

## Rewiring of MAPK cascades in Arabidopsis

Title

### **Dissection of MAPK signaling specificity through protein engineering in a developmental context**

Diego L. Wengier<sup>1\*</sup>, Gregory R. Lampard<sup>1</sup> and Dominique C. Bergmann<sup>1,2,\*</sup>

<sup>1</sup> Howard Hughes Medical Institute and <sup>2</sup>Stanford University, Department of Biology, 371 Serra Mall, Stanford, CA 94305, USA.

\* Corresponding authors: [dbergmann@stanford.edu](mailto:dbergmann@stanford.edu) and [dwengier@dna.uba.ar](mailto:dwengier@dna.uba.ar)

Running title: Rewiring of MAPK cascades in Arabidopsis

Key words: MAPK specificity/Signal Transduction/Network rewiring/Stomatal development

Subject Categories: Synthetic Biology and Plant Biology

Total Character count: 57,660

Figures: 9

Tables: 1

Figure supplements: 7

Supplemental Files: 1

**Footnotes**

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The authors responsible for distribution of materials integral to the findings presented in this article are

Dominique C. Bergmann [dbergmann@stanford.edu](mailto:dbergmann@stanford.edu) and [dwengier@dna.uba.ar](mailto:dwengier@dna.uba.ar)

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### 1 **Abstract**

2  
3 Mitogen-activated protein kinases (MAPK) signaling affects many processes, some of which  
4 have different outcomes in the same cell. In Arabidopsis, activation of a MAPK cascade consisting of  
5 YODA, MKK4/5 and MPK3/6 inhibits early stages of stomatal developmental, but this ability is lost at  
6 the latest stage when guard mother cells (GMCs) transition to guard cells (GCs). Rather than  
7 downregulating cascade components, stomatal precursors must have a mechanism to prevent late stage  
8 inhibition because the same MKKs and MPKs mediate other physiological responses. Here, we  
9 artificially activated the MAPK cascade using MKK7, another MKK that can modulate stomatal  
10 development, and found that inhibition of stomatal development is still possible in GMCs. This suggests  
11 that MKK4/5, but not MKK7, are specifically prevented from inhibiting stomatal development. To  
12 identify regions of MKKs responsible for cell-type specific regulation, we used a domain swap approach  
13 with MKK7 and a battery of *in vitro* and *in vivo* kinase assays. We found that N-terminal regions of  
14 MKK5 and MKK7 establish specific signal-to-output connections like they do in other organisms, but  
15 they do so in combination with previously undescribed modules in the C-terminus. One of these modules  
16 encodes the GMC-specific regulation of MKK5, that when swapped with MKK7's, allows MKK5 to  
17 mediate robust inhibition of late stomatal development. Because MKK structure is conserved across  
18 species, the identification of new MKK specificity modules and signaling rules furthers our understanding  
19 of how eukaryotes create specificity in complex biological systems.

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21

### 22 **Introduction**

23 MAPKs have strategic roles in signal processing, in mediating stress responses, and in guiding  
24 cell fate transitions and development (Keshet and Seger, 2010; Rodriguez et al., 2010; Yang et al., 2013;  
25 Chen and Thorner, 2007). MAPK networks consist of a three-tiered cascade whose kinases--MAPK  
26 kinase kinase or MKKK, MAPK kinase (Mapk/Erk kinases or MEK in animals, MKK in Arabidopsis)

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27 and MAPK (MPK in Arabidopsis) sequentially phosphorylate and activate each other upon signal  
28 perception. Downstream effectors may respond to MPK-mediated phosphorylation by changes in protein  
29 activity, localization or stability, and many of these alterations ultimately alter transcriptional programs.  
30 The MKK level is a bottleneck in many species. In humans, at least 25 MKKKs activate the 7 MEKs,  
31 which lie upstream of 14 MAPKs (Keshet and Seger, 2010). In *Arabidopsis thaliana*, more than 60  
32 MKKKs are predicted upstream of 10 MKKs and 23 MPKs (Dóczy et al., 2012). Evidence exists that  
33 MKKs can activate more than one MPK, and a given MPK may have more than one upstream MKK  
34 (Andreasson and Ellis, 2010). Intuitively, this arrangement could facilitate signal integration, as multiple  
35 signals could converge on a single effector. The use of common components, however, could also lead to  
36 erroneous cross-activation.

37         When expressing multiple MAPK network components and responding to multiple sources of  
38 information, how do cells generate an appropriate output to a particular signal? One strategy is to make  
39 downstream effectors available in only certain cells (Lampard et al., 2008) or under certain conditions  
40 (Bao et al., 2004; Brückner et al., 2004; Chou et al., 2004). Alternatively, signaling networks can be  
41 insulated through the use of scaffolds, subcellular partitioning of signaling complexes, and by varying  
42 signal amplitude or duration (Murphy et al., 2003; Murphy and Blenis, 2006; Raman et al., 2007; Keshet  
43 and Seger, 2010; Good et al., 2011; Dóczy et al., 2012). Some MAPK network components encode  
44 regions that allow them to establish these connections or localizations. For instance, the animal MEK1/2  
45 uses its proline-rich sequence (PRS) to bind the scaffolds Kinase Suppressor of Ras (KSR) and Mek  
46 Partner 1 (MP1). Binding to MP1, in particular, mediates endosomal localization (Schaeffer et al., 1998;  
47 Teis et al., 2002; McKay et al., 2009). Several human MEKs and all Arabidopsis MKKs lack a PRS,  
48 however, leaving questions about how these smaller MKKs are correctly assembled into restricted MAPK  
49 networks.

50         In Arabidopsis, MAPK signaling has been shown to have a fundamental role in the formation of  
51 stomata, the structures in the epidermis of plants that regulate gas exchange (Bergmann et al., 2004;  
52 Wang et al., 2007). A systematic study of cell stage-specific responses to MAPK activation revealed that

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53 stomatal precursors have mechanisms to limit certain cellular outputs and generate MKK-specific  
54 responses (Lampard et al., 2009, 2014). Only four of the 10 MKKs -MKK4, MKK5, MKK7 and MKK9-  
55 have any capacity to influence stomatal development during lineage initiation, guard mother cell (GMC)  
56 commitment and/or guard cell (GC) formation (for simplicity, only MKK5 and MKK7 are shown in  
57 Figure 1). Expression of any of these four constitutively active (CA) MKKs strongly inhibits stomatal  
58 formation in early development (Figure 1A, C and D). At the last stage of development, however, MKK4  
59 and MKK5 lose their ability to inhibit stomatal formation (Figure 1B and E). MKK7 and MKK9  
60 activation, in contrast, results in stomatal clustering (Figure 1B and F). Loss of function studies with  
61 *MKK4* and *MKK5* indicate that these kinases are endogenously required to limit stomatal production  
62 (Wang et al., 2007), but the MKK7/9 significance is unclear as *mkk9* single mutants do not affect stomata  
63 and in the recent report of true loss of function mutations in *mkk7* mutants no stomata phenotype was  
64 described (Jia et al., 2016).

65

66 **Figure 1: Schematic of stomatal lineage, indicating stages where MKK activation leads to similar**  
67 **and divergent outputs (based on (Lampard et al., 2009)).** (A-B) Diagrams of MAPK signaling cascades,  
68 with each colored circle representing a different kinase level; circles are labeled with relevant kinase  
69 number, with orange circle representing MPK of unknown identity. Constitutive activation of YDA  
70 (MKKK), MKK5 or MKK7 inhibits stomatal lineage initiation (A, SPCH and MUTE stages). Late in the  
71 lineage (B, FAMA stage) YDA and MKK7, but not MKK5, activation leads to stomatal proliferation via  
72 an unidentified MPK. (C-F) Tracings of phenotypes resulting from activation of kinases. In C-D,  
73 constitutively active MKK5 (MKK5<sup>DD</sup>) or MKK7 (MKK7<sup>ED</sup>) inhibit initiation (division of meristemoid  
74 mother cell (MMC) into meristemoid (M)) and lineage progression (conversion of M into guard mother  
75 cell (GMC)). In E-F, MKK5<sup>DD</sup> has no effect (E, WT numbers and distribution of stomata in green), but  
76 MKK7<sup>ED</sup> induces guard cell (GC) overproliferation and clustering (F). Stages are referred to as SPCH,  
77 MUTE and FAMA after the promoters to drive expression of MAPK network components (Lampard et  
78 al., 2009). YDA, YODA; 5, MKK5; 7, MKK7; 3/6, MPK3 and MPK6.

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80 MKK4/5's endogenous role in limiting stomatal production in early stages, but not in late  
81 stomatal lineage cells, provides an excellent test case for examining how cell identity may interface with  
82 signaling response. We created a quantitative phenotypic analysis pipeline that revealed a previously  
83 underappreciated capacity for late stage stomatal lineage cells to be inhibited by MAPK signaling. Then,  
84 taking advantage of divergent responses to constitutively activated MKK5 and MKK7, we implemented a  
85 protein engineering approach to identify structural domains in MKK5 that are responsible for its stage-  
86 specific behaviors. We found that MKK N-terminal regions establish specific signal-to-output  
87 connections, much like they do in other organisms (Won et al., 2011), but this requires coordination with  
88 previously unexplored regions in the C-terminus. We also found that a minimal domain in the C-terminus  
89 encodes the basis for MKK-specific regulation. The location of specificity modules within the plant  
90 proteins corresponds to regions in which their human homologues display high sequence diversity,  
91 suggesting that these regions may contribute to specificity in many situations. Given the global  
92 conservation of MAPK signaling, our findings in the complex multicellular context of plant development  
93 may offer insights into general mechanisms of signaling specificity in complex biological systems.

94

95

### 96 **Materials and Methods**

97

#### 98 **Plant material and growth conditions**

99 All transgenic lines were generated in the Col-0 background. Seeds were plated on 0.5X  
100 Murashige-Skoog media containing 1% agar-agar (Caisson Laboratories, North Logan, UT) and 100  
101  $\mu\text{g/ml}$  Kanamycin (Sigma-Aldrich) or 50  $\mu\text{g/ml}$  Hygromycin B (Life Technologies) when appropriate.  
102 Seedlings were grown under a light intensity of 100  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  in a 16:8 photoperiod at  $22 \pm$   
103  $1^\circ\text{C}$ . Analyses were performed in 15 day after germination (dag) cotyledons unless stated otherwise. The

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104 *mpk6-3* allele was Salk\_127507 (Müller et al., 2010). Mutant alleles for *EDR1* (Salk\_127158), *MAP3KδI*  
105 (Salk\_048985) and *MAP3Kδ5* (Salk\_029929 and Salk\_036615) were obtained from the ABRC.

106

### 107 **Multiple sequence alignment and structural analysis**

108 Selected mammalian kinases were aligned using ClustalOmega (Sievers et al., 2011) and  
109 structural models are in Supplemental Figure 3B. MKK5 and MKK7 structural predictions were  
110 performed with I-Tasser (Zhang, 2008), using MEK1 (1S9J) to assist the prediction. Models were  
111 explored with Swiss-PdbViewer v4.1 and fit with the Magic Fit button (Guex and Peitsch, 1997).  
112 Structural features were extracted from models and overlaid on the primary sequence (Supplemental  
113 Figures 3-5).

