MPK3/6-induced degradation of ARR1/10/12 promotes salt tolerance in *Arabidopsis*

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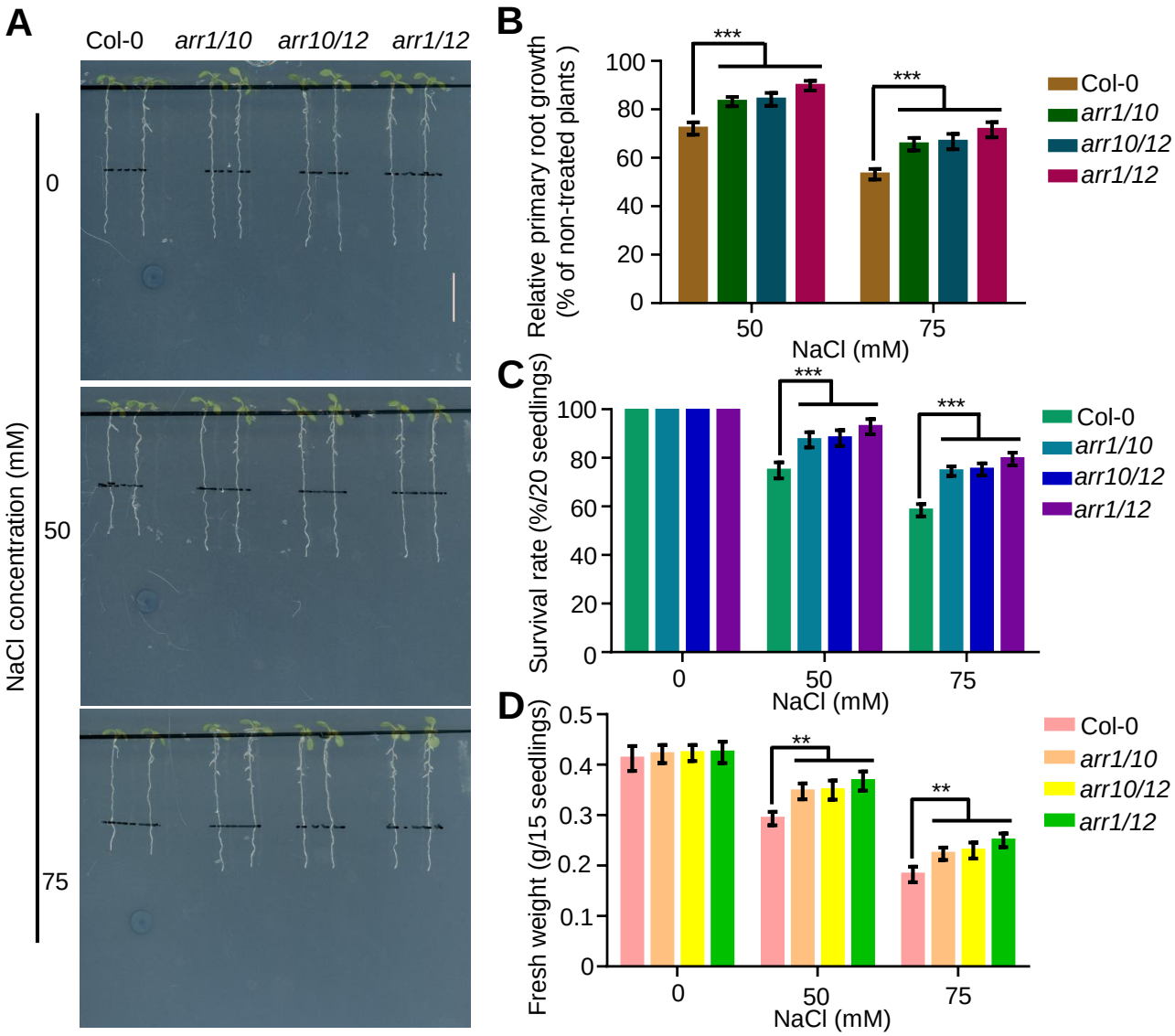
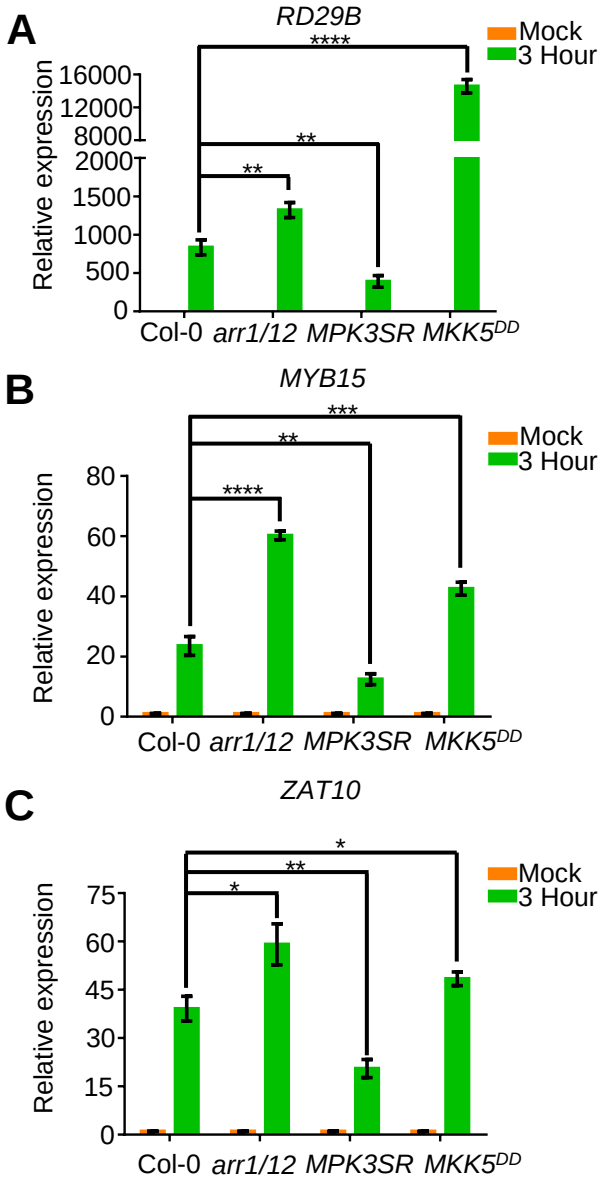


Appendix Fig S1. ARR1/10/12 negatively regulate plant tolerance to salt stress.

Four-day-old seedlings of Col-0, *arr1/10*, *arr10/12* and *arr1/12* mutants were transferred to 1/2 MS medium with or without 50 and 75 mM NaCl. After 3 days of salt treatment, changes in plant phenotype (A) and primary root length (B) were determined. Changes in survival rate (C) and fresh weight (D) were examined after 10 days of salt treatment. Scale Bar, 1 cm.

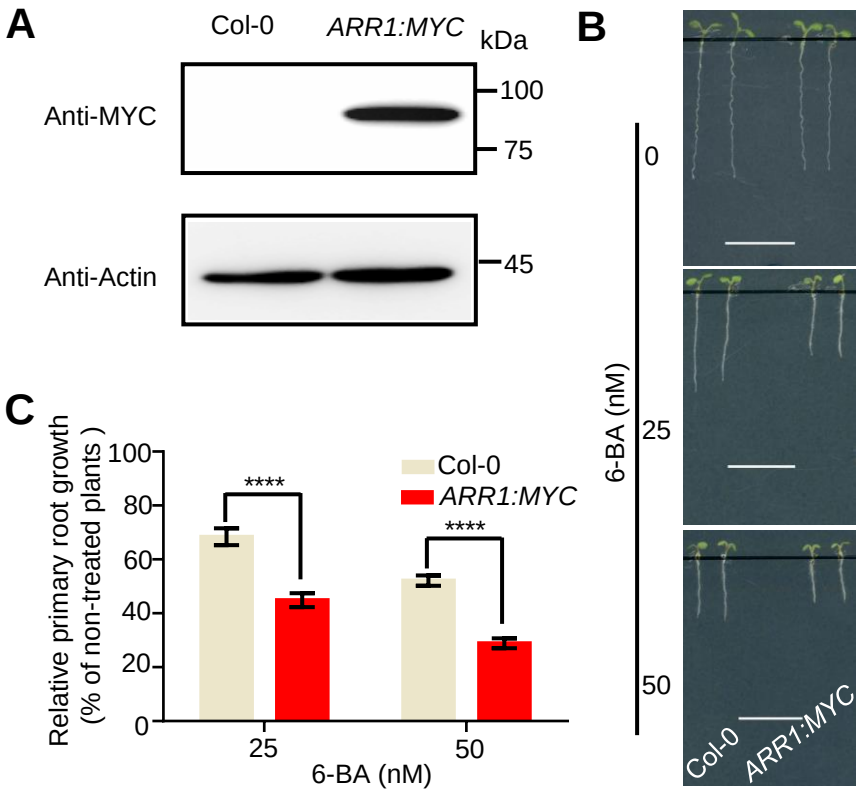
Data information: In (B-D), data are means of three biological replicates ± SD (n=60 for B, and n=12 for C and D). **, and *** indicate significant difference to the corresponding controls with $P<0.01$, and $P<0.001$, respectively (student's *t*-test).



Appendix Fig S2. Expression analysis of salt-responsive genes.

A-C Seven-day-old seedlings were treated with or without 200 mM NaCl for 3 h. Transcript levels of *RD29B*, *MYB15*, and *ZAT10* in Col-0, *arr1/12*, *MPK3SR*, and *MKK5*^{DD} were examined by qRT-PCR. *Actin2* gene and *UBQ1* gene were analyzed as internal controls.

Data information: Data shown are means ± SD of three biological replicates. *, **, ***, and **** indicate significant difference to the corresponding controls with $P<0.05$, $P<0.01$, $P<0.001$, and $P<0.0001$, respectively (student's *t*-test).

