM 20140210	ANAC55_2	3.56e-9	4.02e-7
	ArabidopsisPBM 20140210	AHL12_2	2.54e-8	2.87e-6
	ArabidopsisPBM 20140210	AHL20_3ary	6.96e-8	7.87e-6
	ArabidopsisPBM 20140210	WOX13_2	1.98e-7	2.24e-5
	ArabidopsisPBM 20140210	AHL12_3ary	2.37e-7	2.68e-5
	ArabidopsisPBM 20140210	KAN4_2	3.35e-7	3.79e-5
	ArabidopsisPBM 20140210	AHL25_2	5.88e-7	6.64e-5
	ArabidopsisPBM 20140210	ANAC55	6.37e-7	7.20e-5
	ArabidopsisPBM 20140210	AHL25_3ary	1.05e-6	1.19e-4
	ArabidopsisPBM 20140210	WRKY38	1.49e-6	1.68e-4
	ArabidopsisPBM 20140210	WRKY45	1.74e-6	1.97e-4
	ArabidopsisPBM 20140210	HSFB2A	1.79e-6	2.02e-4
	ArabidopsisPBM 20140210	WRKY12	6.38e-6	7.21e-4

Logo	Database	Name	*p*-value	Adjusted *p*-value
	ArabidopsisPBM 20140210	AHL12	1.07e-5	1.21e-3
	ArabidopsisPBM 20140210	AHL20_2	1.07e-5	1.21e-3
	ArabidopsisPBM 20140210	AHL25	1.59e-5	1.79e-3
	ArabidopsisPBM 20140210	WRKY18	1.90e-5	2.14e-3
	ArabidopsisPBM 20140210	AHL20	2.31e-5	2.60e-3
	ArabidopsisPBM 20140210	RVE1_2	4.87e-5	5.48e-3
	ArabidopsisPBM 20140210	ANAC46	5.88e-5	6.62e-3
	ArabidopsisPBM 20140210	KAN4	8.86e-5	9.97e-3
	ArabidopsisPBM 20140210	YAB5	9.12e-5	1.03e-2
	ArabidopsisPBM 20140210	CCA1	1.79e-4	2.00e-2
	ArabidopsisPBM 20140210	Dof5.7	1.89e-4	2.11e-2
	ArabidopsisPBM 20140210	YAB1	2.36e-4	2.63e-2
	ArabidopsisPBM 20140210	HSFC1_2	2.44e-4	2.72e-2

Appendix Figure S5. *Cis* regulatory Elements Enriched for *MAPKKK5*-Dependent Genes.

319 genes showing reduced induction or suppression in *mapkkk5* compared to wild type were selected. The 1000-bp upstream of the transcription start sites of the selected genes was tested for enrichment of *cis* elements against those of the whole Arabidopsis genes using AME (adjusted p-value < 0.05). Names of transcription factors and sequence logo of their biding sites are shown.