114

### 115 **Construction of constitutively active MKK and synthetic chimeras**

116 Domain swap constructs were assembled by fusion PCR from DNA amplicons (blocks) generated  
117 with Phusion® High-Fidelity DNA Polymerase following manufacturer's instructions (New England  
118 Biolabs, Ipswich, MA). To generate blocks, MKK5<sup>DD</sup> and MKK7<sup>ED</sup> cloned into pENTR without stop  
119 codons or other chimeras were used as templates (Lampard et al., 2009). Blocks were designed to contain  
120 attL1 and attL2 functional sequences from pENTR to ease the cloning procedure through the Gateway  
121 strategy (Supplemental File 1). For domain swaps assembled from two blocks, 5' blocks contained the  
122 M13 forward priming site and attL1 recombination site before the MKK sequence; and 3' blocks  
123 contained the MKK sequence followed by attL2 recombination site and M13 reverse priming site. To  
124 facilitate fusion of the blocks, reverse primers for 5' blocks and forward primers for 3' blocks were  
125 designed as chimeras of the two blocks to be fused, containing at least 15 bases from each block, and  
126 were completely complementary to each other. PCR products were gel extracted using QIAquick Gel  
127 Extraction Kit (QIAGEN Inc., Valencia, CA) and 1:1 molar ratio mix were used as templates on fusion  
128 PCR reactions using M13 forward and reverse primers. Domain swap constructs were gel purified and

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129 cloned into pJET 1.2 according to CloneJET PCR Cloning Kit instructions (Thermo Life Sciences,  
130 Pittsburgh, PA). For domain swap constructs assembled from 3 blocks, 5' and 3' were generated with the  
131 same strategy as above, while internal block was amplified with forward and reverse chimeric primers. As  
132 domain swaps became more elaborate, first domain swap constructs were used as templates for generating  
133 new blocks. Primers, templates and sequences for each domain swap are listed in Supplemental Table 1,  
134 A and B.

135 To build constructs for expression under SPCH and FAMA promoters, 2.5-kb fragments  
136 previously described (Ohashi-ito and Bergmann, 2006; Macalister et al., 2007) were first adapted to the  
137 Multisite Gateway system. Promoters were shuttled from pENTR to pDONR P4 P1R (Life Technologies,  
138 Grand Island, NY) by PCR amplification using promoter shuttling primers (Supplemental File 1)  
139 followed by BP recombination performed under manufacturer's instructions. Promoters flanked by attL4  
140 and attR1 recombination sites (pDONR-promoter) were used in Multisite recombination reactions with  
141 domain swap constructs in pJET and R4pGWB440 [destination vector carrying the Gateway cassette  
142 flanked by attR4 and attR2 recombination sites, in frame C-terminal fusion to enhanced YFP and  
143 kanamycin selection in plants (Nakagawa et al., 2008)]. Recombination reactions were performed in a  
144 two-step protocol. First, 1  $\mu$ l of LR Clonase II was added to 4  $\mu$ l vector mix (containing 150 ng of  
145 pDONR-promoter and 150 ng pJET-domain swap construct) and incubated at 25°C for 5 hours. Then, 150  
146 ng of R4pGWB440 in 4  $\mu$ l solution were added to the reactions along 1  $\mu$ l of LR Clonase II. Reactions  
147 were incubated for additional 16 hours at 25°C and then stopped after the addition of 1  $\mu$ l of Proteinase K  
148 and incubation for 10 min at 37°C. Constructs were confirmed by sequencing and introduced in  
149 Arabidopsis by *Agrobacterium tumefaciens*-mediated transformation.

150 Mutants *edr1*, *map3k $\delta$ 1* and *map3k $\delta$ 5* were transformed with *FAMA<sub>pro</sub>:MKK5<sup>DD</sup>* in pHGY,  
151 previously used in (Lampard et al., 2009). MPK3 and MPK6 clones were provided by Jean Colcombet  
152 (INRA Versailles-Grignon, France) (Berriri et al., 2012).

153

154 **Scoring phenotypes and data analysis**



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155 Seedlings (15 dag) were fixed in 7:1 ethanol:acetic acid, and cleared in Hoyer's medium.  
156 Cotyledons were imaged by differential interference contrast microscopy on a Leica DM2500 microscope  
157 at  $\times 20$  magnification ( $0.320 \text{ mm}^{-2}$  field of view). One picture per independent transgenic seedling was  
158 taken from the distal tip of the cotyledons, within the vascular loop, on the abaxial epidermis. Phenotypes  
159 at the FAMA stage were as follows: (1) normal phenotype, only single stomata with tolerance for 1  
160 stomatal cluster per field of view; (2) stomatal inhibition, no stomata present or inhibited precursors  
161 coexisted with normal stomata and appeared in at least two independent fields of view per sample; (3)  
162 large stomatal cluster, at least two stomatal clusters (4 or more stomata in contact) per field of view; (4)  
163 small stomatal cluster, clusters contained 2-3 stomata in contact. When a sample contained a mixed  
164 population of clusters, the presence of large clusters defined the classification for this category.  $\text{MKK7}^{\text{ED}}$ ,  
165 clusters were systematically bigger than any chimera and often were delayed in development; to confirm  
166 clustering, older epidermis (near apical hydathode or in older plants) was scored. SPCH stage phenotypes  
167 were quantified as (1) inhibited (no stomata per field of view) or (2) not inhibited (2 or more stomata per  
168 field of view). To enable us to score phenotypes in T1 seedlings that must be grown on antibiotic  
169 selection, we grew 35S:YFP lines with the same antibiotic resistance as the MKK variants under the same  
170 conditions and scored these as the equivalent of WT controls.

171 A binomial distribution for phenotypic data was assumed and the percentages of each phenotype  
172 were calculated with a confidence of 95%. This analysis is similar to others done on data of comparable  
173 nature (Wang et al., 2011). Linear regressions between *in vitro* and *in vivo* data; and between SPCH and  
174 FAMA data in Figure 7 and Supplemental Figure 7 were done with Microsoft Excel. To cluster chimeras,  
175 hierarchical clustering was performed on phenotypic data at the FAMA stage using the function `pclus` in  
176 the statistical software R. Percentages of each one of the four phenotypes -Inhibited, Normal, Small  
177 Clusters and Large Clusters- were converted to frequencies (e.g. dividing by 100). The distance matrix  
178 was obtained by calculating the dissimilarities between all chimeras in their four phenotypes with the  
179 Manhattan method. Clustering was performed with the `ward.d` method and the number of bootstrap  
180 replications was 1000. To statistically determine how similar chimeras were within clusters, a Chi-

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181 squared test of independence was implemented to compare phenotype distributions. Frequencies were  
182 compared to YFP (inactive), MKK5<sup>DD</sup>, MKK7<sup>ED</sup> (inhibition of stomatal formation and stomatal clustering)  
183 and N5-MKK7<sup>ED</sup> (strong stomatal inhibition). Chimeras that were not statistically different are noted with  
184 the same number in Figure 7B. Unnumbered chimeras were statistically different from the rest ( $p < 0.05$ ).

185

### 186 **Confocal microscopy**

187 Confocal images were collected using a Leica SP5 confocal microscope with excitation/emission  
188 spectra of 514/520 to 540 for YFP and 565/580 to 610 for propidium iodide counterstaining. ImageJ (NIH)  
189 was used to build Z-stacks from confocal images. To improve resolution of cell outlines, layers were  
190 summed rather than averaged. Z-stacks were then split into single channels and only the channel for the  
191 cell outlines was conserved, transformed into a grey-scale image and colors were inverted. Bright and  
192 contrast were modified to improve image quality. For localization, color channels in Z-stacks were  
193 maintained and images were cropped. Localization of MKK-YFP chimeras was investigated in stable  
194 (T2-T3) lines with the exception of chimeras that induced stomatal inhibition, where localization was  
195 determined in T1s on antibiotic selection.

196

### 197 **Kinase-inactive MPK3 and MPK6 phosphorylation**

198 *In vitro* kinase assays to assess the ability of MKK variants to phosphorylate either kinase  
199 inactive (KI) MPK3 or MPK6 were performed as described in (Lampard et al., 2014). KI-MPKs were  
200 used to avoid autophosphorylation of the substrates. Band intensity was detected and analyzed using  
201 ImageJ (NIH). Each reaction was performed in triplicate. The ratio of phosphorylated KI-  
202 MPK/unphosphorylated KI-MPK detected by p42/44 antibody (Cell Signaling, Cat. No. 9102) was used  
203 to estimate MKK activity. Background signal corresponds to 1. Mean and standard error was calculated  
204 for each sample.

205

### 206 **Yeast two-hybrid assays**

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207 Yeast two-hybrid assays was performed with the matchmaker Two-Hybrid System 3 (Clontech)  
208 using a modified set of plasmids compatible with Gateway technology and conditions specified by the  
209 manufacturer. MKKs and chimeras were cloned as DNA Binding Domain fusions and MPKs were cloned  
210 as Activation Domain fusions. Three independent yeast colonies were tested for each pairwise  
211 comparison at 1, 1:10 and 1:100 dilutions after incubation of 2, 3 and 4 days in plates containing 1 mM 3-  
212 amino-1/2/4-triazole. Experiments were repeated three times with yeast cultures at OD600 of 1, 2 and 4.  
213 Interactions were evaluated as positive if significant growth was detected in 1:100 dilution at day 3.

214

### 215 Accession Numbers

216 Arabidopsis Genome Initiative locus identifiers for the genes studied in this work are: SPCH,  
217 AT5G53210; FAMA, AT3G24140; MKK5, AT3G21220; MKK6, AT5G56580; MKK7, AT1G18350;  
218 MPK3, AT3G45640; MPK6, AT2G43790; EDR1, AT1G08720; MAP3Kδ1, AT1G11850; MAP3Kδ5,  
219 AT4G24480.

220

221

### 222 Results

223

#### 224 Stomatal development is inhibited by MAPK activation at the FAMA stage

225 To carefully define the range of phenotypes in our system, we re-analyzed the inhibitory effect of  
226 CA-MKK expression in FAMA stage cells (Figure 1B) using more sensitive and quantitative  
227 measurements than in our previous studies (Lampard et al., 2009, 2014). For simplicity, we selected one  
228 representative MKK from each MKK4/5 and MKK7/9 pair as previous studies showed that MKK4  
229 mirrors MKK5 activity, and MKK7 mirrors MKK9 activity, in every stage of stomatal development  
230 (Lampard et al., 2009, 2014). Because MKKs were to be analyzed *in planta*, we selected MKK5 and  
231 MKK7, both of which can be easily detected as YFP fusion proteins, thereby providing a control for  
232 expression. FAMA promoter (FAMApro) was used to drive the expression of constitutively active MKKs

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233 which are made dominantly active by replacing the regulatory S/T residues of the activation loop with  
234 phosphomimetic D/E residues (MKK5<sup>DD</sup> = MKK5<sup>T215D,S221D</sup> and MKK7<sup>ED</sup> = MKK7<sup>S193E,S199D</sup>) (Lampard  
235 et al., 2009; Popescu et al., 2009). To be able to observe all epidermal phenotypes produced by different  
236 MKK expression levels, such as inhibition of stomatal development and stomatal clustering, phenotypes  
237 were quantified in cotyledons of independent primary transformants (T1s in Table 1, Figure 2A). We paid  
238 special attention to evidence of seedling lethality, a typical result of inhibition of stomatal development.