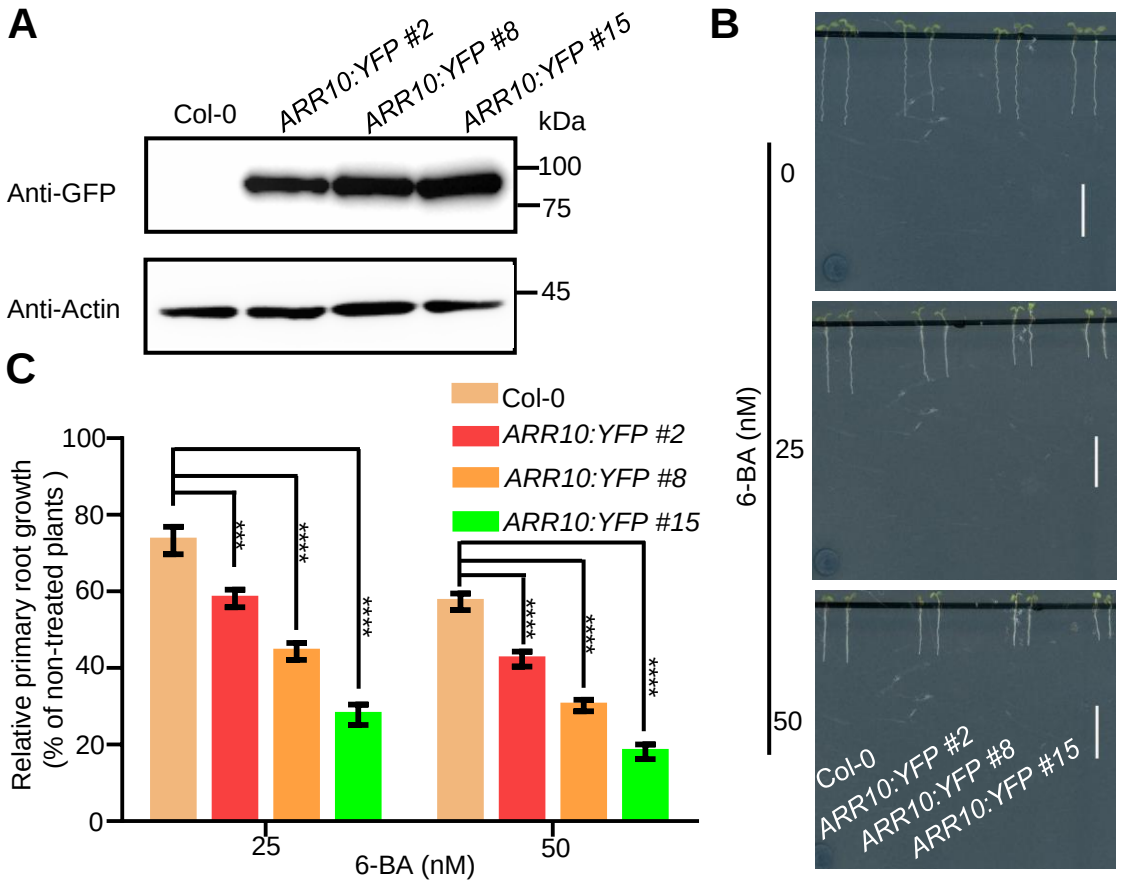


Appendix Fig S3. Transgenic plant overexpressing ARR1 shows hypersensitivity to 6-BA compared with that of wild type.

A ARR1 protein level in *35S:ARR1:MYC* transgenic plant. Total proteins were extracted from 7-day-old seedlings. Anti-MYC antibody was used to detect ARR1 protein. Actin was used as a loading control.

B-C Cytokinin response of Col-0, *35S:ARR1:MYC* seedlings grown on 1/2 MS medium with or without 25 nM or 50 nM 6-BA for 5 days. Scale Bar, 1 cm.

Data information: In (C), data are means of three biological replicates ± SD (n=60). **** indicates significant difference to the corresponding controls with $P<0.0001$ (student's *t*-test).

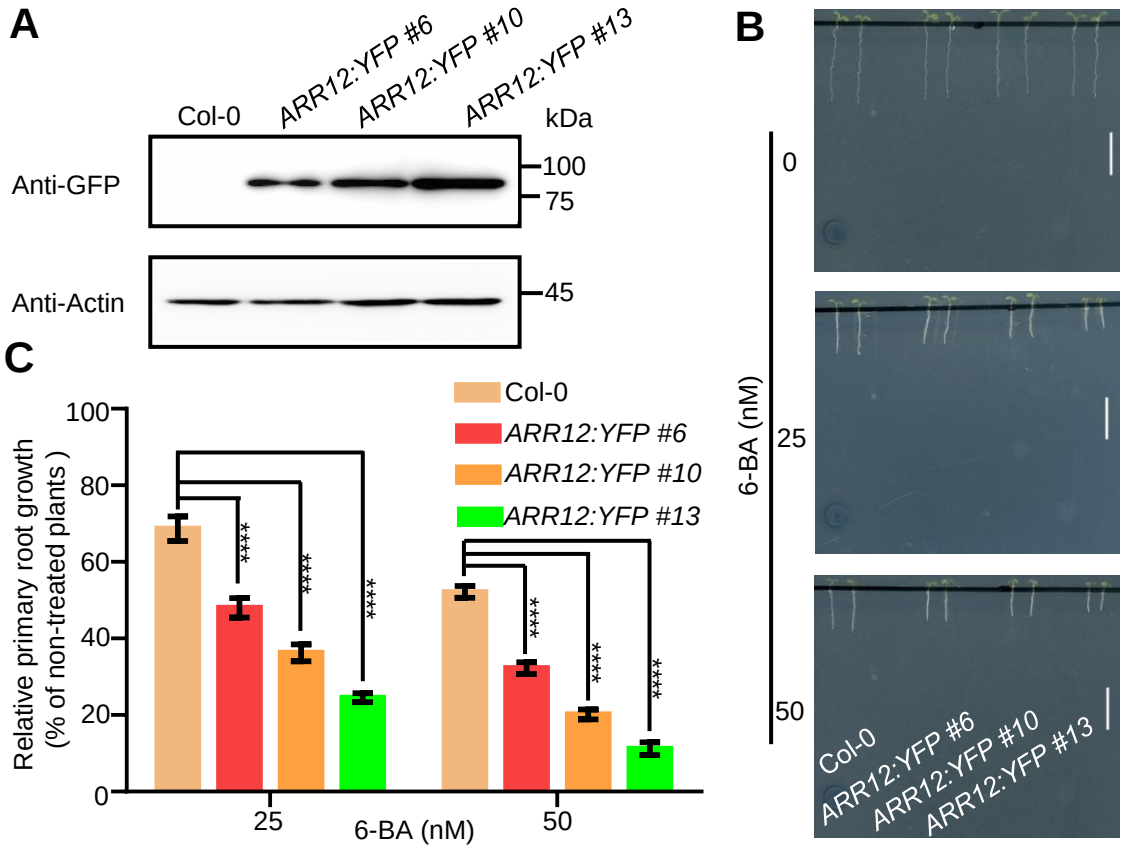


Appendix Fig S4. Transgenic plants overexpressing ARR10 all show hypersensitivity to 6-BA compared with that of wild type.

A ARR10 protein levels in *35S:ARR10:YFP* transgenic plants. Total proteins were extracted from 7-day-old seedlings. Anti-GFP antibody was used to detect ARR10 protein. Actin was used as a loading control.

B-C Cytokinin response of Col-0, *35S:ARR10:YFP #2, 35S:ARR10:YFP #8,* and *35S:ARR10:YFP #15* seedlings grown on 1/2 MS medium with or without 25 nM or 50 nM 6-BA for 5 days. Scale Bar, 1 cm.

Data information: In (C), data are means of three biological replicates ± SD (n=60). ***, and **** indicate significant difference to the corresponding controls with $P<0.001$, and $P<0.0001$, respectively (student's *t*-test).

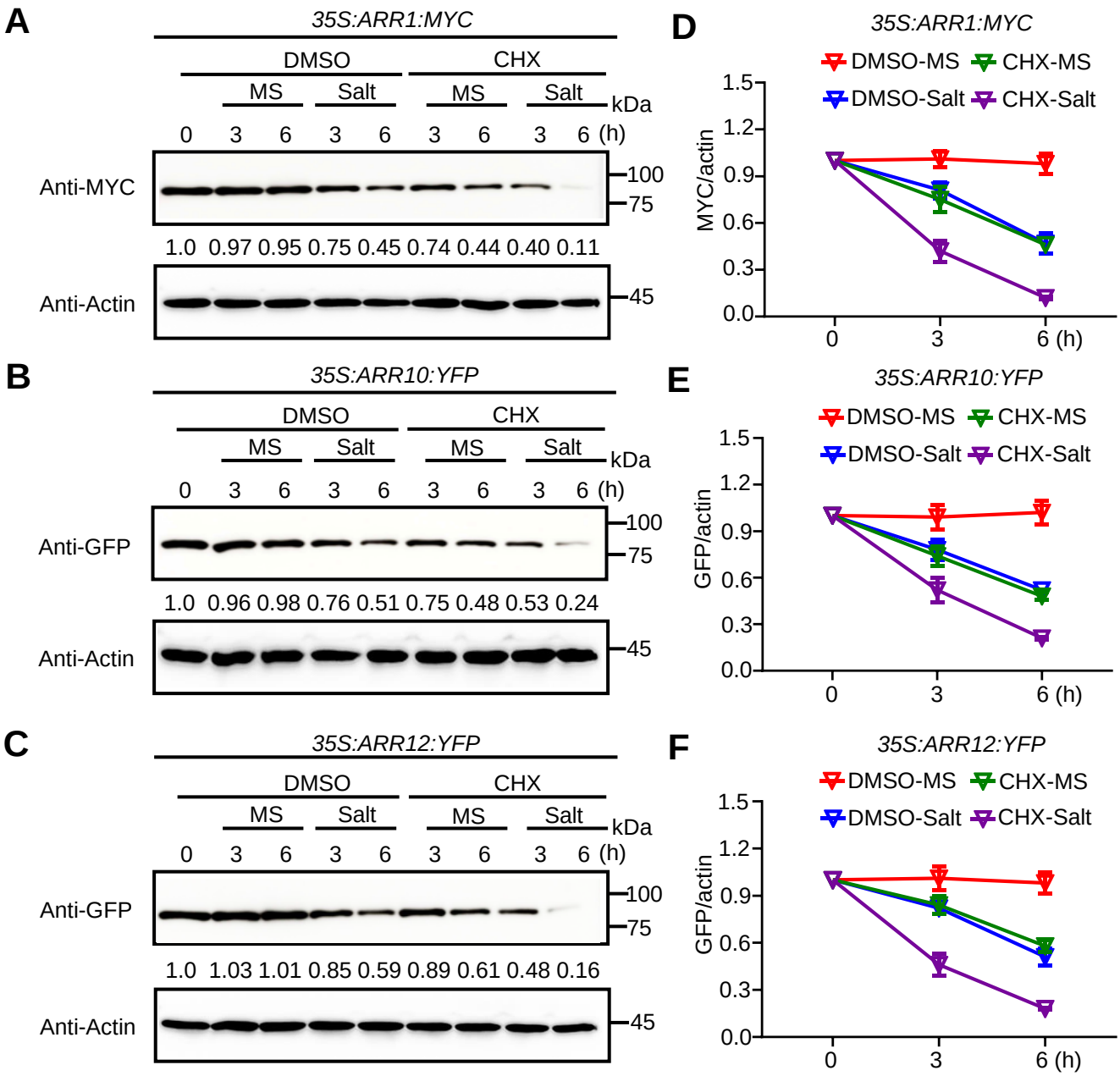


Appendix Fig S5. Transgenic plants overexpressing ARR12 all show hypersensitivity to 6-BA compared with that of wild type.