A

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  1 MRWLPQISFSSPSSSPSSSLKPVASYSESPDPDRNQDRDRFHRRLFRFNRGRLTRQRKLR  60
 61 HLTDDDVLLGERRASTSSSTFDSGLTRSPSAFTAVPRSPSAVPLPLPLPLPEVAGIRNAA 120
121 NARGLDDRDRDPERLISDRTSSGPPLTSVNGGFARDSRKATENSSYQDFSPRNRNGYWVN 180
181 IPTMSAPTSPYMSPVPSPQRKSTGHDLPFFYLPPKSNQAWSAPDMPLDTSGLPPPAFYDI 240
241 TAFSTDNSPIHSPQPRSPRKQIRSPQPSRPSSPLHSVDSSAPPRDSVSSPLHPRLSTDVT 300
301 NGRRDCCNVHPLPLPPGATCSSSSAASVPSPQAPLKLDSFPMNSQWKKGKLIGRGTFGSV 360
361 YVASNSETGALCAMKEVELFPDDPKSAECIKQLEQEIKLLSNLQHPNIVQYFGSETVEDR 420
421 FFIYLEYVHPGSINKYIRDHCGTMTESVVRNFTRHILSGLAYLHNKKTVHRDIKGANLLV 480
481 DASGVVKLADFGMAKHLTGQRADLSLKGSPYWMAPELMQAVMQKDSNPDLAFAVDIWSLG 540
541 CTIIEMFTGKPPWSEFEGAAAMFKVMRDSPPIPESMSPEGKDFLRLCFQRNPAERPTASM 600
601 LLEHRFLKNSLQPTSPSNSDVSQLFNGMNITEPSSRREKPNFKLDQVPRARNMTSSESES 660
661 GQQQQQQQYRSPDLTGTVNRLSPRSTLEAIPSPCPSQRPKPSSSDRRRTGVTSDHL     716
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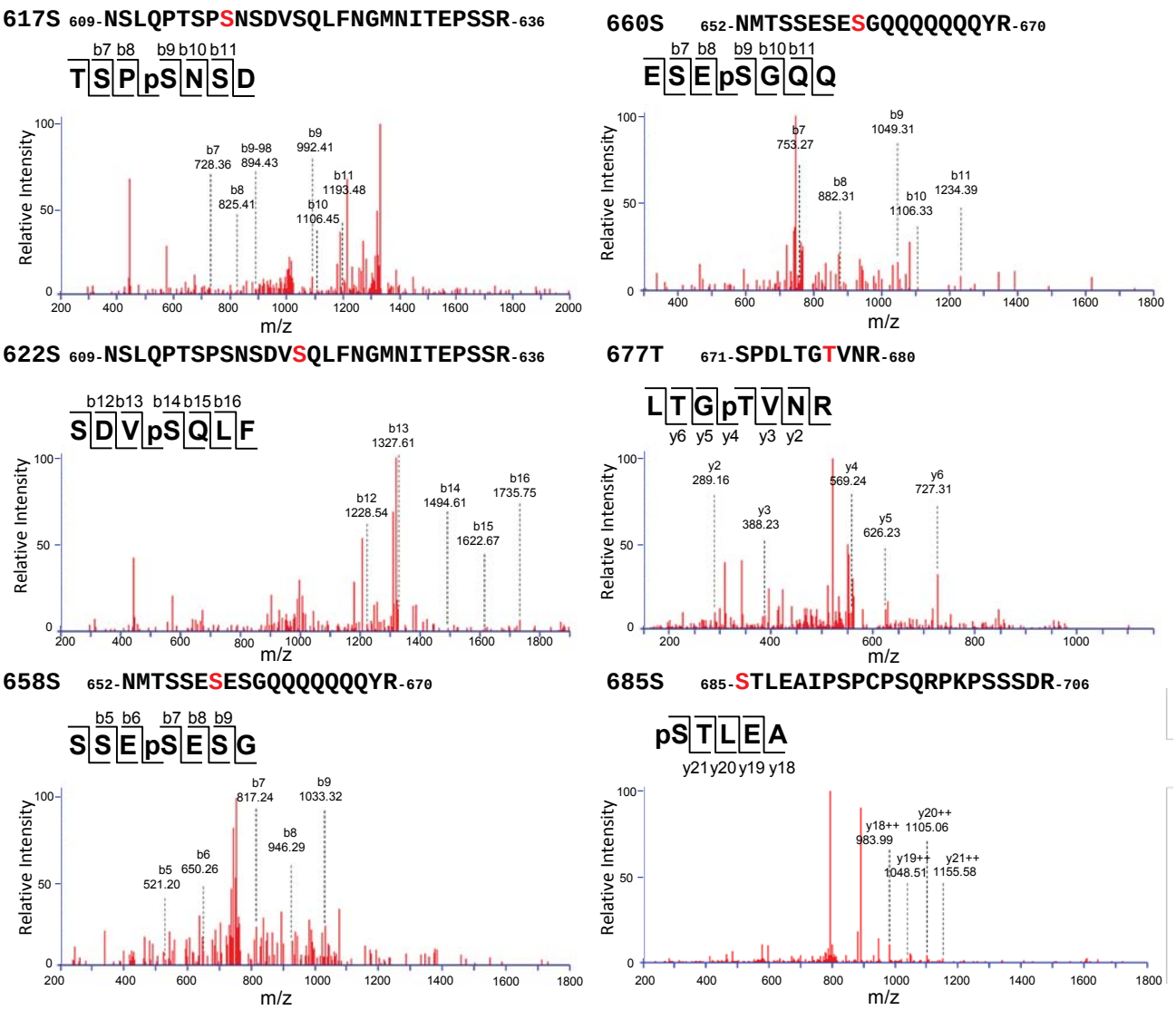
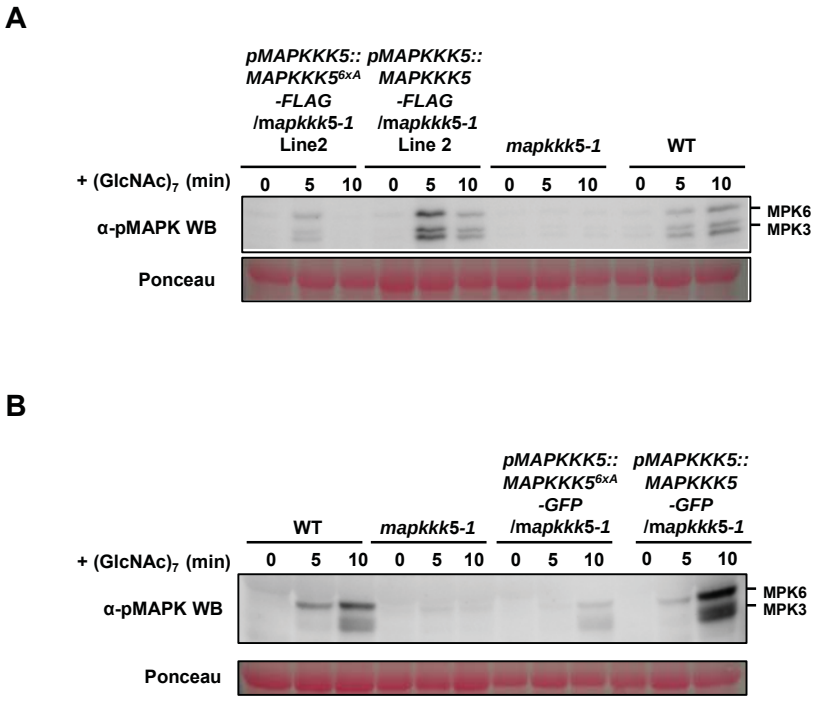
B