239

240 **Figure 2: Differences phenotypic output between MKK5 and MKK7.** (A) Micrographs of phenotypes  
241 resulting from FAMA-stage expression of MKK5<sup>DD</sup> and MKK7<sup>ED</sup>: inhibited GMCs, where precursors  
242 exit the stomatal lineage and do not make GCs (arrowheads); Normal (single stomata comprised of two  
243 GCs) or small (2-3) or large (4+) clusters of adjacent stomata resulting from overproliferation of  
244 precursors before they become GCs. Scale bars are 10  $\mu$ m. (B) Quantification of phenotypes, percentage  
245 of seedlings showing one of four phenotypes. >19 independent transformants were scored per genotype  
246 and stage (Ns reported in Table 1). 35Spro:YFP was used as a negative control (see Methods). Error bars  
247 correspond to 95% confidence intervals.

248

249

250 **Table 1: Quantification of *in vivo* and *in vitro* phenotypes conferred by chimeras.**

Name	Kinase Activity (normalized to MKK5 <sup>DD</sup> )				SPCH stage inhibition of lineage initiation		FAMA stage phenotypes (as percentage of total T1 seedlings)					
	KI-MPK3		KI-MPK6		n	%	n	Inhibited	WT	GC clusters		
	AVG	SE	AVG	SE						Small	Large	Total
YFP control							24	0.00	91.67	8.33	0.00	8.33
MKK5 <sup>DD</sup>	34.485	9.052	38.259*	2.308	69	78.26	25	0.00	76.00	24.00	0.00	24.00
MKK7 <sup>ED</sup>	6.836	4.808	10.867	5.726	93	100.00	50	20.00	26.00	0.00	54.00	54.00
N7-MKK5 <sup>DD</sup>	5.500	1.894	21.448	9.176	75	26.67	125	8.80	32.00	48.80	10.40	59.20
N5-MKK7 <sup>ED</sup>	15.889	7.052	35.235*	1.457	22	86.36	47	93.62	6.38	0.00	0.00	0.00
MKK5 <sup>DD</sup> -C7	13.888	9.753	25.666*	2.530	47	87.23	44	65.91	29.55	4.55	0.00	4.55
MKK5 <sup>DD</sup> -7A	45.980	22.674	26.287	4.947	114	12.28	98	0.00	97.96	2.04	0.00	2.04
MKK5 <sup>DD</sup> -7B	46.746	22.671	38.718*	0.750	20	10.00	70	50.00	50.00	0.00	0.00	0.00
MKK7 <sup>ED</sup> -C5	3.877	2.091	0.728	0.148	43	13.95	19	0.00	78.95	21.05	0.00	21.05
MKK7 <sup>ED</sup> -CDR5	2.451	1.242	1.303*	0.312	89	98.81	30	96.67	3.33	0.00	0.00	0.00
MKK7 <sup>ED</sup> -CPR5	15.844	11.444	5.904	2.422	105	0.00	36	2.78	91.67	5.56	0.00	5.56
MKK7 <sup>ED</sup> -5A	15.440	12.606	5.219	4.017	125	88.00	54	81.48	14.81	3.70	0.00	3.70
MKK7 <sup>ED</sup> -5B	7.238	4.545	1.317	0.200	198	16.67	60	0.00	48.33	40.00	11.67	51.67
N7-MKK5 <sup>DD</sup> -C7	3.526	1.153	2.411	0.915	91	54.95	43	2.33	13.95	13.95	69.77	83.72
N5-MKK7 <sup>ED</sup> -C5	4.724	2.876	1.439	0.673	51	72.55	46	0.00	71.74	28.26	0.00	28.26
MPK3, not shown					49	0.00	50	0.00	82.00	18.00	0.00	18.00
MPK3 <sup>D193G/E197A</sup>					18	0.00	52	0.00	80.77	19.23	0.00	19.23
MPK3 <sup>T119C</sup> , not shown					43	0.00	89	0.00	86.52	13.48	0.00	13.48
MPK6, not shown					122	0.00	45	0.00	84.44	15.56	0.00	15.56
MPK6 <sup>D218G/E222A</sup>					6	100.00	27	0.00	77.78	22.22	0.00	22.22
MPK6 <sup>Y144C</sup> , not shown					35	0.00	21	0.00	90.48	9.52	0.00	9.52

251

252 Notes: Kinase activity is determined in triplicates with the exception of those marked with “\*” that were done in duplicates. Averages (AVG) and

253 Standard Errors (SE) are represented in the table. Phenotypic categories for FAMA stage phenotypes are described in Methods.

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254 Previously, expression of MKK7<sup>ED</sup> was shown to lead to stomatal hyperproliferation (Lampard et  
255 al., 2009), and we could confirm that result: 54% T1s showed large stomatal clusters (Figure 2A and B,  
256 Table 1); however, 26% were WT (most of which showed no YFP signal) and 20% had stomatal  
257 precursors that failed to complete their development into GCs (Figure 2A and B, Inhibited). This third  
258 class died as seedlings. Among MKK5<sup>DD</sup>-YFP transformants, there were no seedling lethals: 76% T1s  
259 had a phenotype indistinguishable from controls (Figure 2B, Table 1) and 24% exhibited one to three  
260 small clusters (2-3 stomata in contact) per 0.32 mm<sup>2</sup>. Among control seedlings grown in parallel, ~8%  
261 exhibited similar small clusters (Table 1). Phenotype distributions for MKK5<sup>DD</sup>-YFP and MKK7<sup>ED</sup>-YFP  
262 were statistically different in Chi-squared test of independence ( $p \ll 0.05$ ).

263 These results indicate that MAPK activation through MKK7<sup>ED</sup>, besides driving stomatal  
264 clustering, can also lead to inhibition of stomatal development at the FAMA stage. The failure of  
265 MKK5<sup>DD</sup> to inhibit this stage transition is puzzling, since MKK5 is the endogenous kinase, whereas  
266 MKK7 is not normally expressed in FAMA stage cells (Adrian et al., 2015). Moreover, MKK5<sup>DD</sup> is an  
267 effective inhibitor of earlier stages (Lampard et al., 2009) and *MKK4MKK5RNAi* lines show excess  
268 mature GCs (Wang et al., 2007). Also, when compared to MKK7<sup>ED</sup>, MKK5 exhibits stronger interactions  
269 with MPK3/6 in Y2H (Supplemental Figure 1) and stronger kinase activity *in vitro* (Supplemental Figure  
270 2). MKK5, therefore appears to be subject to an additional level of *in vivo* regulation that blocks its  
271 inhibitory effect, while MKK7 seems to escape this regulation. We reasoned that structural differences  
272 between MKK5 and MKK7 could be probed to define the nature and the source of this differential  
273 regulation.

274

### 275 **Predicted tertiary structures of MKK5 and MKK7 suggest sources of MKK identity and specificity**

276 We reasoned that the domains most likely to confer the FAMA-stage differential responses would  
277 be surface exposed (thus available for interactions with partners) and would exhibit the greatest structural  
278 and sequence divergence among MKKs. To facilitate the identification of such regions, we modeled plant  
279 MKK folds based on the X-ray crystal structures of human orthologs MEK1 and MEK2 using I-Tasser

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280 (Zhang, 2008) (Figure 3) and used structural information from several other mammalian kinases (Hanks  
281 and Hunter, 1995; Kannan and Neuwald, 2004; Goldsmith et al., 2007; Knight et al., 2007) to identify  
282 conserved features.

283       Structural alignment of MKK5 and MKK7 to mammalian kinases confirmed the conservation of  
284 the kinase fold (Knight et al., 2007) (Figure 3B and D, with primary sequence in Supplemental Figure  
285 3A). The core catalytic domains of MKK5 and MKK7 are quite similar, but the N- and C-termini are  
286 variable. This is similar to MEK1/2, where catalytic domains are similar and well resolved, but the  
287 flanking N- and C- terminal extensions and a region containing the PRS are not. In addition, MKK5  
288 possesses, but MKK7 completely lacks, sequences at positions comparable to the C-terminal extension in  
289 MEK1/2 (Supplemental Figure 3C). Previously, we showed that this C-terminal extension does not  
290 contribute to MKK5 activity or specificity, but that N-termini have an important role in MKKs activities,  
291 possibly through the presence of D-docking domains that mediate interactions with downstream MPKs  
292 (Lampard et al., 2014).

293  
294 **Figure 3: Differences in protein structure between MKK5 and MKK7.** (A-D) Schematic and  
295 predicted structures of MKK5 (blue) and MKK7 (red). CPR, C-terminal proximal region. CDR, C-  
296 terminal Distal Region. (C-D) Four regions important for this study (N-termini, Loop A, Loop B and the  
297 C-termini) are bolded. Conserved  $\alpha$  helices A, F and G are labeled in grey.

298  
299       In addition to the distinct C-terminal distant region (CDR), divergent surface-exposed regions of  
300 the plant MKKs include a C-terminal proximal region (CPR) between conserved subdomains VIII and X  
301 (Figure 3, Supplemental Figure 3 and 4). The CPR contains two loops. Loop A starts immediately  
302 downstream of the YM(S/A)PER sequence, a MKK signature (Rodriguez et al., 2010), and ends before  
303 the highly conserved  $\alpha$ -helix F. The sequence of loop A is similar between kinases with identical  
304 functions and different between kinases with divergent functions in both Arabidopsis and humans  
305 (Supplemental Figure 4). For example, loop A in MKK4 and MKK5 is identical, whereas it differs

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306 between MKK5 and MKK7. Loop B is downstream of  $\alpha$ -helix F and displays a high tolerance for  
307 sequence variability (Figure 3, Supplemental Figure 3 and 4). Among CMGC (Cyclin-dependent kinases,  
308 MAPK, Glycogen synthase kinase and Cyclin-dependent kinase-like kinase) group kinases, loop B  
309 contains an insert that binds interacting proteins (Kannan and Neuwald, 2004), and a different insert in  
310 MEK1/2 mediates binding to MAPK scaffolds MP1 and KSR (Schaeffer et al., 1998; Brennan et al.,  
311 2011). This region is shorter in plant MKKs (making them resemble human MEK3-MEK7), but the  
312 sequence divergence among MKKs is consistent with this loop being a specificity or identity determinant.  
313 It is therefore a prime region to target in our dissection of specificity.