A ARR12 protein levels in *35S:ARR12:YFP* transgenic plants. Total proteins were extracted from 7-day-old seedlings. Anti-GFP antibody was used to detect ARR12 protein. Actin was used as a loading control.

B-C Cytokinin response of Col-0, *35S:ARR12:YFP #6*, *35S:ARR12:YFP #10*, *and 35S:ARR12:YFP #13* seedlings grown on 1/2 MS medium with or without 25 nM or 50 nM 6-BA for 5 days. Scale Bar, 1 cm.

Data information: In (C), data are means of three biological replicates ± SD (n=60). **** indicates significant difference to the corresponding controls with $P<0.0001$ (student's *t*-test).

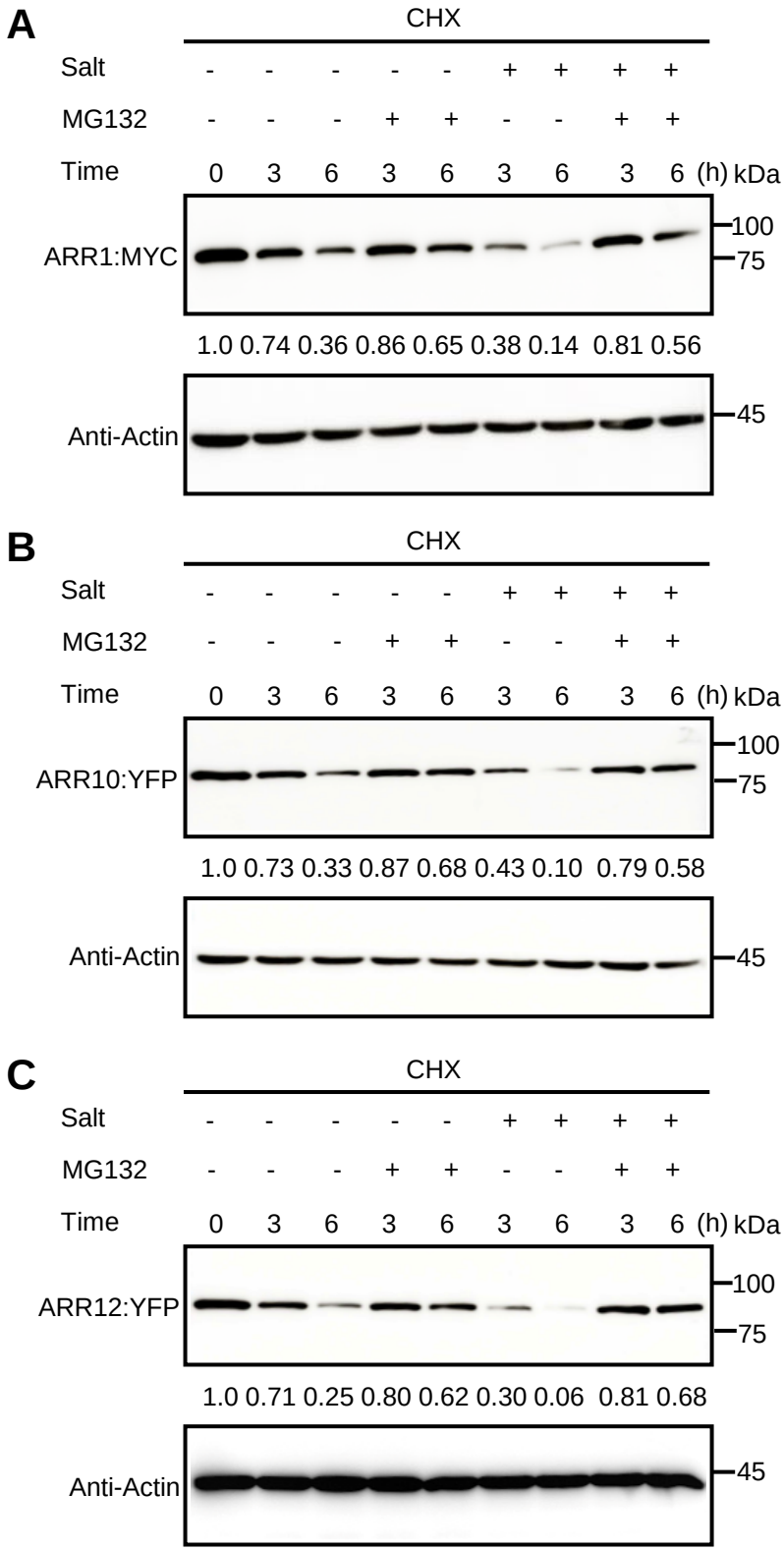


Appendix Fig S6. Salt stress induces ARR1/10/12 protein degradation.

A-C Immunoblot analysis of the levels of ARR1:MYC, ARR10:YFP, and ARR12:YFP proteins. Seven-day-old *35S:ARR1:MYC*, *35S:ARR10:YFP*, and *35S:ARR12:YFP* seedlings were treated with or without 50 μM cycloheximide (CHX) for 3 h, followed by treatment with or without 200 mM NaCl for the indicated amount of time. Actin was used as a control. ARR1 was detected with anti-MYC antibody, ARR10 and ARR12 were detected with anti-GFP antibody. The relative intensity of band detected by anti-MYC or anti-GFP antibody to that by anti-actin antibody without treatment was set to 1.0.

D-F Quantitative analysis of ARR1(D), ARR10 (E), and ARR12 (F) protein level were obtained by ImageJ software.

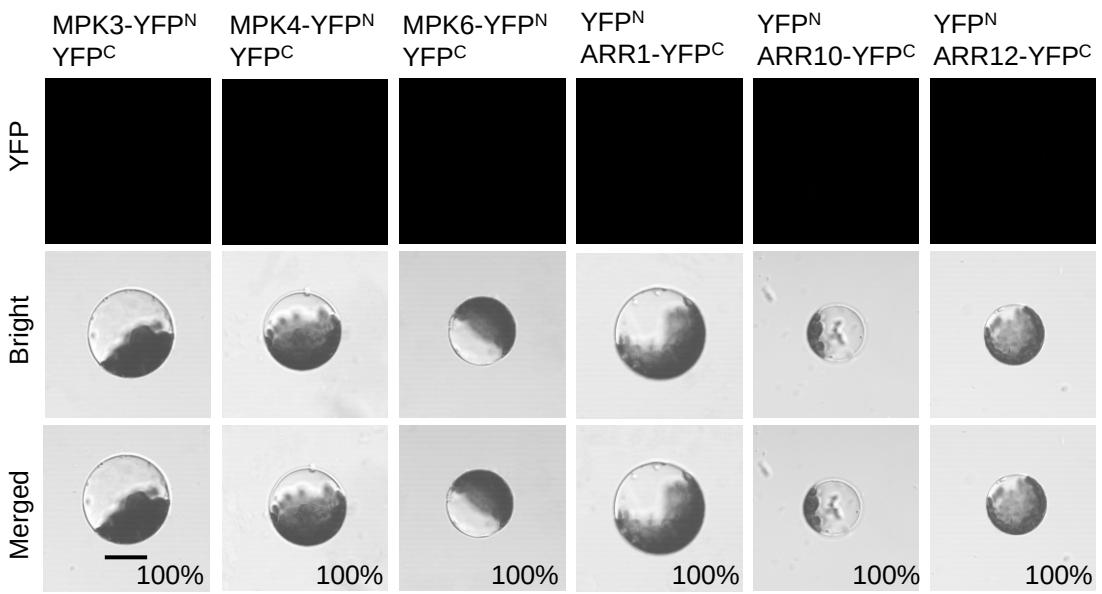
Data information: In (D-F), data shown are means ± SD of three biological replicates.