Appendix Figure S6. Identification of amino acid residues of MAPKKK5 phosphorylated by PB27.

A Amino acid residues phosphorylated by PBL27 are indicated in red. Yellow region denotes the C-terminal domain of MAPKKK5.

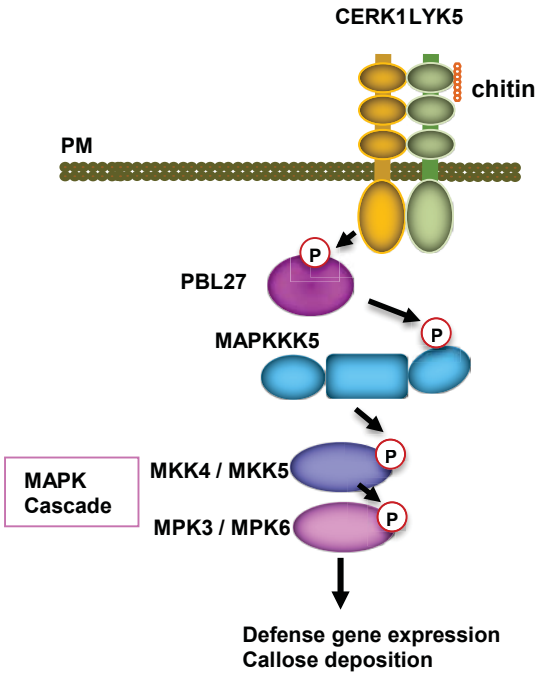
B Assigned spectrum of each phosphorylated peptide. b-fragments in 609-636 and 652-670 peptides and y-fragments in 671-680 and 685-706 peptides were labelled to three or four spectrums around the phosphorylated sites. In 685-706 peptides, divalent spectrums were labelled (++).



Appendix Figure S7. Chitin-Induced MAPK Activation in Additional *mapkkk5* Mutant Lines Expressing *MAPKKK5^{6xA}* Tagged with FLAG or GFP.

A Immunoblot analysis for $(GlcNAc)_7$ -triggered MAPK activation in a *mapkkk5* mutant expressing MAPKKK5^{6xA} tagged with FLAG. Total proteins were extracted from seedlings treated with 10 μM $(GlcNAc)_7$. Activation of each MAPK was analyzed by immunoblot with α-pMAPK antibody. The protein loading control was shown by Ponceau staining (bottom). The experiments were performed three times with similar results.

B Immunoblot analysis for $(GlcNAc)_7$ -triggered MAPK activation in a *mapkkk5* mutant expressing MAPKKK5^{6xA} tagged with GFP. Ponceau-stained loading controls are shown (bottom).



Appendix Figure S8. Model for the Phospho-Signaling Pathway from the Chitin Receptor Complex to MAPKs.

Upon perception of chitin, CERK1 phosphorylates PBL27, and then PBL27 phosphorylates the C-terminal domain of MAPKKK5. MAPKKK5 phosphorylates MKK4 and MKK5, which results in MPK3- and MPK6-mediated activation of immune responses such as defense gene expression and callose deposition.