314

### 315 **N-termini link specific MKKs to specific phenotypes**

316 Different *in vivo* behaviors of MKK5<sup>DD</sup> and MKK7<sup>ED</sup> make it possible to begin to correlate  
317 unique sequences and structures with unique functions. Informed by the structural analysis, we made  
318 chimeric MKKs based on dividing the MKKs into N- terminal (N), kinase domain (MKK) and C-terminal  
319 (C) regions, and further dividing the C domain into CDR and CPR (and within CPR, domain A and B)  
320 (Figure 3). To assay the function of these domains, we measured the phenotypes induced by chimeras at  
321 the FAMA stage in T1s and compared to those obtained for intact MKK5<sup>DD</sup> and MKK7<sup>ED</sup>. Expression  
322 and subcellular localization of YFP-tagged MKKs was verified by confocal microscopy. We predicted  
323 that certain domain combinations could result in non-functional chimeras. Because expression of  
324 MKK5<sup>DD</sup> and non-functional chimeras would give essentially the same phenotype at the FAMA stage (no  
325 effect on stomatal development), it was important to discriminate MKK5<sup>DD</sup>-like chimeras from non-  
326 functional chimeras. We took two approaches to verify that kinases were still active. First, we measured  
327 the intrinsic kinase activity against MPK3/6 in *in vitro* kinase assays (Supplemental Figure 2). Second, we  
328 took advantage of the fact that both MKK5<sup>DD</sup> and MKK7<sup>ED</sup> drive robust inhibition of stomatal initiation at  
329 the SPCH stage (Lampard et al., 2009) to create an *in vivo* assay for kinase activity. We expressed the  
330 chimeras at the SPCH stage and quantified the degree of inhibition of stomatal initiation (Table 1).



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331 To characterize the role of the N-terminus in MKK5, we replaced it with the N-terminus of  
332 MKK7 (N7-MKK5<sup>DD</sup>) and the chimera was expressed in FAMA stage cells. ~59% of T1 transformants  
333 produced stomatal clusters (Figure 4B and G), though clusters were smaller than those generated by  
334 MKK7<sup>ED</sup>. In addition, 9% of T1 transformants showed inhibition of stomatal formation (Figure 4G). We  
335 also noticed that N7-MKK5<sup>DD</sup> partially relocalized to mitochondria (Figure 4B) similar to MKK7<sup>ED</sup>  
336 (Lampard et al., 2014). Our *in vitro* and *in vivo* controls for activity both indicated that N7-MKK5<sup>DD</sup> was  
337 less active than intact MKK5<sup>DD</sup>; only ~27% of T1s inhibited stomatal initiation (Figure 4H) and *in vitro*  
338 kinase activity was lower, especially towards MPK3 (Figure 4I). This dramatic output alteration  
339 (aphenotypic MKK5<sup>DD</sup> to a weak MKK7<sup>ED</sup>-like behavior) suggests that the N-terminus is more than just a  
340 structural/regulatory region required for protein activity. Instead, it appears to channel the MKK towards  
341 specific phenotypic outcomes. This specificity behavior resembles that observed in yeast where MKKs  
342 involved in other cellular processes were engineered to interact with components of the mating pathway,  
343 but were only able to transduce a mating signal when their N-termini were replaced with the N-terminus  
344 from Ste7, the mating specific MKK (Won et al., 2011).

345

346 **Figure 4: N-termini link MKKs to their phenotypic outputs and the C-terminal Loop B is required**  
347 **for FAMA stage-specific regulation of MKK5.** (A-F) Paired micrographs of representative major  
348 phenotype (left) and subcellular localization (right), of specific MKK5<sup>DD</sup>- and MKK7<sup>ED</sup>-YFP variants  
349 (diagramed above). Black brackets mark clusters and asterisks indicate inhibition. YFP is in green and  
350 cell outlines in magenta. For example, in (A) MKK5<sup>DD</sup> expression results in a WT phenotype and the  
351 protein is cytoplasmic and in (B) mitochondrial/cytoplasmic N7-MKK5<sup>DD</sup> induces stomatal clustering.  
352 Scale bars are 50  $\mu$ m in phenotype images and 10  $\mu$ m for localization images. (G) Quantification of  
353 phenotypes in (A-F). (H) SPCH stage inhibition of lineage initiation. Error bars in (G) and (H) correspond  
354 to 95% confidence interval. (I) *In vitro* kinase activity towards kinase inactive MPK3 and MPK6. Kinase  
355 assays were performed in triplicates, normalized to unphosphorylated KI-MPK and averaged; error bars  
356 represent standard errors.

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357

358           If the N-terminus enforces MKK specific activities, then replacement of N7 by N5 in MKK7<sup>ED</sup>  
359 should reveal the endogenous response to MKK5 activation. With the N5-MKK7<sup>ED</sup> chimera we found  
360 efficient inhibition of lineage initiation at the SPCH stage and a normal ability to phosphorylate MPK3  
361 and MPK6 *in vitro* (Figure 4H and I). Like MKK5<sup>DD</sup> the chimera was cytoplasmic localized (compare A  
362 and C in Figure 4). Unlike MKK5<sup>DD</sup>, N5-MKK7<sup>ED</sup> completely inhibited GC production (Figure 4C and  
363 G). Thus, with this manipulation, we were finally able to recapitulate the stomatal lineage inhibition  
364 phenotype we had expected from MKK5<sup>DD</sup> based on its ability to inhibit stomatal development at earlier  
365 stages (Lampard et al., 2009) and the loss of function stomatal cluster phenotype (Wang et al., 2007).

366

### 367 **Loop B prevents MKK5<sup>DD</sup> from inhibiting stomata formation at the FAMA stage**

368           Demonstrating that development of FAMA-stage cells could be inhibited, however, raised the  
369 question of why intact MKK5<sup>DD</sup> is unable to do so. We hypothesized that sequences in the MKK5 C-  
370 terminus act as negative regulatory regions. To test this idea, we first replaced the entire C-terminal  
371 region of MKK5, creating MKK5<sup>DD</sup>-C7. FAMA-stage expression did result in a partially penetrant  
372 inhibition of stomatal formation where inhibited precursors coexisted with normal stomata (Figure 4D  
373 and G). MKK5<sup>DD</sup>-C7 displayed high activity in SPCH-stage lineage inhibition (Figure 4H), but was less  
374 efficient than MKK5<sup>DD</sup> in *in vitro* kinase assays, particularly towards MPK3 (Figure 4I). Because  
375 previously reported MKK5 deletions in the CDR portion of the C-terminus did not significantly change  
376 MKK5<sup>DD</sup> activities (Lampard et al., 2014), we reasoned that putative regulatory regions were located in  
377 the CPR.

378           The largest sequence differences between C5 and C7 reside in loop A and B in the CPR.  
379 Substitution of loop A (MKK5<sup>DD</sup>-7A) resulted in a chimera that did not affect stomatal development at  
380 the FAMA stage (Figure 4E and G), but substitution of loop B (MKK5<sup>DD</sup>-7B) led to inhibition of stomatal  
381 formation at high frequency (Figure 4F and G). This result suggests that MKK5's loop B is a region that  
382 blocks MKK5 from participating in stomatal inhibition at the FAMA stage. Interestingly, SPCH stage

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383 activity was markedly reduced for both chimeras (Figure 4H), but *in vitro* activities of MKK5<sup>DD</sup>-7A and  
384 MKK5<sup>DD</sup>-7B were at least as high as that of MKK5<sup>DD</sup> (Figure 4I). These observations suggest that  
385 MKK5<sup>DD</sup>-7A and MKK5<sup>DD</sup>-7B are catalytically active kinases but cannot generate appropriate signals *in*  
386 *vivo*.

387

### 388 **Loop B is required for robust MKK7<sup>ED</sup> activity**

389 If there was truly a discrete domain of MKK5 that was subject to negative regulation, then  
390 transferring it to MKK7<sup>ED</sup> should dampen the stomatal clustering phenotype at the FAMA stage (Figure  
391 5A). We initially swapped the entire C-terminus, and the resulting MKK7<sup>ED</sup>-C5 only produced normal  
392 stomata, similarly to MKK5<sup>DD</sup> (Figure 5B and G). This could suggest that C5 is able to block MKK7  
393 inhibitory function at FAMA stage. However, monitoring other indicators of MKK activity (SPCH stage  
394 lineage inhibition and *in vitro* phosphorylation of MPK3 and MPK6) suggested that C7 was essential for  
395 overall activity (Figure 5H and I). Thus this phenotype is the result of creating a generally inactive MKK7  
396 chimera, more than an effect due to the presence of MKK5 regulatory sequences. Thus, we split C5 into  
397 CDR5 and CPR5, and determined if we could restore MKK7<sup>ED</sup> activity *in vitro* and in the SPCH stage.  
398 Activity was restored in MKK7<sup>ED</sup>-CDR5: this chimera completely inhibited lineage initiation at the SPCH  
399 stage and was indistinguishable from MKK7<sup>ED</sup> in *in vitro* kinase assays (Figure 5H and I). Rather than  
400 decreasing MKK7<sup>ED</sup> function, however, MKK7<sup>ED</sup>-CDR5 had a strikingly stronger inhibitory effect on  
401 stomatal development at the FAMA stage than MKK7<sup>ED</sup> (Figure 5C and G). In fact, it resembled the  
402 strong phenotype produced by N5-MKK7<sup>ED</sup> (Figure 4C). This implies that CDR5, like N5, channels  
403 MKK activity to inhibition of stomatal development. In contrast, MKK7<sup>ED</sup>-CPR5 was largely inactive at  
404 both SPCH and FAMA stages (Figure 5D and G), and in *in vitro* phosphorylation assays against MPK3  
405 and MPK6 (Figure 5H and I), indicating that CPR7 is necessary for MKK7<sup>ED</sup> catalytic activity.

406

### 407 **Figure 5: Loops A and B are required for specific and robust FAMA stage MKK7 activities.** (A-F)

408 Paired micrographs of representative major phenotype (left) and subcellular localization (right), of

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409 specific MKK7<sup>ED</sup>-YFP variants diagramed above. Black brackets mark clusters and asterisks indicate  
410 inhibition. YFP is in green and cell outlines in magenta; white arrowheads point to mitochondrial  
411 localization. For example, in (A) MKK7<sup>ED</sup> induces stomatal clustering (brackets) and is mitochondrial  
412 localized (white arrowheads). Scale bars are 50  $\mu\text{m}$  for phenotype images and 10  $\mu\text{m}$  for localization  
413 images. (G) Quantification of phenotypes in (A-F). (H) SPCH stage inhibition of lineage initiation. Error  
414 bars in (G) and (H) correspond to 95% confidence interval. (I) *In vitro* kinase activity towards kinase  
415 inactive MPK3 and MPK6. Kinase assays were performed in triplicates, normalized to unphosphorylated  
416 KI-MPK and averaged; error bars represent standard errors.