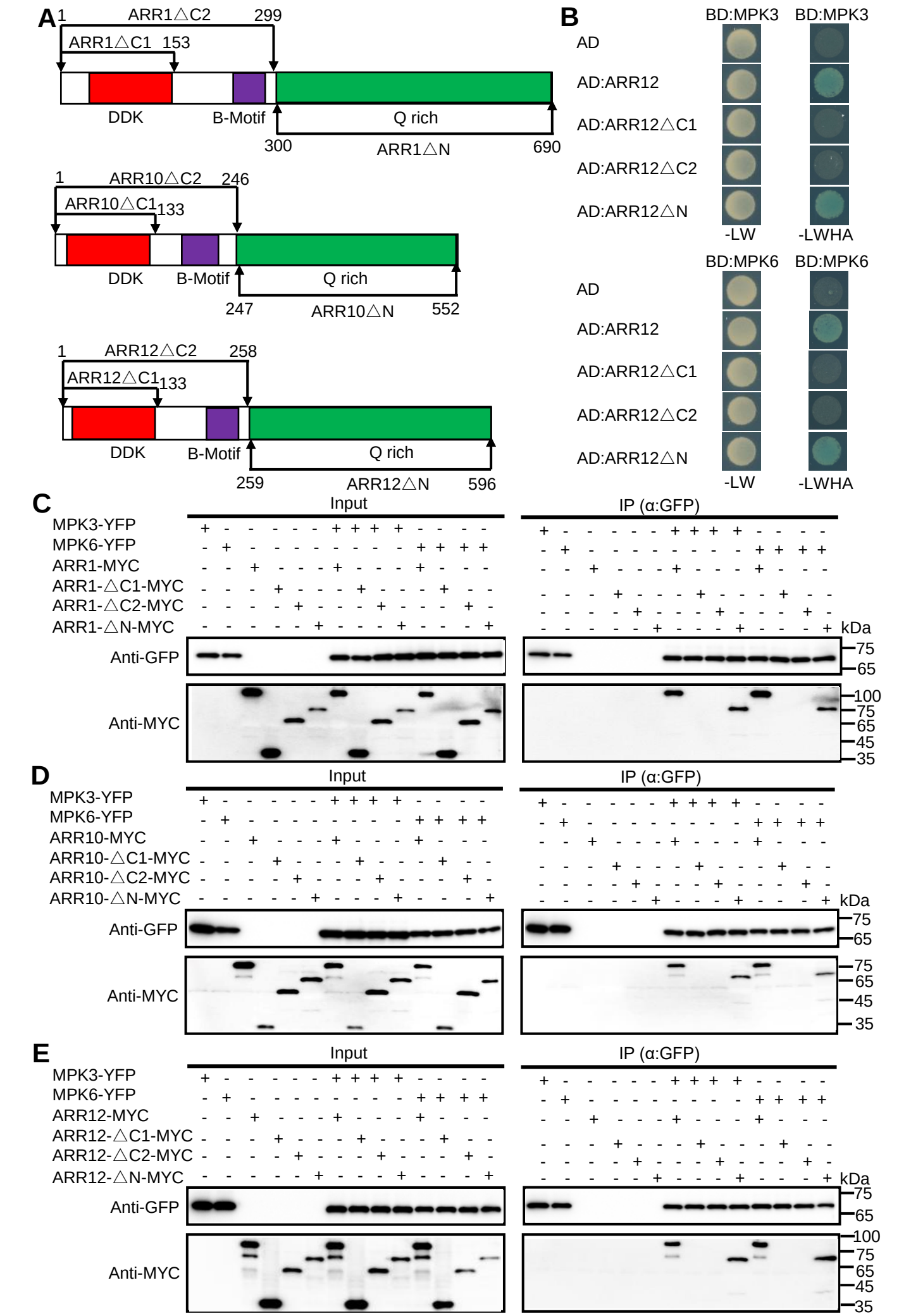
Appendix Fig S7. Salt stress-induced ARR1/10/12 protein degradation is through the 26S proteasome pathway.

A-C Immunoblot analysis of the levels of ARR1:MYC, ARR10:YFP, and ARR12:YFP proteins. Seven-day-old *35S:ARR1:MYC*, *35S:ARR10:YFP*, and *35S:ARR12:YFP* seedlings were treated with 50 μM cycloheximide (CHX) for 3 h, followed by treatment with DMSO, 50 μM MG132, 200 mM NaCl, or 200 mM NaCl, together with 50 μM MG132 for the indicated amount of time. ARR1 was detected with anti-MYC antibody, ARR10 and ARR12 were detected with anti-GFP antibody. Actin was used as a control. The relative intensity of band detected by anti-MYC or anti-GFP antibody to that by anti-actin antibody without treatment was set to 1.0.

Data information: Similar results were obtained with three biological repeats.



Appendix Fig S8. The negative control of BiFC analysis of interaction between MPK3/4/6 and ARR1/10/12. Partial yellow fluorescence protein (YFP) constructs were fused to MPK3/4/6 or ARR1/10/12. YFP^N and YFP^C are empty vectors. The fusion constructs were co-expressed transiently in *Arabidopsis* protoplasts cells. The interaction between MPK3/4/6 and ARR1/10/12 were visualized by fluorescence microscopy. The number labelled in the image means the probability of present cell in cells observed. Bar, 50 μm.


A
1
ARR1△C2
299
ARR1△C1
153
DDK
B-Motif
Q rich
300
ARR1△N
690
1
ARR10△C2
246
ARR10△C1
133
DDK
B-Motif
Q rich
247
ARR10△N
552
1
ARR12△C2
258
ARR12△C1
133
DDK
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Q rich
259
ARR12△N
596
B
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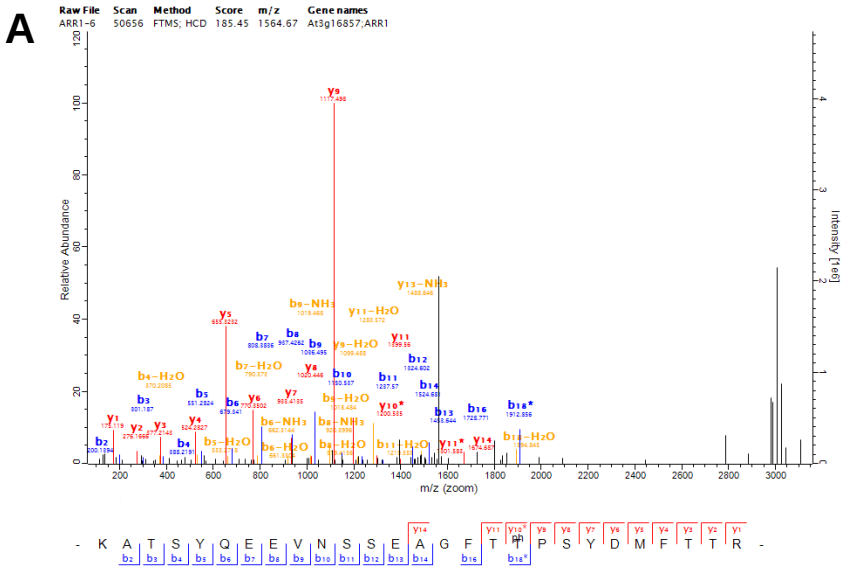
BD:MPK3
AD
AD:ARR12
AD:ARR12△C1
AD:ARR12△C2
AD:ARR12△N
-LW
-LWHA
BD:MPK6
BD:MPK6
AD
AD:ARR12
AD:ARR12△C1
AD:ARR12△C2
AD:ARR12△N
-LW
-LWHA
C
Input
IP (α:GFP)
MPK3-YFP
MPK6-YFP
ARR1-MYC
ARR1-△C1-MYC
ARR1-△C2-MYC
ARR1-△N-MYC
kDa
Anti-GFP
Anti-MYC
75
65
100
75
65
45
35
D
Input
IP (α:GFP)
MPK3-YFP
MPK6-YFP
ARR10-MYC
ARR10-△C1-MYC
ARR10-△C2-MYC
ARR10-△N-MYC
kDa
Anti-GFP
Anti-MYC
75
65
75
65
45
35
E
Input
IP (α:GFP)
MPK3-YFP
MPK6-YFP
ARR12-MYC
ARR12-△C1-MYC
ARR12-△C2-MYC
ARR12-△N-MYC
kDa
Anti-GFP
Anti-MYC
75
65
100
75
65
45
35

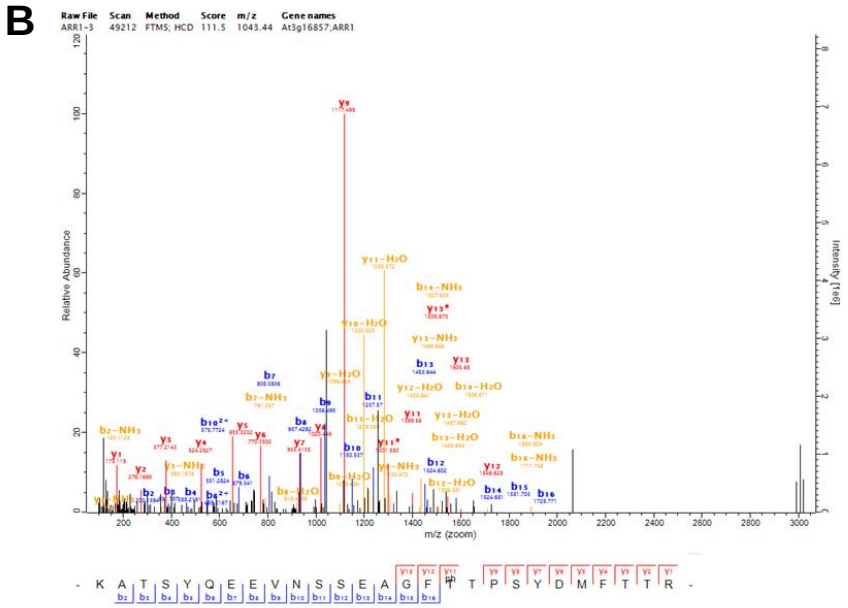
Appendix Fig S9. MPK3/6 interact with the Q-rich domain of ARR1/10/12.

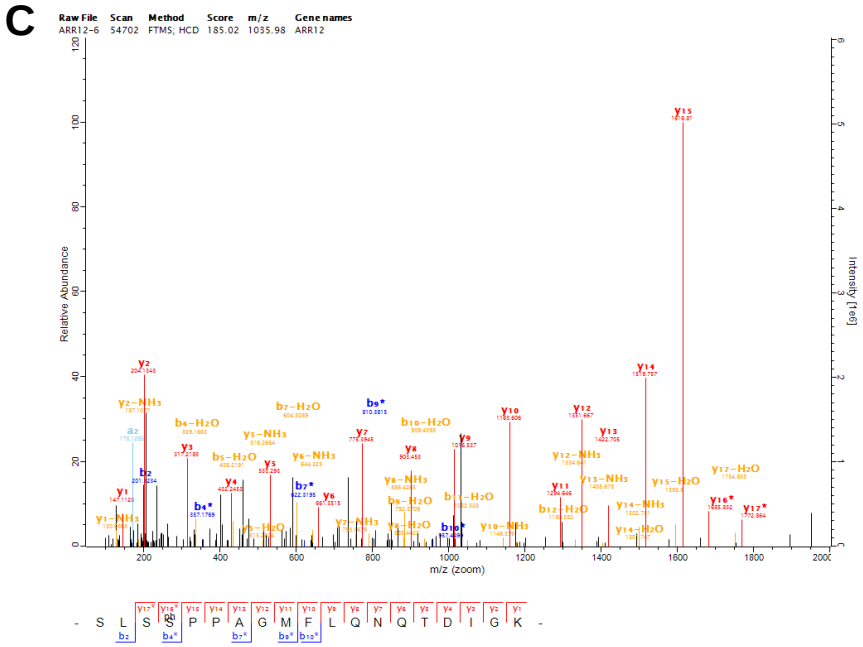
A Diagram of ARR1/10/12-truncated proteins used for the yeast two-hybrid assay descried in (B) or Co-IP descried in (C-E).