APPENDIX TABLE S1. PCR primers used in this study

Objective	Name	5'-sequence-3'
Constructs of MAPKKK5 domain	pMAPKKK5-F	CACCGCATCGTTAAGTATGTTGTTACTTC
	pMAPKKK5-R	GAGAAGAAGAAGGAGACGAGAACGAG
	MAPKKK5-Full-F	CACCATGCGTTGGCTTCCGCAAATC
	MAPKKK5-Full ⊿stop-R	AAGGTGATCTGAAGTGACGCCCG
	MAPKKK5-Full-R	TCAAAGGTGATCTGAAGTGACGCC
	Pst I -MAPKKK5-Full-R	TGGTTCTGCAGTCAAAGGTGATCTGAAGTGACG
	Pst I -MAPKKK5-N-R	TGGTTCTGCAGCTGGGAATTCATTGGGAATGAATCC
	EcoR I -MAPKKK5-KD-F	CACCGAATTCCCGAGGTTGTCTACTGATGTTACG
	Pst I -MAPKKK5-KD-R	TGGTTCTGCAGTTGAGAGACATCACTGTTGCT
	EcoR I -MAPKKK5-C-F	CACCGAATTCGCATCTATGTTGCTAGAACA
Point mutation	$MAPKKK5^{K375M}$-F	TGTGTGCGATGATGGAAGTTGAGCTATTTC
	$MAPKKK5^{K375M}$-R	GGAAATAGCTCAACTTCCATCATCGCACAC
	$MKK1^{K97R}$-F	CGCTCTTAGAGTCATTCAATTGAACAC
	$MKK1^{K97R}$-R	GTGTTCAATTGAATGACTCTAAGAGCG
	$MKK2^{K99R}$-F	TCGCCTTGAGAGTCATTCAACTA
	$MKK2^{K99R}$-R	TAGTTGAATGACTCTCAAGGCGA
	$MKK2^{T229A, S235A}$ F	GACAAACGCTGCAGGTTTAGCAAACGCTTTTGTGG
	$MKK2^{T229A, S235A}$ R	CCACAAAAGCGTTTGCTAAACCTGCAGCGTTTGTC
	$MKK4^{K108R}$-F	GTGATATACGGTAACCACGAGGAGACT
	$MKK4^{K108R}$-R	CTCGTGGTTACCGTATATCACTCTAAGTGC
	$MKK4^{T224A, S230A}$-F	CTTGGCTCAGGCTATGGATCCGTGTAATGCTTCTGTTGG
	$MKK4^{T224A, S230A}$-R	GGTTCCAACAGAAGCATTACACGGATCCATAGCCTGAGCC
	$MKK5^{K99R}$-F	GTGATTTACGGAAACCACGAAGATACCG
	$MKK5^{K99R}$-R	CTTCGTGGTTTCCGTAAATCACTCTGAGAGC
	$MKK5^{T215A, S221A}$-F	GGCACAAGCTATGGATCCTTGTAATGCTTCTGTTGG
	$MKK5^{T215A, S221A}$-R	CCAACAGAAGCATTACAAGGATCCATAGCTTGTGCC
	$MAPKKK5^{S617A, S622A}$-F	CCAACCTCACCAGCTAACAGTGATGTCGCTCAATTA
	$MAPKKK5^{S617A, S622A}$-R	TTAAATAATTGAGCGACATCACTGTTAGCTGGTGAGG
	$MAPKKK5^{S658A, S660A}$-F	TCCTCAGAGGCTGAAGCTGGGCAACAG
	$MAPKKK5^{S658A, S660A}$-R	CTGCTGTTGCCCAGCTTCAGCCTCTGAG
	$MAPKKK5^{T677A, S685A}$ -F	CTAACAGGAGCTGTGAACCGTCTGTCTCCTCGTGCTACTCTGGA
	$MAPKKK5^{T677A, S685A}$ -R	CCTCCAGAGTAGCACGAGGAGACAGACGGTTCACAGCTCCTGTTA