417

418 So far, the domains from MKK5 that dampened MKK7<sup>ED</sup> activity at the FAMA stage also  
419 decreased the activity of the chimeras *in vitro* and at the SPCH stage (MKK7<sup>ED</sup>-C5 and MKK7<sup>ED</sup>-CPR5).  
420 From these results, it appears that the CPR region is important for MKK7<sup>ED</sup> catalytic activity and thus we  
421 created smaller domain swaps (loops A and B) to attempt to transfer negative regulatory sequences from  
422 MKK5 into MKK7<sup>ED</sup> without affecting kinase functionality. MKK7<sup>ED</sup>-5A and MKK7<sup>ED</sup>-5B were active  
423 *in vivo* (inhibited lineage initiation at the SPCH stage, Figure 5H) and *in vitro* (phosphorylated MPK3/6,  
424 Figure 5I), although to different degrees. At the FAMA stage, MKK7<sup>ED</sup>-5A inhibited stomatal formation  
425 to a greater extent than MKK7<sup>ED</sup> (Figure 5E and G). This behavior is similar to N5-MKK7<sup>ED</sup> (Figure 4C)  
426 and MKK7<sup>ED</sup>-CDR5 (Figure 5C), suggesting that N5, loop 5A and CDR5 restrict MKK7<sup>ED</sup> activity to  
427 inhibition of stomatal development. In contrast, MKK7<sup>ED</sup>-5B's ability to cause stomatal clustering and  
428 inhibition of stomatal development at the FAMA stage was markedly reduced when compared to  
429 MKK7<sup>ED</sup> (Figure 5G). Because MKK7<sup>ED</sup>-5B also showed reduced activities in other indicators of MKK  
430 activity (Figure H and I), we concluded that the negative regulation of MKK5<sup>DD</sup> is restricted to loop 5B  
431 but cannot be transferred without affecting MKK7<sup>ED</sup> catalytic activity. Nevertheless, the same results  
432 highlight that loop 7B is required for robust MKK7<sup>ED</sup> activity.

433

434 **Swapping domains allows specificity to be changed in MKK5<sup>DD</sup> and MKK7<sup>ED</sup>**

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435 Our results show that the N-terminus, CPR region (loops A and B) and CDR region modulate  
436 MKK activity. We showed that N7 and CPR7 are necessary for MKK7<sup>ED</sup>-mediated GC clustering at the  
437 FAMA stage, but when CDR5 is incorporated into MKK7<sup>ED</sup>, GC production is inhibited. If our “wiring  
438 diagram” for specificity is correct, then a chimera that contains the GC promoting domains from MKK7  
439 but not the inhibitory CDR5 (i.e., N7-MKK5<sup>DD</sup>-C7) should mimic MKK7<sup>ED</sup>. Indeed, when we constructed  
440 N7-MKK5<sup>DD</sup>-C7, it resembled MKK7<sup>ED</sup> both qualitatively and quantitatively (Figure 6A and C).  
441 Likewise, N5-MKK7<sup>ED</sup>-C5 should match MKK5<sup>DD</sup> activities, and it does *in planta* (Figure 6B and C).  
442 Interestingly, robust rewiring *in vivo* (Figure 6C and D) appears to be uncoupled from kinase activity *in*  
443 *vitro*, as both rewired proteins were much less capable of phosphorylating MPK3 and MPK6 than  
444 MKK5<sup>DD</sup> and MKK7<sup>ED</sup> (Figure 6E). One interpretation of these swaps is that specificity lies only outside  
445 of the kinase domain. If this were true, then we should be able to generate a chimera that resembles N7-  
446 MKK5<sup>DD</sup>-C7 and MKK7<sup>ED</sup> using the kinase domain from another MKK. We selected the kinase domain  
447 of MKK6 that can also phosphorylate MPK3 and MPK6 *in vitro* (Popescu et al., 2009), and created N7-  
448 MKK6<sup>DD</sup>-C7. Expression of N7-MKK6<sup>DD</sup>-C7 at the FAMA stage, however, did not produce any  
449 noticeable phenotype (Supplemental Figure 6). This suggests that although kinase domains in MKK5 and  
450 MKK7 are not differential, they still contain stomatal fate-enabling regions.

451  
452 **Figure 6: MKK5 and MKK7 activity and localization in the stomatal lineage can be reciprocally**  
453 **rewired.** (A-B) paired micrographs of representative major phenotype (left) and subcellular localization  
454 (right), specific MKK-YFP variants diagramed above. Black brackets mark clusters; YFP is in green and  
455 cell outlines in magenta; white arrowheads point to mitochondrial localization. (A) N7-MKK5<sup>DD</sup>-C7  
456 mimics MKK7<sup>ED</sup> in that it produces clusters and can localize to mitochondria. (B) N5-MKK7<sup>ED</sup>-C5  
457 mimics MKK5<sup>DD</sup> in that it has a WT phenotype and localizes in the cytoplasm. Scale bars are 50  $\mu$ m in  
458 phenotype images and 10  $\mu$ m for localization images. (C) Quantification of phenotypes (A-B). (D) SPCH  
459 stage inhibition of lineage initiation. Error bars in (C) and (D) correspond to 95% confidence interval. (E)

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460 *In vitro* kinase activity towards kinase inactive MPK3 and MPK6. Kinase assays were performed in  
461 triplicates, normalized to unphosphorylated KI-MPK and averaged; error bars represent standard errors.

462

### 463 **Comprehensive analysis of chimeras reveals functions of MKK domains**

464 We repeatedly observed that the ability of native and chimeric MKKs to phosphorylate their  
465 targets *in vitro* does not predict their activities *in vivo*. In fact, when chimera data are considered together,  
466 *in vitro* versus *in vivo* data have no statistical correlation (Supplemental Figure 7). In contrast, when only  
467 *in vivo* data were compared, activities in SPCH and FAMA stages were positively correlated (Figure 7A).  
468 Interestingly, native and chimeric MKKs were distributed in two subpopulations. MKKs closer to the  
469 regression line promoted stomatal clustering (red dots) or inhibited stomatal formation (black dots).  
470 MKKs further from the regression line had no effect in stomatal development at the FAMA stage (blue  
471 dots), but had a broad range of activities at the SPCH stage (shaded area in Figure 7A). This behavior  
472 might be reflecting the additional regulation that some of the MKKs showed at the FAMA stage.

473

474 **Figure 7: MKK5 defines a cluster of chimeras with low activity at FAMA stage, whereas clustering**  
475 **and inhibiting chimeras group in two independent clusters.** (A) SPCH and FAMA stage activities  
476 show a weak positive correlation. Shaded area corresponds to activities at the FAMA stage lower than  
477 30%. Data points are colored according to the cluster they belong to in (B). MKK activities at the FAMA  
478 stage are separated into three clusters (B): cluster 1 contains MKKs that show no phenotypic effect and  
479 contain loop 5B; cluster 2 contains MKKs that lead to stomatal clustering and contain N7; and, cluster 3  
480 contains MKKs that lead to inhibition of stomatal development and contain loop 7B. Numbers next to  
481 nodes correspond to approximately unbiased *p*-values with bootstrap replications set to 1000. Chimeras  
482 not significantly different in Chi-squared tests if independence are indicated with an identical number ( $p >$   
483 0.05).

484

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485           We reasoned that MKKs subject to the same regulation would share structural similarities. To test  
486 this hypothesis and generate an overall picture of the relationship between MKK structural domains and  
487 *in vivo* functions, we clustered 15 native and chimeric MKKs and controls according to their quantitative  
488 phenotype data at the FAMA stage (clustering detailed in methods). Constructs robustly fell into three  
489 clusters (Figure 7B): Cluster 1, no phenotypic effect; Cluster 2, induces stomatal proliferation; and  
490 Cluster 3, inhibits stomatal formation (the presumed endogenous role for MKK5). Within clusters,  
491 however, not all MKKs were identical. We performed sequential tests of independence to determine how  
492 similar the distribution of phenotype frequencies was between chimeras in each cluster. Cluster 1 was  
493 composed of MKKs similar to MKK5<sup>DD</sup> (group 1) and MKKs similar to inactive YFP (group 2). Cluster 2  
494 was statistically separated into weak MKK7<sup>ED</sup>-like chimeras (group 3) and two MKKs that induced strong  
495 clustering, yet were different from each other. Cluster 3 was statistically separated into strong inhibitors  
496 of stomatal formation (group 4) and weak inhibitors (different from each other).

497           To summarize, when analyzing native and chimeric MKK structures across Clusters, we see that  
498 loops A and B have discrete functions in selecting MKK-specific outputs and in kinase activity *in vivo*.  
499 Loop A can be thought of as a “channel selector”, that, together with N-terminus and CDR, selects  
500 between the normal role of arresting stomatal progression and the artificial role of promoting stomatal  
501 clustering. Loop B is a “volume control” with the 7B version increasing, and 5B decreasing, the  
502 phenotypes specified by the other domains of the MKKs

503

### 504 **MPK6 mediates GC inhibition, but like MKK5, is prevented from doing so at the FAMA stage.**

505           Previous loss- and gain-of-function experiments placed MPK3 and MPK6 downstream of an  
506 activated MKK4/5 homologue (NtMEK2), suppressing stomatal formation (Wang et al., 2007) in the  
507 early stages of the stomatal lineage, but this assay could not address the potential for MPK3 and MPK6 to  
508 mediate FAMA-stage activities. Our chimeras that drive stomatal inhibition at the FAMA-stage, however,  
509 could be used to see whether either mediated such late stage inhibition. We used N5-MKK7<sup>ED</sup> which, in  
510 WT led to complete inhibition of stomatal development (Fig 8C, Table 1). When expressed in the loss of



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511 function *mpk6-3* background, N5-MKK7<sup>ED</sup> failed to promote complete inhibition in 19 independent T1s  
512 (Figure 8D) indicating that MPK6 is likely downstream. This led us to the question of whether MPK6,  
513 like MKK5<sup>DD</sup>, would also be actively inhibited from effecting fate at the FAMA stage. To test this, we  
514 created a constitutively active MPK6 (MPK6<sup>DE</sup>) (Berriri et al., 2012) and tested its ability to suppress  
515 stomatal formation. Expression of MPK6<sup>DE</sup> (but not MPK3<sup>DE</sup>, Figure 8A, Table 1), inhibited stomatal  
516 progression at the SPCH stage, indicating that MPK6<sup>DE</sup> is active in this assay. When expressed at the  
517 FAMA stage, however, MPK6<sup>DE</sup> did not affect stomatal development (Figure 8B, Table 1), a phenotypic  
518 output remarkably similar to that of MKK5<sup>DD</sup> (early, but not late, inhibition). We hypothesized that  
519 MKK5 and MPK6 normally repress stomatal development, but are actively prevented from having this  
520 effect at the FAMA stage.

521  
522 **Figure 8: MPK6 inhibits stomatal development at the FAMA stage in a MKK-specific manner.** (A  
523 and B) Micrographs indicating the requirement for MPK6 as a downstream factor in N5-MKK7<sup>ED</sup>-  
524 mediated FAMA-stage inhibition (asterisks). In *mpk6* mutants (B), stomata (arrowhead) coexist with  
525 precursors that exit the lineage. (C-and D) DIC micrographs showing results of expression of MPK6<sup>DE</sup> at  
526 SPCH and FAMA stages, stomata are highlighted in green. MPK3<sup>DE</sup> does not affect development at any  
527 stage (Table 1). Scale bars in are 50  $\mu$ m.