B The Q-rich domain of ARR12 interacts with MPK3/6 in yeast. Yeast cells were plated on selection medium: SD-LW (-Leu/Trp) or SD-LWHA (-Leu/Trp/His/Ade) supplemented with AbA (Aureobasidin A) and X-α-gal (5-bromo-4-chloro-3-indolyl-b-d-galactopyranoside). BD: pGBKT7, AD: pGADT7.

C-E *In vivo* Coimmunoprecipitation assay shows the interactions of the Q-rich domain of ARR1/10/12 with MPK3/6. MPK3/6 proteins and ARR1/10/12-truncated proteins were co-expressed in *Arabidopsis* protoplast cells. Protein extracts (Input) were immunoprecipitated with GFP Trap magnetic agarose beads. Immunoblots were performed with anti-MYC antibody to detect ARR1/10/12-truncated proteins and with anti-GFP antibody to detect MPK3/6.





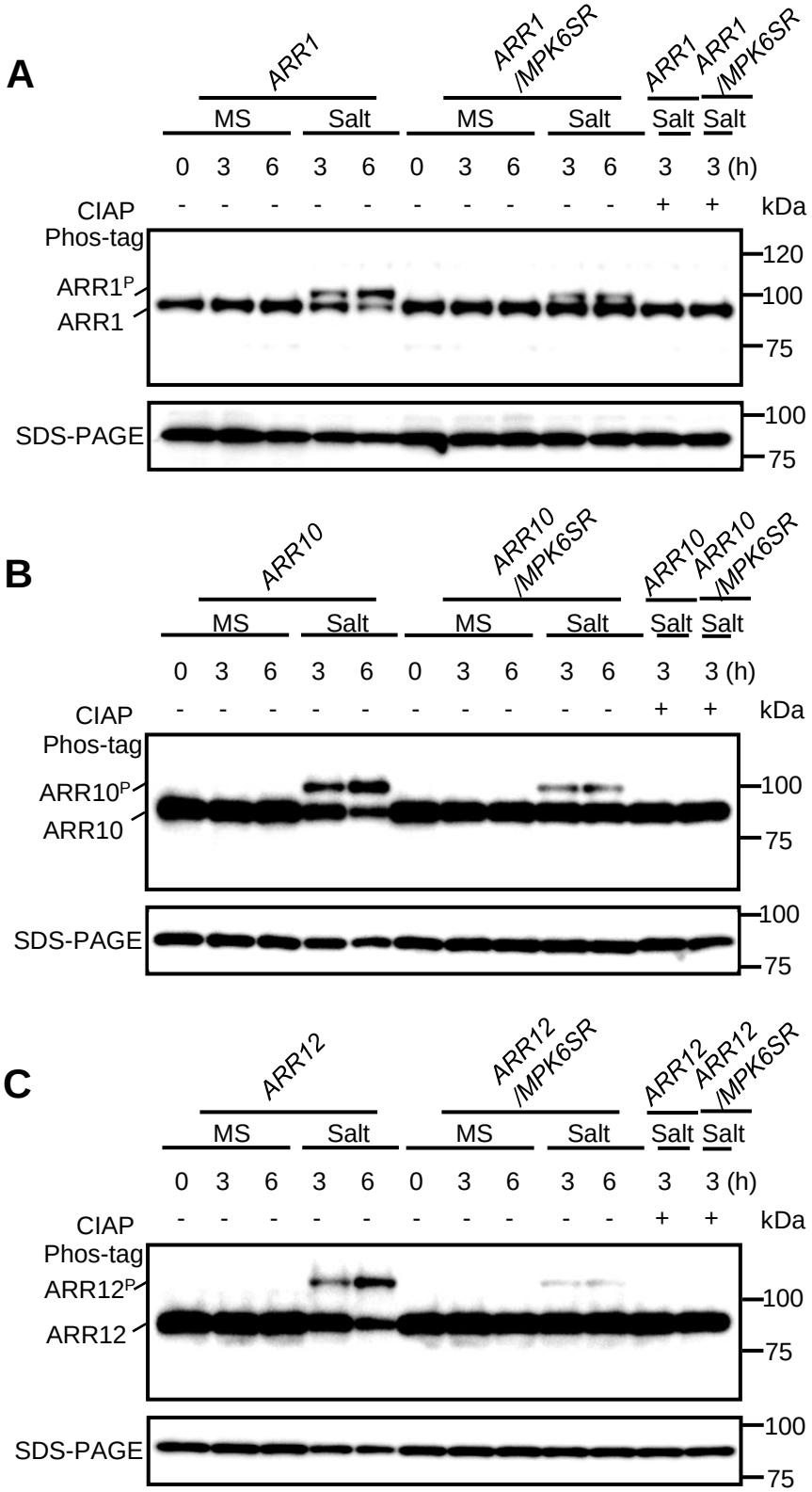


Appendix Fig S10. MPK3/6 phosphorylation sites on ARR1/10/12.

A Mass spectrometry analysis of MPK6 phosphorylation site on ARR1.

B Mass spectrometry analysis of MPK3 phosphorylation site on ARR1.

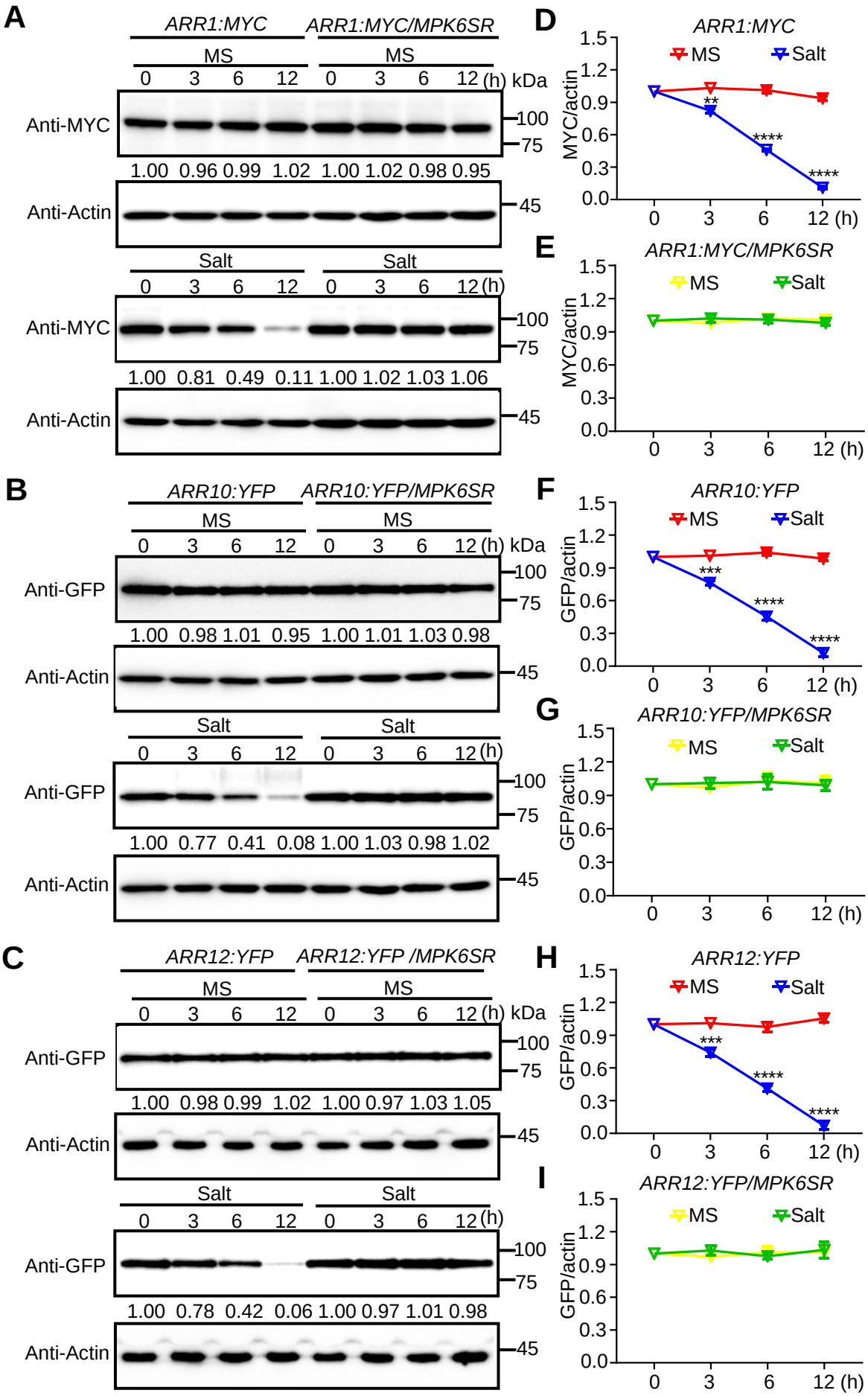
C Mass spectrometry analysis of MPK6 phosphorylation site on ARR12.



Appendix Fig S11. MPK3/6-mediated ARR1/10/12 phosphorylation *in vivo*.

A-C Immunoblot analysis with anti-MYC antibody or anti-GFP antibody showing the upshift of ARR1, ARR10, and ARR12 in a phos-tag gel. The upshift of ARR1, ARR10, and ARR12 were abolished after treatment with calf intestinal alkaline phosphatase (CIAP). Seven-day-old seedlings of *35S:ARR1:MYC* (A), *35S:ARR10:YFP* (B), and *35S:ARR12:YFP* (C) in Col and *MPK6SR* backgrounds were treated with or without 200 mM NaCl for indicated time points. Total proteins were extracted and subjected to immunoblot analysis. ARR1, ARR10, and ARR12 proteins separated on SDS-PAGE gel without phos-tag were detected as loading controls.