mapkkk5-1 T-DNA genotyping	T-DNA primer	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC
	gene specific primer	CATGGTGCCGCAATGATCACGG
mapkkk5-2 T-DNA genotyping	T-DNA primer	TGGTTCACGTAGTGGGCCATCG
	gene specific primer	CACCATGCGTTGGCTTCCGCAAATC
Semi-RT-PCR	ACTIN8-F	GCTGAGAGATTCAGGTGCCC
	ACTIN8-R	GAGATCCACATCTGCTGG
	GFP-tag specific primer	GTTCACGTCGCCGTCCAGCTCGACCA
	FLAG-tag specific primer	TCCTTGTAGTCGCTGTTATCAACCAC
Quantitative RT-PCR	MAPKKK5-F	CGCCGGGATTCGAAATG
	MAPKKK5-R	TCGGGATCTCGATCTCTGTCA
	BON3-F	TTACAGACATGGCGGGTACA
	BON3-R	TCTAACCGCCGACCATTATC
	FRK1-F	CAGAAACAGCGCGAAACGA
	FRK1-R	GGTCGGGCGGTCTGAAA
	GSTU3-F	TCAAGAGCCCGTTGCTACTTC
	GSTU3-R	TGTGAACCAAAACAGGGACTTTT
	BGLU27-F	AATCATGACAAGCCCAATTGG
	BGLU27-R	TGCGTTGTTTTCTTTTCTCCATT
	ACTIN2-F	AGTGTCTGGATCGGTGGTTC
	ACTIN2-R	CCCCAGCTTTTTAAGCCTTT
Protein expression	Nde I -BIK1-F	CACCCATATGGGTTCTTGCTTCAGTTCTC
	Sal I -BIK1-R	GTCGACCTACACAAGGTGCCTGCCAAAAGG
	Nde I -MAPKKK5-Full-F	CACCCATATGATGCGTTGGCTTCCGCAAATC
	Sal I -MAPKKK5-Full-R	GTCGACTCAAAGGTGATCTGAAGTGACGCCC
	Sal I -MAPKKK5-N-R	GTCGACTCACTGGGAATTCATTGGGAATGAATC
	Nde I -MAPKKK5-KD-F	CACCCATATGTGGAAGAAAGGGAAGCTAATAGG
	Sal I -MAPKKK5-KD-R	GTCGACTCATAGGAACCGGTGTTCTAGCA
	Nde I -MAPKKK5-C-F	CACCCATATGATGGCATCTATGTTGCTAGAACACC
	Nde I -MKK1-F	CACCCATATGAACAGAGGAAGCTTATGC
	Sal I -MKK1-R	GTCGACTCACCATTGCGAGATGAAGGAGC
	Nde I -MKK2-F	CACCCATATGAAGAAAGGTGGATTCAGCA
	Sal I -MKK2-R	GTCGACTTACACGGAGAACGTACCAGACA
	Nde I -MKK4-F	CACCCATATGAGACCGATTCAATCGCCTC
	Xho I -MKK4-R	CTCGAGCTATGTGGTTGGAGAAGAAGACGAG
	Nde I -MKK5-F	CACCCATATGAAACCGATTCAATCTCCTTC
	Xho I -MKK5-R	CTCGAGCTAAGAGGCAGAAGGAAGAGGACG
Yeast two-hybrid assay	MAPKKK1-KD-F	CACCATGCAAGATTTCTTCGGCTC
	MAPKKK1-KD-R	TCAATCTGTAACGGTAGTGGAG
	MAPKKK2-KD-F	CACCATGGTTTTCGCCAAATCCCAGT
	MAPKKK2-KD-R	TCACAACTCTGATGGTAAAGGACTG