528

529

## 530 Discussion

531 In multicellular organisms, coordinated development requires constant communication between  
532 cells and the evaluation of environmental conditions. All this information is integrated to decide from a  
533 spectrum of possible outputs, and the spectrum is frequently limited by a cell's identity. In previous, more  
534 superficial studies, FAMA stage cells appeared to lose the ability to inhibit stomatal development upon  
535 MAPK activation (Lampard et al., 2009). Here we show that these cells do not lack the capacity to be  
536 inhibited, but rather that MKK5 (and possibly MPK6) may be actively prevented from participating in



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537 this cellular outcome. Structural analysis and engineered chimeras revealed that this regulation and other  
538 specific responses rely on distinct MKK domains.

539 We found that Arabidopsis MKKs behave as modular proteins with four discrete regions: N-  
540 terminus, CDR and two loops (A and B) in the CPR. N-termini contribute to subcellular localization  
541 (Figure 4 and in (Lampard et al., 2014)), to phenotypic output (Figure 4) and may mediate interactions  
542 with downstream MPKs through their docking domains. In particular, we hypothesize that N7 has the  
543 ability to bind different types of MPK. Throughout development, MKK7 inhibits stomatal development  
544 by recruiting MPK3/6, but a yet unknown proliferative MPK mediates stomatal clustering at the FAMA  
545 stage (Figure 9A). In the C-termini, Arabidopsis MKKs 1, 2, 4, 5 and 6 contain an extension that could be  
546 equivalent to the MKKK-interacting domain for versatile docking (DVD) in human MEKs 1, 3, 4, 6 and 7  
547 (Supplemental Figure 4) (Takekawa et al., 2005). Arabidopsis MKK7, 8, 9, and 10, however, lack this  
548 domain, making it unclear how they engage the appropriate MKKK. In fact, the addition of CDR5 to  
549 MKK7<sup>ED</sup> restricted this kinase's activity to an inhibitory output (Figure 5C and G), suggesting that CDR5  
550 interferes with MKK7<sup>ED</sup> interactions. Upstream of the CDR, Loop A and B are two surface-exposed  
551 modules in the CPR that may contribute to establishing interactions with other network components. In  
552 our experiments, swapping loop A in MKK7<sup>ED</sup> restricted its phenotypic output such that MKK7<sup>ED</sup>-5A  
553 only inhibited stomata formation (Figure 5E, G). We propose then, that loop A promotes certain MKK-  
554 MPK interactions or, alternatively, restricts how MPKs contact MKKs. This hypothesis is supported by  
555 sequence similarities between human MEKs that share the same downstream MAPKs (Keshet and Seger,  
556 2010). For example, ERK kinases MEK1 and MEK2 have identical loop As, and p38 kinases MEK3 and  
557 MEK6 differ at only one site (Supplemental Figure 4). Interestingly, human MEK7, which can  
558 phosphorylate both JNK and p38, shares some residues with MEK3 and MEK6 and others with the JNK  
559 kinase MEK4.

560 The function of loop B seems to be associated to MKK-specific regulation. Our data shows that  
561 loop B is required for robust MKK7<sup>ED</sup> activity, but it prevents MKK5<sup>DD</sup>-mediated inhibition at the FAMA  
562 stage (Figure 4F-G and 5F-G). Based on these phenotypes, we propose that loop B mediates interactions

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563 with different scaffolds (Figure 9). A signal-promoting scaffold binds loop 7B and enforces MKK7  
564 interactions with its cognate MKKK and MPK (Figure 9A). Such a scaffold would also explain why *in*  
565 *vivo* activity of MKK7<sup>ED</sup> was always stronger than that of MKK5<sup>DD</sup>, even though *in vitro* assays showed  
566 an opposite pattern. On the other hand, we predict that a distinct scaffold recruited by loop 5B prevents  
567 MKK5<sup>DD</sup> from inhibiting stomatal development at the FAMA stage (inhibitory scaffold) (Figure 9B).  
568 This prediction could also partially explain the behavior of certain chimeras. For example, the inhibitory  
569 scaffold would bind MKK7<sup>ED</sup>-5B through loop 5B and dampen MKK7<sup>ED</sup> activities. Likewise, a signal-  
570 promoting scaffold would bind MKK5<sup>DD</sup>-7B through loop 7B, releasing the inhibition of MKK5<sup>DD</sup>.

571

572 **Figure 9: A new model derived from activities during stomatal development for the endogenous**  
573 **MKK5 pathway and another activated by MKK7.** (A) In the MKK7 pathway, MKK7 mimics MKK5  
574 in early development, forcing precursors to exit the stomatal lineage. At the last stage, MKK7 induces  
575 two phenotypes. Firstly, MKK7 induces stomatal clustering by means of an unknown MKK7-specific  
576 MPK (orange circle). This proliferation depends on MKK7 mitochondrial localization (Lampard et al.,  
577 2014). Secondly, MKK7 forces precursors to exit the lineage by escaping MKK5-specific regulation. An  
578 MKK7 scaffold enforces interactions between MKK7 and other components (MPKs and YODA, gray  
579 circles) within the network. (B) In the endogenous pathway, MKK5 and MPK3/6 (black circles) are  
580 involved in transducing developmental signals that inhibit stomatal lineage initiation and GMC  
581 commitment, and initiating defense and stress responses. At the last stage of development, a stage-  
582 specific MKK5 scaffold prevents MKK5 (and possibly MPK3/6) from inhibiting stomatal formation, only  
583 allowing defense and stress responses.

584

585 The existence of signal-enhancing and inhibiting MAPK scaffolds in plants is supported by recent  
586 findings (Zhao et al., 2014; Cheng et al., 2015; Zhang et al., 2015). While the relevance of the first type is  
587 quite intuitive, the second type is more controversial. The inhibitory scaffold EDR1 was identified in the  
588 context of pathogen defense. Current hypotheses are that EDR1 provides a failsafe against inadvertently

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589 activating defense or cell death programs when other (not pathogen-induced) cues activate MAPK  
590 signaling. EDR modulates MPK3 activity indirectly by interacting with MKK4/5 and regulating their  
591 abundance (Zhao et al., 2014). Interestingly, we observed in FAMA stage cells that MPK6 was only  
592 inhibited when the upstream MKK was also inhibited. The clearest evidence that MKK5-MPK3/6 are  
593 scaffolded in FAMA stage cells would be, of course, to identify the scaffold. We tested whether EDR1  
594 worked to inhibit FAMA-stage expressed MKK5<sup>DD</sup>, however we failed to see a loss of inhibition in *edr1*  
595 (none seen in 20 independent T1s). EDR1 is a member of the large Raf-like MKKKs family (Ichimura et  
596 al., 2002) and potentially any of the 48 members of this family (alone or in combination) could serve as a  
597 stomatal scaffold. As testing the entire family is not technically feasible, we used stomatal lineage  
598 expression data (Adrian et al., 2015) to identify two close homologues of EDR1 expressed at the FAMA  
599 stage, MAP3K $\delta$ 1 (At1g11850) and MAP3K $\delta$ 5 (At4g24480). *MAP3K $\delta$ 1* and *MAP3K $\delta$ 5* mutants, however,  
600 were indistinguishable from WT in their response to FAMA stage MKK5<sup>DD</sup> expression (0/20 independent  
601 T1s for each). Thus, the molecular identity of the stomatal scaffold remains of great future interest.

602 MKK4/5 face the problem of being used in early stages to repress stomatal progression, but being  
603 required for physiological regulation in guard cells. At the FAMA stage, MKK4/5 must therefore be  
604 actively rerouted from their previous role inhibiting stomatal development to allow terminal  
605 differentiation of guard cells (Figure 9). A negative scaffold acting late in the stomatal lineage to redirect  
606 MKK4/5 would provide an elegant solution to a complex signal integration problem.

607

608

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- 717



## Rewiring of MAPK cascades in Arabidopsis

### 718 **Supplemental Figure legends**

719

### 720 **Supplemental Figure 1: Yeast two-hybrid assay with native and chimeric MKKs and MPK3/MPK6.**

721 Representative yeast two-hybrid assay between MKK chimeras and MPK3/6 at 3 days of growth on  
722 control (-LW) and interaction (-LWH); three patches are serial (10 fold) dilutions.

723

### 724 **Supplemental Figure 2: *In vitro* kinase assays of MKKs and chimeras using kinase inactive (KI)**

725 **MPK3 and MPK6 as substrates.** Phosphorylation assays were performed as described in methods.

726 Samples were separated in SDS-PAGE, transferred to PVDF membranes and probed with anti-pERK

727 antibody. Western blots were quantified and average of experiments was used as an estimation of *in vitro*

728 activity. Each lane is labeled with the figure where the data is presented (grey bar marked *Figures*), the

729 kinase used with a graphic representation and with its full name in bottom panel. Samples unassigned to

730 any figure (labeled as “-“ in *Figure*) were not presented in the main figures of this manuscript for the sake

731 of brevity, but are included here to allow us to keep the blots intact. Red squares highlight samples that

732 were not considered for quantification due to detection artifacts.

733

### 734 **Supplemental Figure 3: Multiple sequence alignment (Sievers et al., 2011) of catalytic domains in**

735 **MPK kinases from mouse, rat, human and *Arabidopsis thaliana*.** A, Conserved subdomains (**Subdom.**)

736 and consensus sequences (**Consen.**) are represented on top of the alignment and follow the same codes

737 and convention for an alignment of 60 different kinases by Hanks and Hunter (Hanks and Hunter, 1995).

738 In the consensus line: *uppercase letters*, invariant residues; *lowercase residues*, nearly invariant residues;

739 *o*, positions conserving nonpolar residues; \*, positions conserving polar residues; +, positions conserving

740 small residues with near neutral polarity. Mammalian representative kinases selected for the alignment

741 have been crystalized and belong to **AGC group** [cAMP-dependent kinase, cGMP-dependent kinase,

742 etc.], **CAMK group** [Calcium-calmoduline-dependent protein kinase], **CMGC group** [cyclin-dependent

743 kinase, mitogen-activated kinase, glycogen synthase kinase and cyclin-dependent-like kinase] and **STE**

## Rewiring of MAPK cascades in Arabidopsis

744 **group** [homologues of STE11 and STE20]. Gray boxes show CMGC insert (Goldsmith et al., 2007) in  
745 ERK2, CDK2 and p38, and Pro-rich sequence (PRS, involved in binding the scaffold MP1) in MEK1 and  
746 MEK2 were included in the alignment and cause an expansion of subdomain X. Secondary structure (2°  
747 **str.**) information is overlaid in the alignment (red for  $\alpha$ -helices and yellow for  $\beta$ -strands). Conserved  $\alpha$ -  
748 helices and  $\beta$ -strands are labeled following convention (Hanks and Hunter, 1995; Knight et al., 2007).  
749 Due to CMGC insert and PRS,  $\alpha$ G helix is located in two different regions of the alignment and was  
750 named differently (residues underlined):  **$\alpha$ G1** for MmPKA, HsARK-1, RnCaMKI, RnERK2, HsCDK2  
751 and Hsp38; and  **$\alpha$ G2** for HsMEK1/2 and AtMKK5/7. In green text, missing residues in crystal structures  
752 from HsMEK1 and HsMEK2 which include the PRS. B, Table provides general information and  
753 structural model names for kinases used in this comparison. C, Multiple sequence alignment of N- and C-  
754 termini from human MEK1/2 and Arabidopsis MKK5/7. Sequences highlighted in gray correspond to  
755 first and last three amino acids of the catalytic domains shown in A.