Data information: Similar results were obtained with three biological repeats.

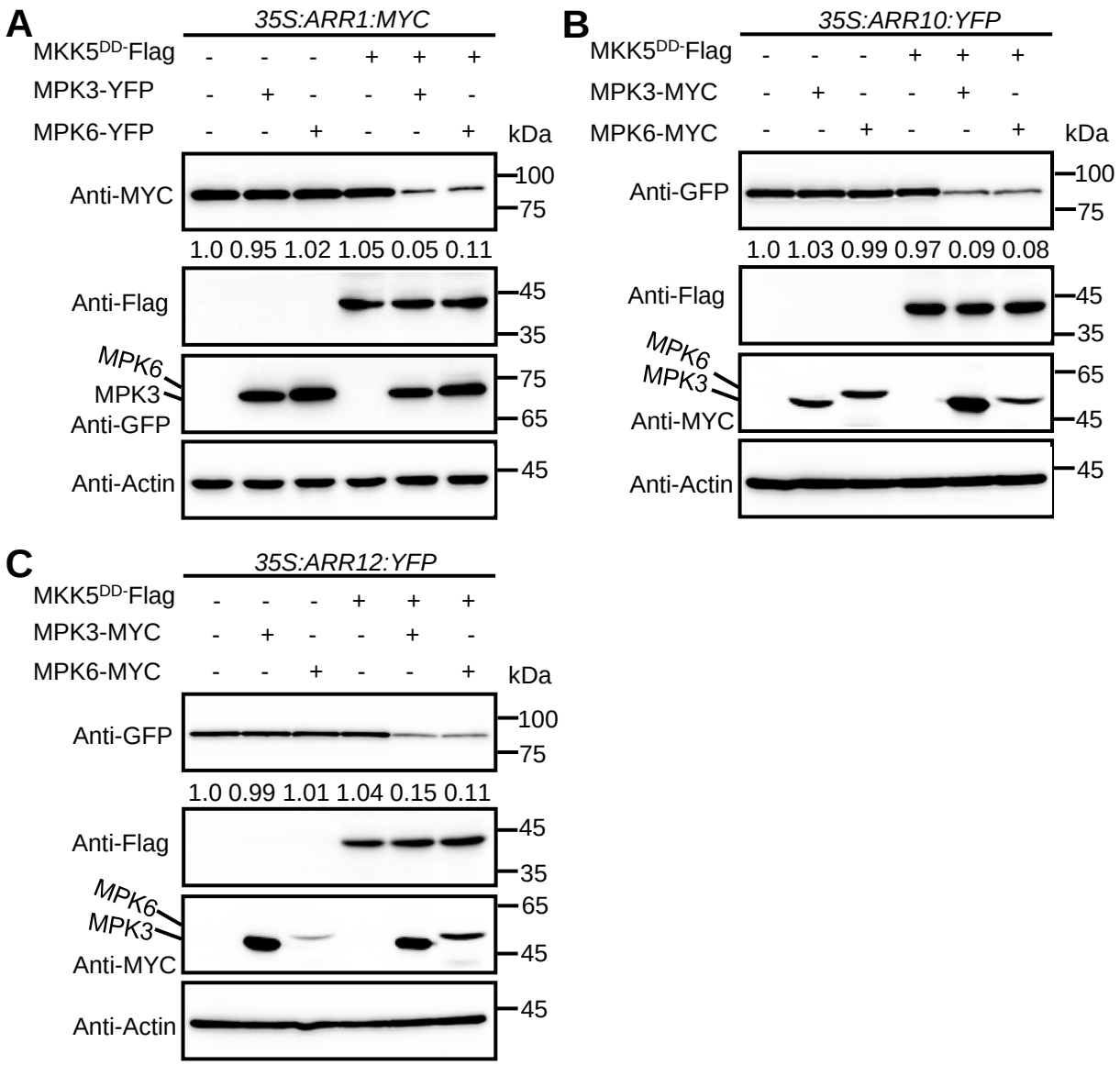

A
ARR1:MYC
ARR1:MYC/MPK6SR
MS
MS
0 3 6 12 0 3 6 12 (h) kDa
Anti-MYC
100
75
1.00 0.96 0.99 1.02 1.00 1.02 0.98 0.95
Anti-Actin
45
Salt
Salt
0 3 6 12 0 3 6 12 (h)
Anti-MYC
100
75
1.00 0.81 0.49 0.11 1.00 1.02 1.03 1.06
Anti-Actin
45
D
ARR1:MYC
MS
Salt
MYC/actin
1.5
1.2
0.9
0.6
0.3
0.0
**
****
****
0 3 6 12 (h)
E
ARR1:MYC/MPK6SR
MS
Salt
MYC/actin
0 3 6 12 (h)
B
ARR10:YFP
ARR10:YFP/MPK6SR
MS
MS
0 3 6 12 0 3 6 12 (h) kDa
Anti-GFP
100
75
1.00 0.98 1.01 0.95 1.00 1.01 1.03 0.98
Anti-Actin
45
Salt
Salt
0 3 6 12 0 3 6 12 (h)
Anti-GFP
100
75
1.00 0.77 0.41 0.08 1.00 1.03 0.98 1.02
Anti-Actin
45
F
ARR10:YFP
MS
Salt
GFP/actin
1.5
1.2
0.9
0.6
0.3
0.0
***
****
****
0 3 6 12 (h)
G
ARR10:YFP/MPK6SR
MS
Salt
GFP/actin
0 3 6 12 (h)
C
ARR12:YFP
ARR12:YFP /MPK6SR
MS
MS
0 3 6 12 0 3 6 12 (h) kDa
Anti-GFP
100
75
1.00 0.98 0.99 1.02 1.00 0.97 1.03 1.05
Anti-Actin
45
Salt
Salt
0 3 6 12 0 3 6 12 (h)
Anti-GFP
100
75
1.00 0.78 0.42 0.06 1.00 0.97 1.01 0.98
Anti-Actin
45
H
ARR12:YFP
MS
Salt
GFP/actin
1.5
1.2
0.9
0.6
0.3
0.0
***
****
****
0 3 6 12 (h)
I
ARR12:YFP/MPK6SR
MS
Salt
GFP/actin
0 3 6 12 (h)

Appendix Fig S12. Salt stress-induced ARR1/10/12 degradation is dependent on MPK3/6.

A-C Immunoblot analysis the effects of MPK3/6 on salt stress-induced ARR1/10/12 protein degradation. Seven-day-old seedlings of *35S:ARR1:MYC*, *35S:ARR10:YFP*, and *35S:ARR12:YFP* in Col-0 and *MPK6SR* backgrounds were treated with or without 200 mM NaCl for indicated time points. Actin was used as the internal reference. The relative intensity of band detected by anti-MYC or anti-GFP antibody to that by anti-Actin antibody without treatment was set to 1.0.

D-I Quantification analysis of MPK3/6 effect on salt stress-induced ARR1/10/12 protein degradation in (A-C).

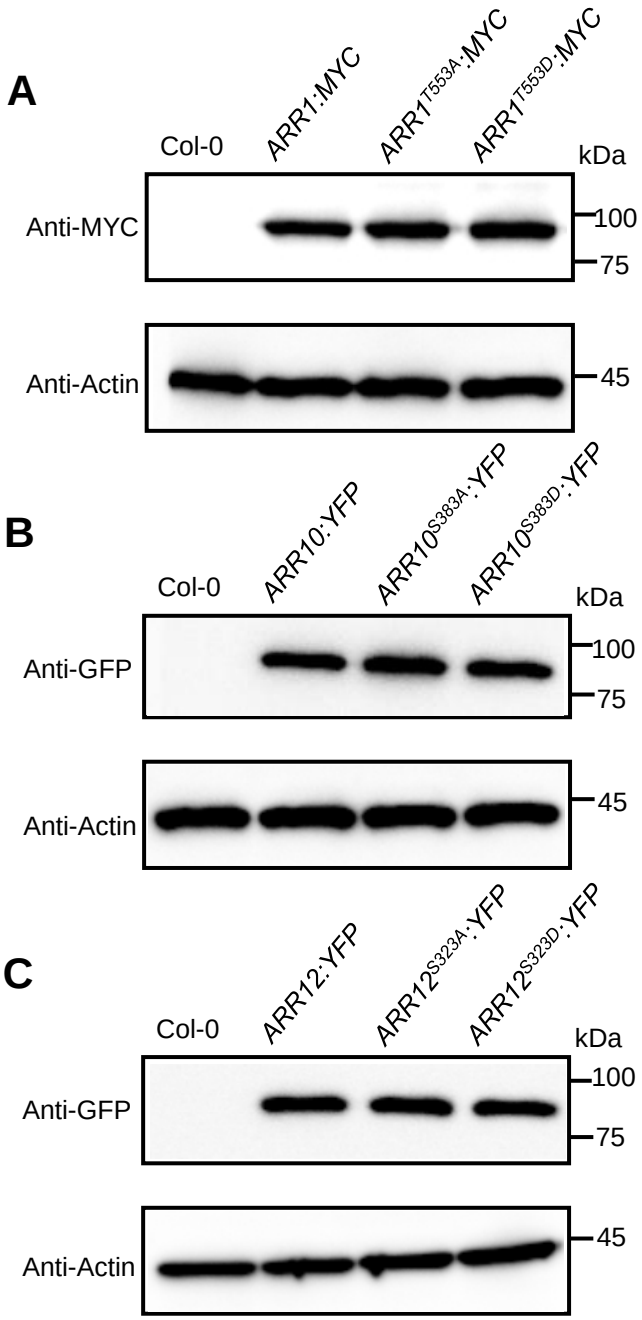
Data information: In (D-I), the statistical analyses of the related density of western blotting bands were performed using ImageJ software based on three independent biological replicates. Values are means ± SD (n=3). **,***, and **** indicate significant difference to the corresponding controls with $P<0.01$, $P<0.001$, and $P<0.0001$, respectively (student's *t*-test).



Appendix Fig S13. MKK5-MPK3/6-mediated phosphorylation induces protein degradation of ARR1/10/12.