Yeast two-hybrid assay	MAPKKK3--F	CACCATGCCTACTTGGTGGGGAAGAAAG
	MAPKKK3--R	CTACACCAGTCTTGATCTCAATGG
	MAPKKK3-N-R	CTACGTGGAAAACCCCGAAGGAGA
	MAPKKK3-KD-F	CACCAGGATTGGAGGAGGCTACGAGA
	MAPKKK3-KD-R	CTATGAGAAGTTTCCATCATAGGAACG
	MAPKKK3-C-F	CACCTCTCAGCTTCTAGAACACCCT
	MAPKKK4--F	CACCATGCCTTGGTGGAGTAAATC
	MAPKKK4--R	TTAGGGTCCTCTGTTTGTTG
	MAPKKK4-N-R	TTATCGCGATCCAGGGCTAACCGTA
	MAPKKK4-KD-F	CACCCGAGCTGGAGGGTCAACTACT
	MAPKKK4-KD-R	TGCAGGCTCGCCACTCACAATA
	MAPKKK4-C-F	CACCATGGAAAGGCCTATTGTGAGTG
	MAPKKK5--F	CACCATGCGTTGGCTTCCGCAAATCT
	MAPKKK5--R	CTAAAGGTGATCTGAAGTGAC
	MAPKKK5-N-R	CTACTGGGAATTCATTGGGAATGA
	MAPKKK5-KD-F	CACCCCGAGGTTGTCTACTGATGTT
	MAPKKK5-KD-R	CTATTGAGAGACATCACTGTTGC
	MAPKKK5-C-F	CACCATGGCATCTATGTTGCTAGAACACCGGTT
Yeast two-hybrid assay	MAPKKK6-KD-F	CACCATGGCGCGACAGATGACGTCAT
	MAPKKK6-KD-R	TCAAGTTTTTGTCACCTCTACTTTTTCTGC
	MAPKKK7-KD-F	CACCATGGCGCGGCAAATGACGTCAT
	MAPKKK7-KD-R	TCAAGTTTTTGATATTCCCACATTTTCTCCTG
	MAPKKK8-F	CACCATGGACAGAATTCTAGCTCG
	MAPKKK8-R	TCATCTACGGAGAAGGGGAGAT
	MAPKKK8-KD-F	CACCATGGGAGCTAGGTTTATCCAG
	MAPKKK8-KD-R	TCATCTACGGAGAAGGGGAGAT
	MAPKKK9-F	CACCATGAAGAAGTCGTCGGATAAGTCACC
	MAPKKK9-R	TCATCTACGGATTAGCGGAGAT
	MAPKKK9-KD-F	CACCACGGGAGATATGTTTATCCA
	MAPKKK9-KD-R	TCATCTACGGATTAGCGGAGAT
	MAPKKK10-F	CACCATGGACGTAACTGCTATCTTCG
	MAPKKK10-R	TCAAAGATTTATAACAAATGGATGGTGCAGCAG
	MAPKKK10-KD-F	CACCATGGAGTTCCCGGATCGCATC
	MAPKKK10-KD-R	TCAAAGATTTATAACAAATGGATGGTGCAGCAG
	MAPKKK11-F	CACCATGGCCAATTCAACTGAGTTGATGC
	MAPKKK11-R	TCATCCACGGAGGAGCGAAGATA
	MAPKKK11-KD-F	CACCATGTTTATCCCATTGGGGGAT
	MAPKKK11-KD-R	TCATCCACGGAGGAGCGAAGATA
	MAPKKK12-KD-F	CACCATGCAGGATATTCTCGGATCGG
	MAPKKK12-KD-R	CTAGCTTCCCAGTTCACAGACA
	MAPKKK13-KD-F	CACCATGTTATCCTCACCATCTTC
	MAPKKK13-KD-R	TTATTCTTCTTCTAAGTCAAACCCCG
	MAPKKK14-KD-F	CACCATGGAGAAACAGAACATTATCTC
	MAPKKK14-KD-R	TTACGGAGACGACTCAGTGAAGAAC
	MAPKKK15-F	CACCATGGAGGAACAAAACTGGATAAG
	MAPKKK15-R	TCAATTGTAATTAGTAATCTC
	MAPKKK16-F	CACCATGGAGATTAATTGGACAAGAGGACC
	MAPKKK16-R	TCAGACTTGGTTAGTGAATTTAAC
	MAPKKK17-F	CACCATGGAATGGACTAGAGGAAGAATCC
	MAPKKK17-R	TTACAATTCCCCCACCAATAC
	MAPKKK18-F	CACCATGAATTGGACTAGAGGAAAAAC
	MAPKKK18-R	CTAATTCCGTCGAACCGTGATCC
	MAPKKK19-F	CACCATGGACTGGGTTCGAGGAGATACC
	MAPKKK19-R	CTACCTCAAGGTGATCCAGCTCC
	MAPKKK20-F	CACCATGGAGTGGGTTCGAGGAGAAA
	MAPKKK20-R	TCACCGGACTGTAAGCCAACT
	MAPKKK21-F	CACCATGGAGTGGATTCGTAGAGAAAC
	MAPKKK21-R	TCACCTGACGTTGACCCAATCAC
	BIK1-F	CACCATGGGTTCTTGCTTCAGTTCTC
	BIK1-R	CTACACAAGGTGCCTGCCAAAAGG
	PBS1-F	CACCATGGGTTGTTTCTCGTGTTTTG
	PBS1-R	CTACCCGGTACTGTTGCTCTCTGAAG
	PBL1-F	CACCATGGGTTCTTGTCTCAGTTCTCG
	PBL1-R	CTACAATCCAACGGTTTTTTTG