756

757 **Supplemental Figure 4: Multiple sequence alignment of C-termini in Arabidopsis and human**  
758 **MKKs.** Top panel, partial sequences for all Arabidopsis and human MKKs were aligned with  
759 ClustalOmega. Loops A and B defined for Arabidopsis MKKs are highlighted in gray. Number of first  
760 amino acid of the partial sequence is noted to the left of each sequence. The long C-terminal extension of  
761 AtMKK3 is truncated in this figure. Bottom panel, genes used in the alignment. Name of genes for  
762 Arabidopsis correspond to Arabidopsis Genome Initiative (AGI) codes and GeneCards (GC) for humans.  
763 NCBI GI, National Center for Biotechnology Information protein sequence identifier.

764

765 **Supplemental Figure 5: Multiple sequence alignment for MKK4, MKK5, MKK7 and MKK9.**  
766 Domains described in (Lampard et al., 2014) are highlighted in blue and green.

767

768 **Supplemental Figure 6: Representative phenotype and subcellular localization of transgenic**  
769 **seedlings expressing FAMap:N7-MKK6<sup>EE</sup>-C7.**

## Rewiring of MAPK cascades in Arabidopsis

770

771 **Supplemental Figure 7: *In vitro* kinase activity of MKKs does not correlate with their *in vivo***  
772 **activity.** Linear regressions for MPK3 or MPK6 activity versus SPCH stage (A) or FAMA stage (B)  
773 activities, with their formulas and  $R^2$ , are displayed in the figure. FAMA stage activity was calculated as  
774 the addition of phenotypes different than normal (Inhibited, Small and Large clusters in Table 1).

Figure 1

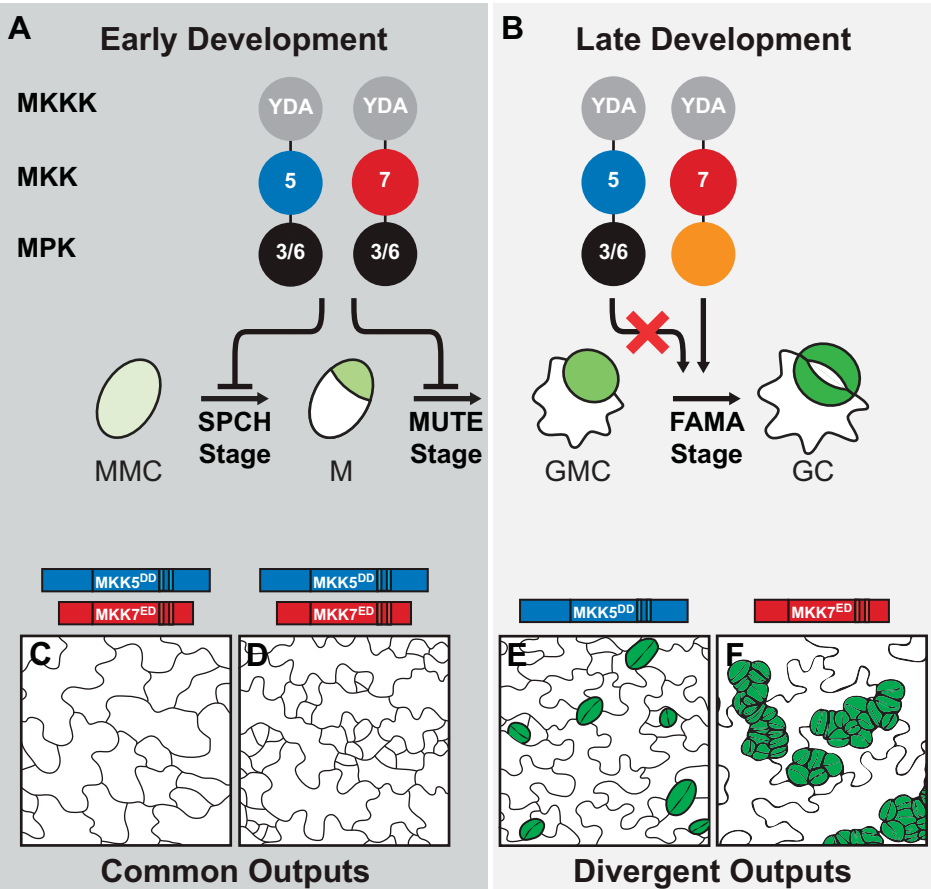
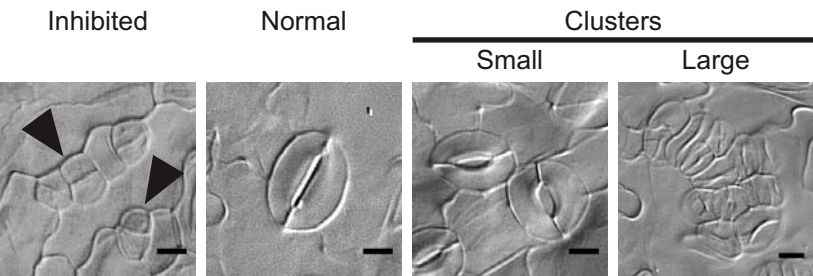