Arabidopsis protoplast transient expression assay showed that MKK5^{DD}-activated MPK3/6 promoted degradations of ARR1 (A), ARR10 (B), and ARR12 (C). ARR1 was detected with anti-MYC antibody, ARR10 and ARR12 were detected with anti-GFP antibody. MPK3 and MPK6 were detected with anti-GFP or anti-MYC antibody, MKK5^{DD} was detected with anti-Flag antibody. Actin was used as a control.

Data information: Similar results were obtained with three biological repeats.

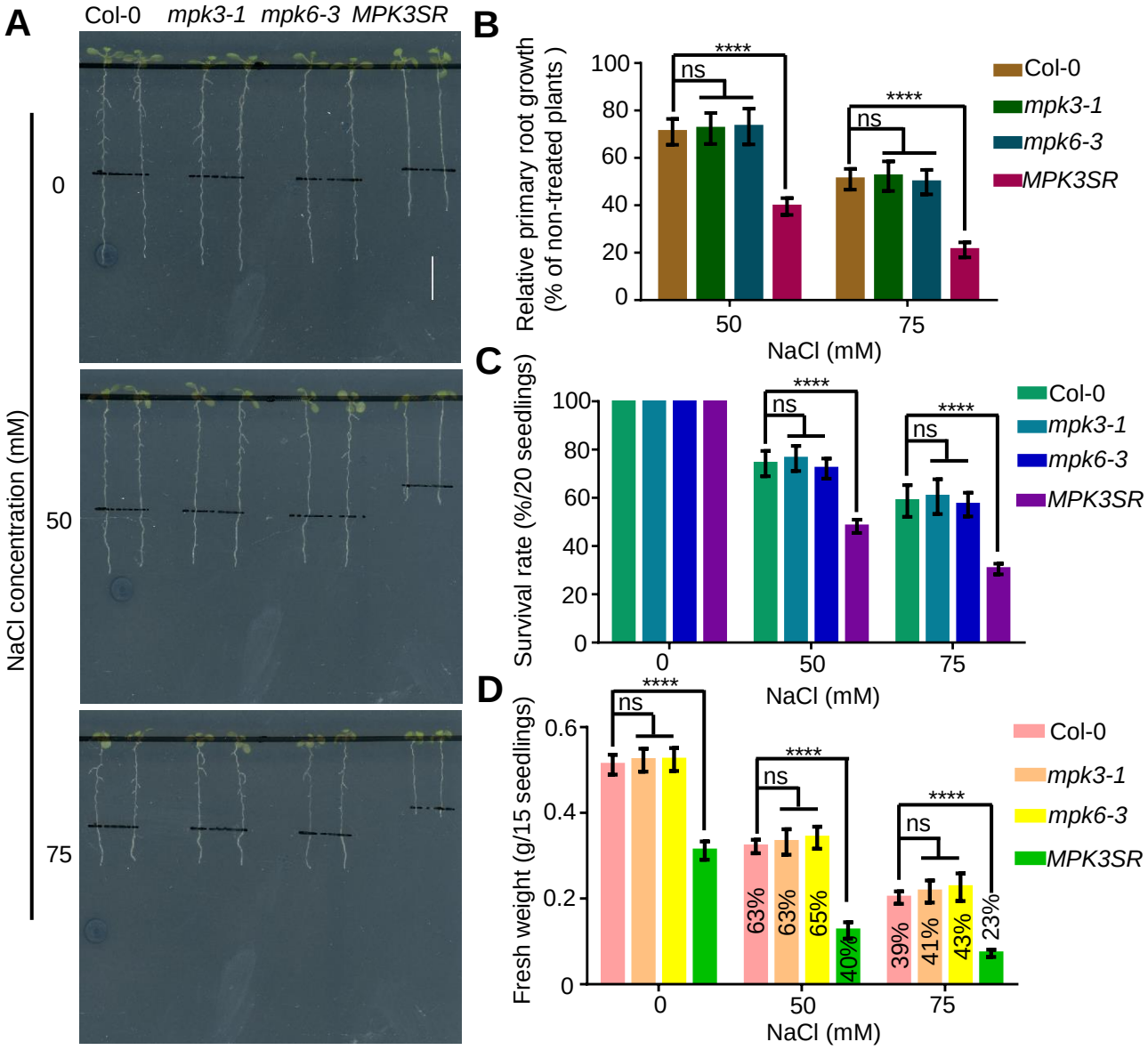


Appendix Fig S14. Immunoblot analysis the level of three forms of ARR1/10/12 proteins.

A The level of ARR1:MYC, ARR1^{T553A}:MYC, and ARR1^{T553D}:MYC proteins. Total proteins were extracted from seven-day-old *35S:ARR1:MYC* , *35S:ARR1^{T553A}:MYC* , and *35S:ARR1^{T553D}:MYC* transgenic plants. Anti-MYC antibody was used to detect ARR1, ARR1^{T553A} , and ARR1^{T553D} protein. Actin was used as a loading control.

B The level of ARR10:YFP, ARR10^{S383A}:YFP, and ARR10^{S383D}:YFP proteins. Total proteins were extracted from seven-day-old *35S:ARR10:YFP* , *35S:ARR10^{S383A}:YFP* , and *35S:ARR10^{S383D}:YFP* transgenic plants. Anti-GFP antibody was used to detect ARR10, ARR10^{S383A} , and ARR10^{S383D} protein. Actin was used as a loading control.

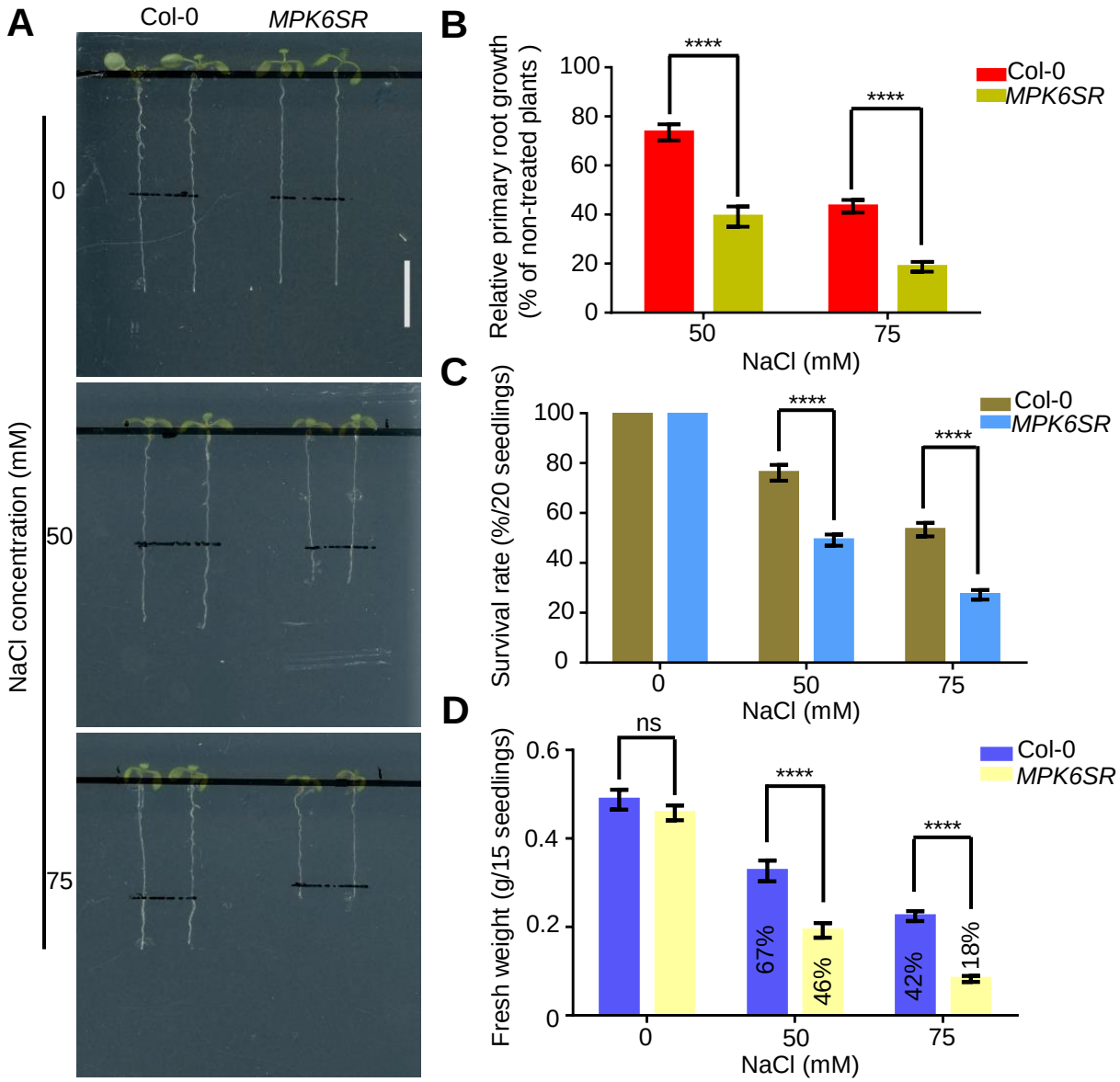
C The level of ARR12:YFP, ARR12^{S323A}:YFP, and ARR12^{S323D}:YFP proteins. Total proteins were extracted from seven-day-old *35S:ARR12:YFP* , *35S:ARR12^{S323A}:YFP* , and *35S:ARR12^{S323D}:YFP* transgenic plants. Anti-GFP antibody was used to detect ARR12, ARR12^{S323A} , and ARR12^{S323D} protein. Actin was used as a loading control.



Appendix Fig S15. MPK3/6 positively regulate plant tolerance to salt stress.

Three-day-old seedlings of Col-0, *mpk3-1*, *mpk6-3*, and *MPK3SR* were transferred to 0.5 μM NA-PP1-containing 1/2 MS medium for 2 d to inhibit MPK3^{TG} in *MPK3SR* and then the plants were transferred to 0.5 μM NA-PP1-containing 1/2 MS medium supplemented with or without 50 mM or 75 mM NaCl. Pictures were taken and the elongated root length was determined 3 days later (A-B). Changes in survival rate (C) and fresh weight (D) were examined and calculated after 10 days of salt treatment. The fresh weight of NaCl treated seedlings/fresh weight of NaCl untreated seedlings ratios were labelled in the columns in (D). Scale Bar, 1 cm.

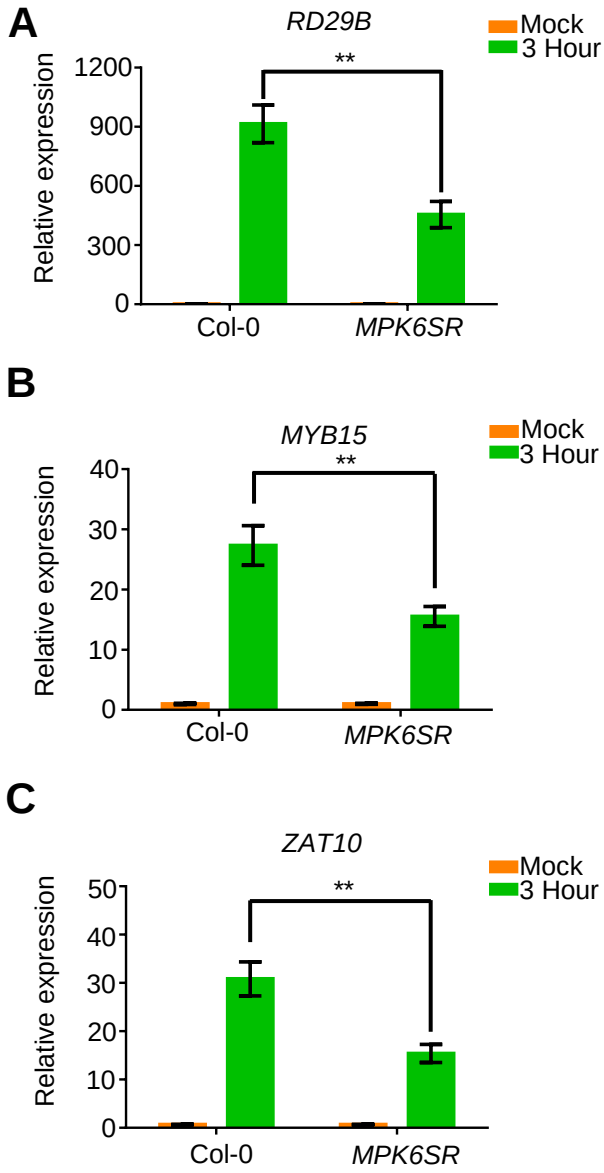
Data information: In (B-D), data are means of three biological replicates ± SD (n=60 for B, and n=12 for C and D). ns indicates no significant difference to the corresponding controls. **** indicates significant difference to the corresponding controls with P<0.0001(student's t-test).



Appendix Fig S16. *MPK6SR* displays hypersensitive to salt stress compared with that of WT.

Three-day-old seedlings of Col-0 and *MPK6SR* were transferred to 0.5 μM NA-PP1-containing 1/2 MS medium for 2 d to inhibit MPK6^{YG} in *MPK6SR* and then the plants were transferred to 0.5 μM NA-PP1-containing 1/2 MS medium supplemented with or without 50 mM or 75 mM NaCl. Pictures were taken and the elongated root length was determined 3 days later (A-B). Changes in survival rate (C) and fresh weight (D) were examined and calculated after 10 days of salt treatment. The fresh weight of NaCl treated seedlings/fresh weight of NaCl untreated seedlings ratios were labelled in the columns in (D). Scale Bar, 1 cm.

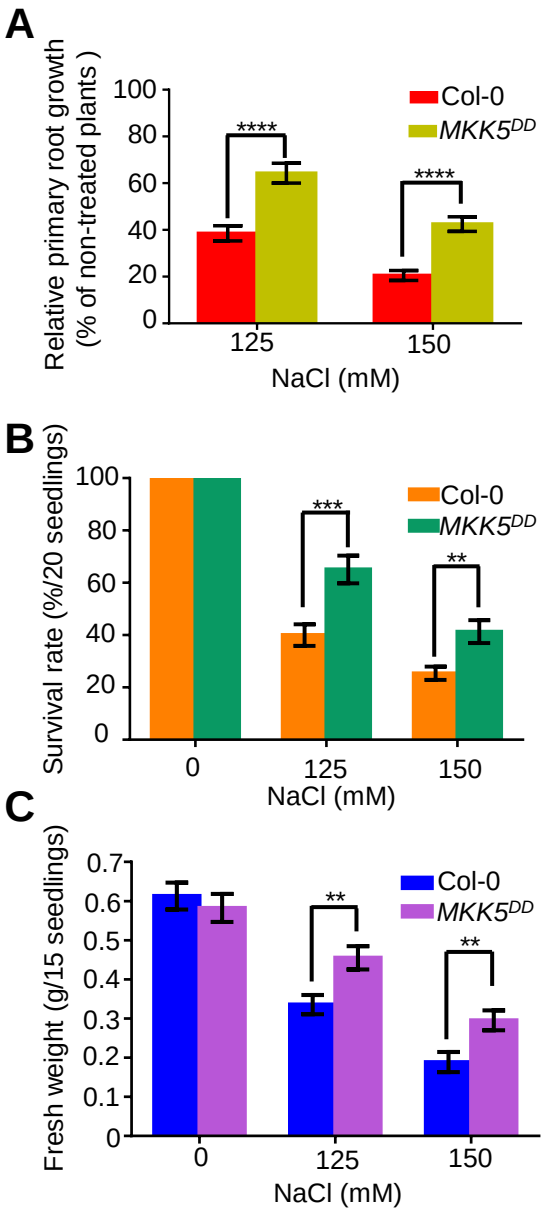
Data information: In (B-D), data are means of three biological replicates ± SD (n=60 for B, and n=12 for C and D). ns indicates no significant difference to the corresponding controls. **** indicates significant difference to the corresponding controls with P<0.0001(student's t-test).



Appendix Fig S17. Expression analysis of salt-responsive genes.

A-C Seven-day-old seedlings were treated with or without 200 mM NaCl for 3 hours. Transcript levels of *RD29B*, *MYB15*, and *ZAT10* in Col-0 and *MPK6SR* were examined by qRT-PCR. *Actin2* gene and *UBQ1* gene were analyzed as internal controls.

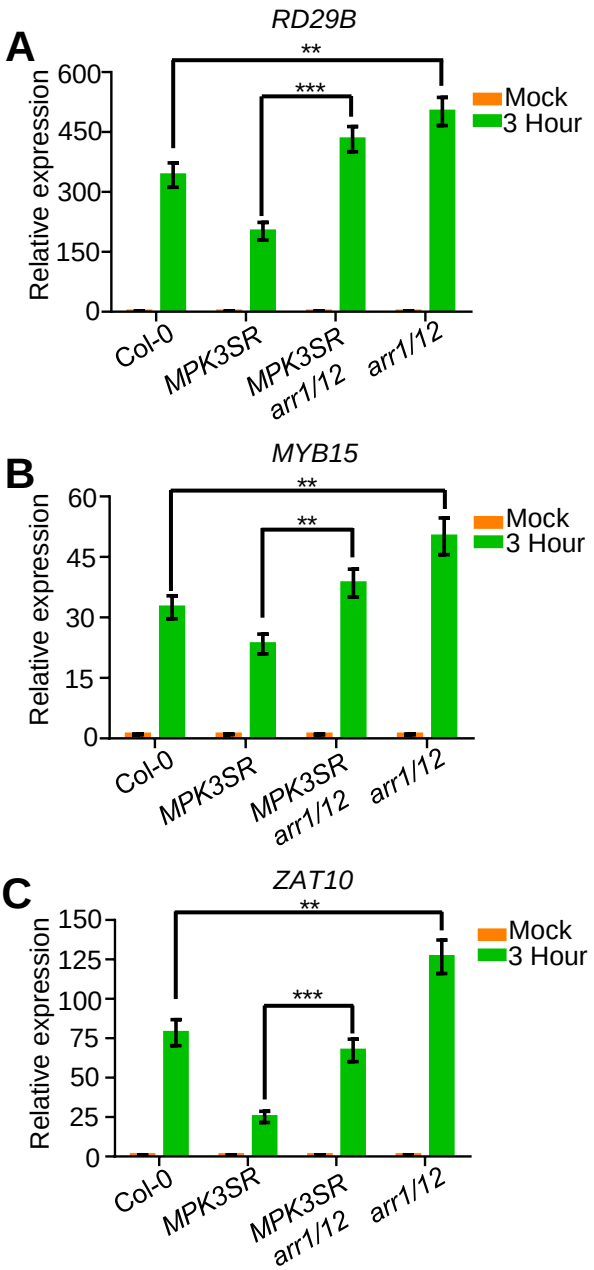
Data information: Data shown are means ± SD of three biological replicates. ** indicates significant difference to the corresponding controls with $P<0.01$ (student's *t*-test).



Appendix Fig S18. MKK5 positively regulates plant tolerance to salt stress.

Three-day-old seedlings of Col-0 and *MKK5*^{DD} were transferred to 0.2 μM dexamethasone (DEX)-containing 1/2 MS medium for 2 d to active MKK5 in *MKK5*^{DD} seedlings and then the plants were transferred to 0.02 μM dexamethasone (DEX)-containing 1/2 MS medium supplemented with 125 mM or 150 mM NaCl. Primary root length was determined 3 d later (A), survival rate (B) and fresh weight (C) were examined 10 d later.

Data information: Data are means of three biological replicates ± SD (n=60 for A, and n=12 for B and C). **, ***, and **** indicate significant difference to the corresponding controls with $P<0.01$, $P<0.001$, and $P<0.0001$, respectively (student's *t*-test).



Appendix Fig S19. Expression analysis of salt-responsive genes.

A-C qRT-PCR showed that the expression level of salt-responsive genes (*RD29B*, *MYB15*, and *ZAT10*) in Col-0, *MPK3SR*, *MPK3SRarr1/12*, and *arr1/12* seedlings. Seven-day-old seedlings were treated with or without 200 mM NaCl for 3 hours. *Actin2* gene and *UBQ1* gene were analyzed as internal controls.

Data information: Data shown are means ± SD of three biological replicates. **, and *** indicate significant difference to the corresponding controls with $P<0.01$, and $P<0.001$, respectively (student's *t*-test).