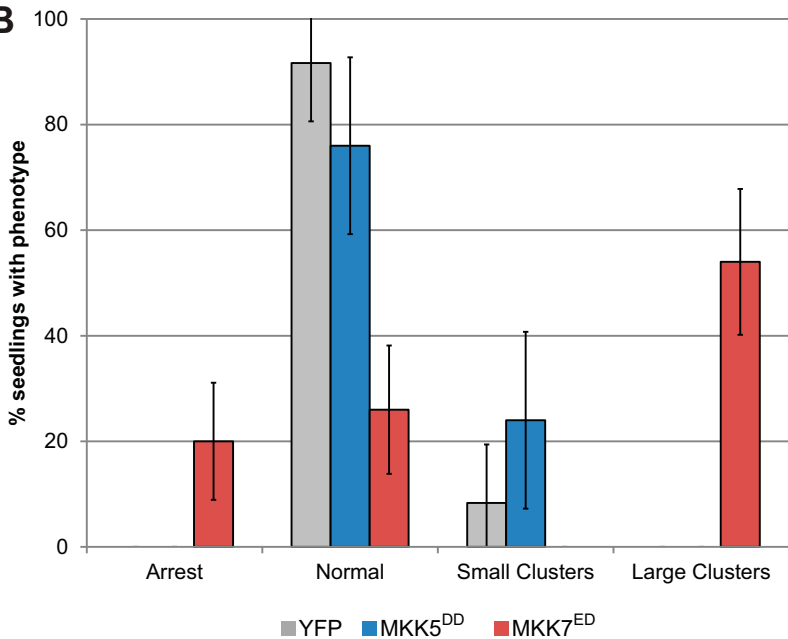
Figure 2

A

## Phenotypes due to FAMA stage expression



B



# Figure 3

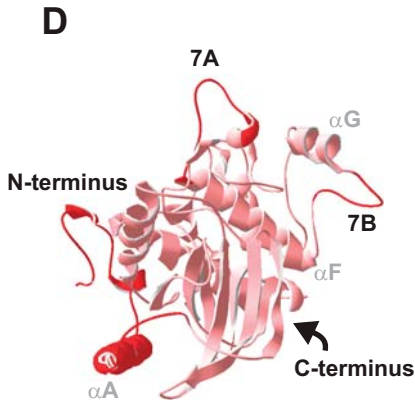
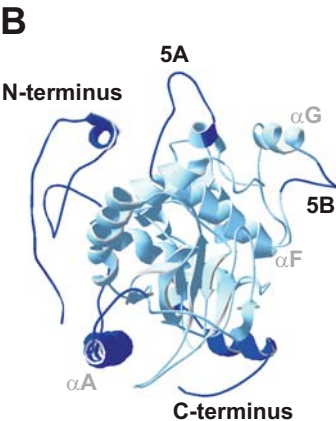
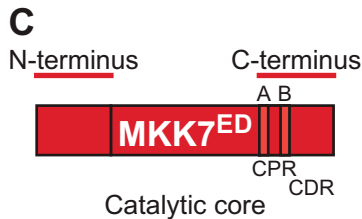
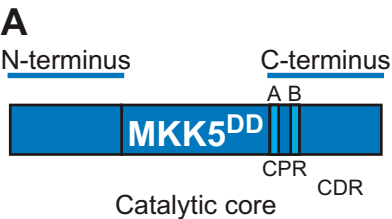
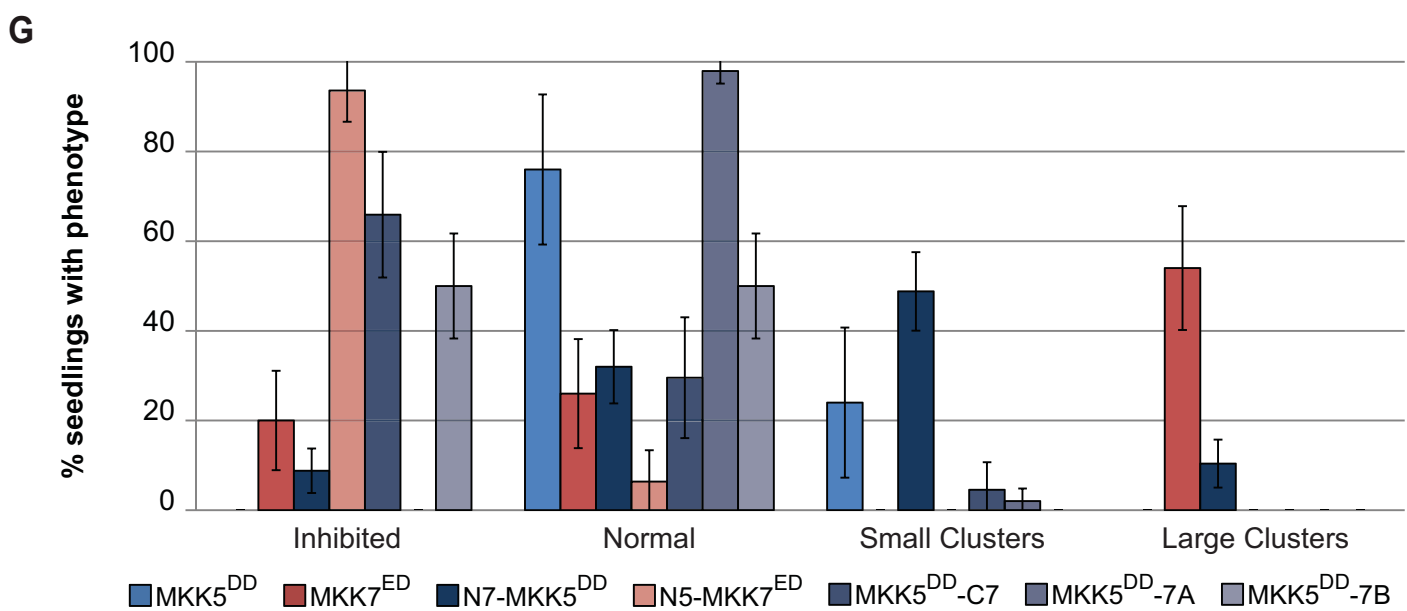
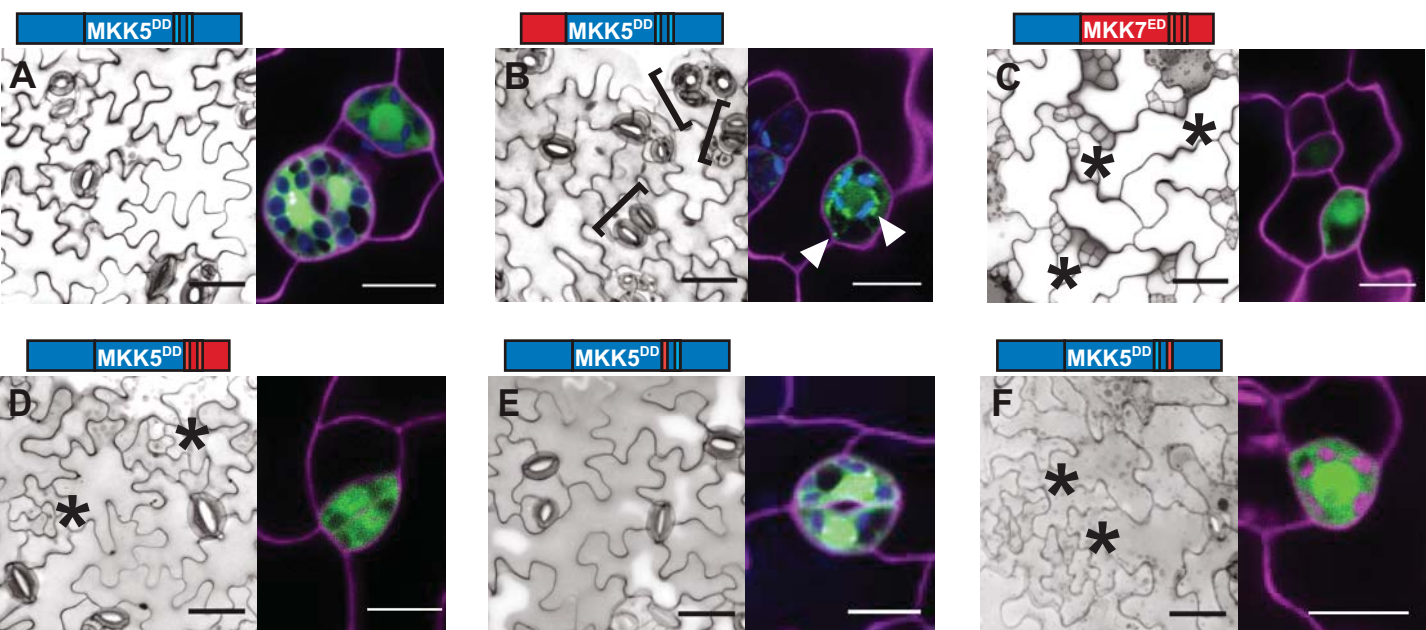
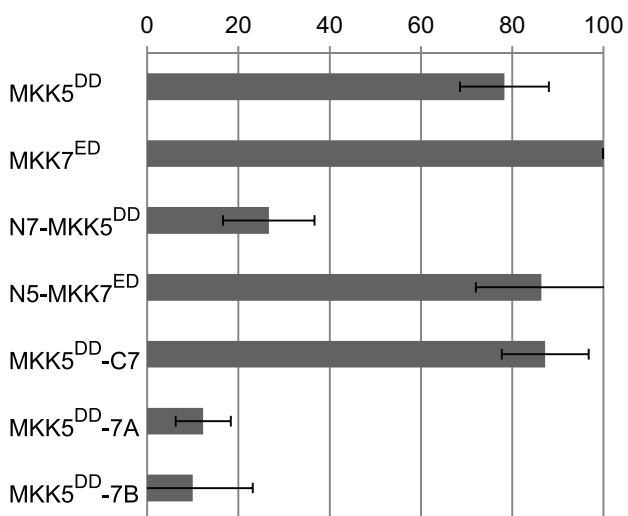


Figure 4



**H** % seedlings with inhibition at SPCH stage



**I** Relative Kinase Activity

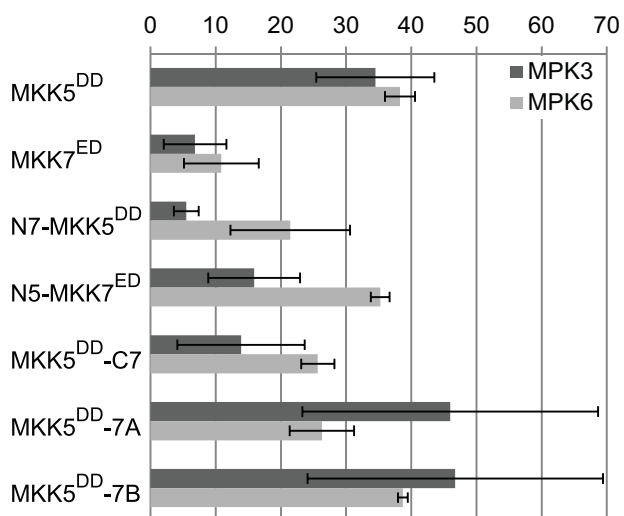


Figure 5

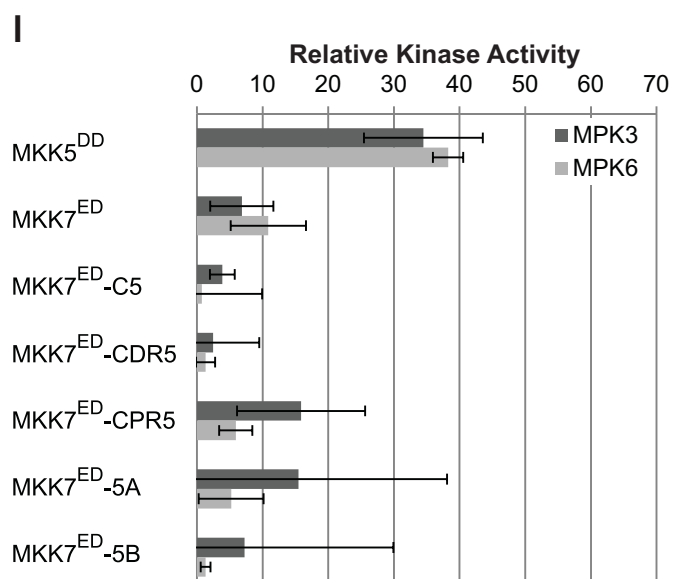
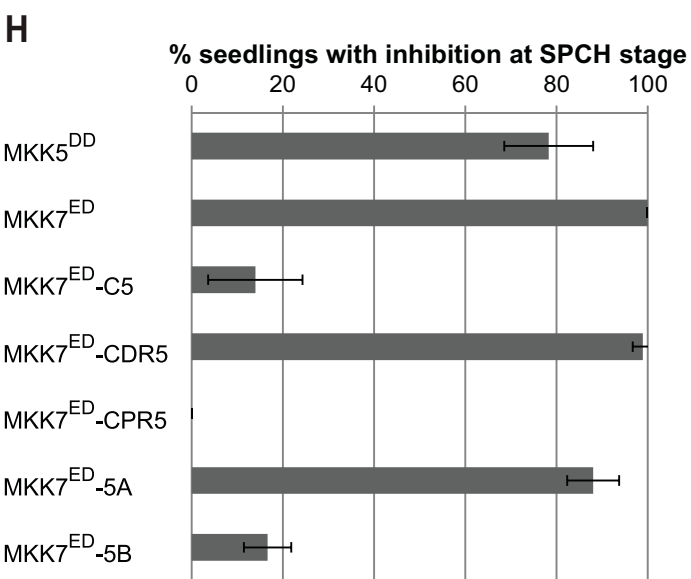
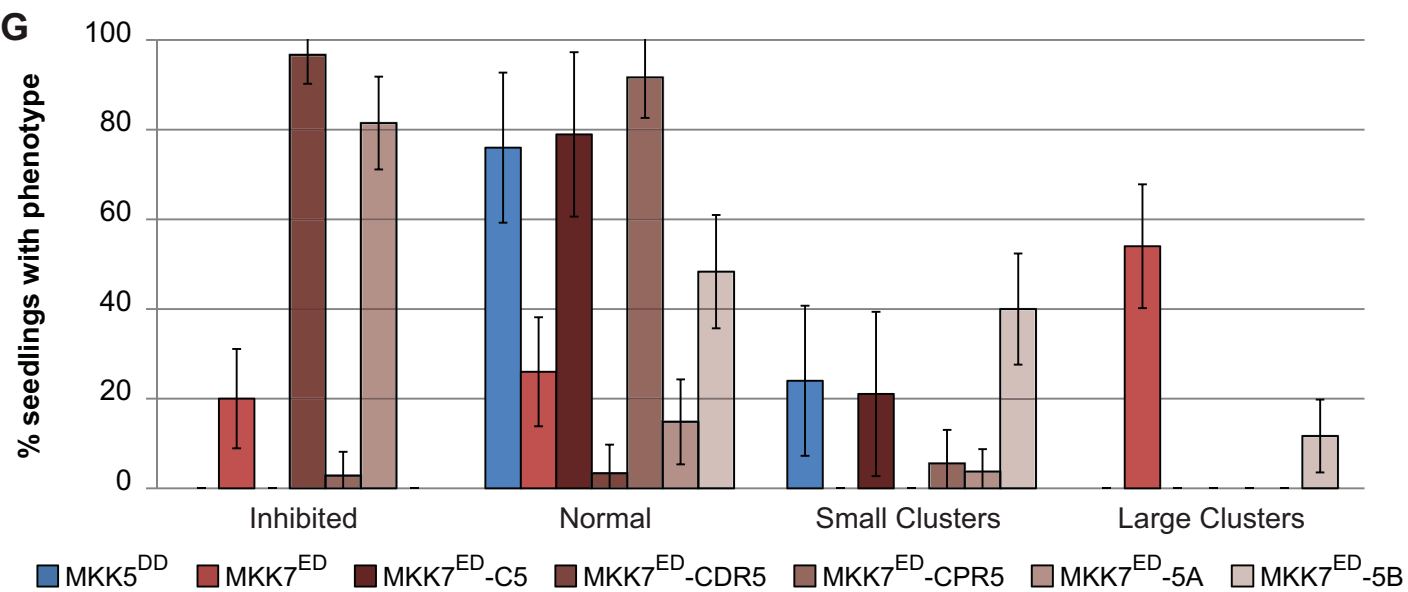
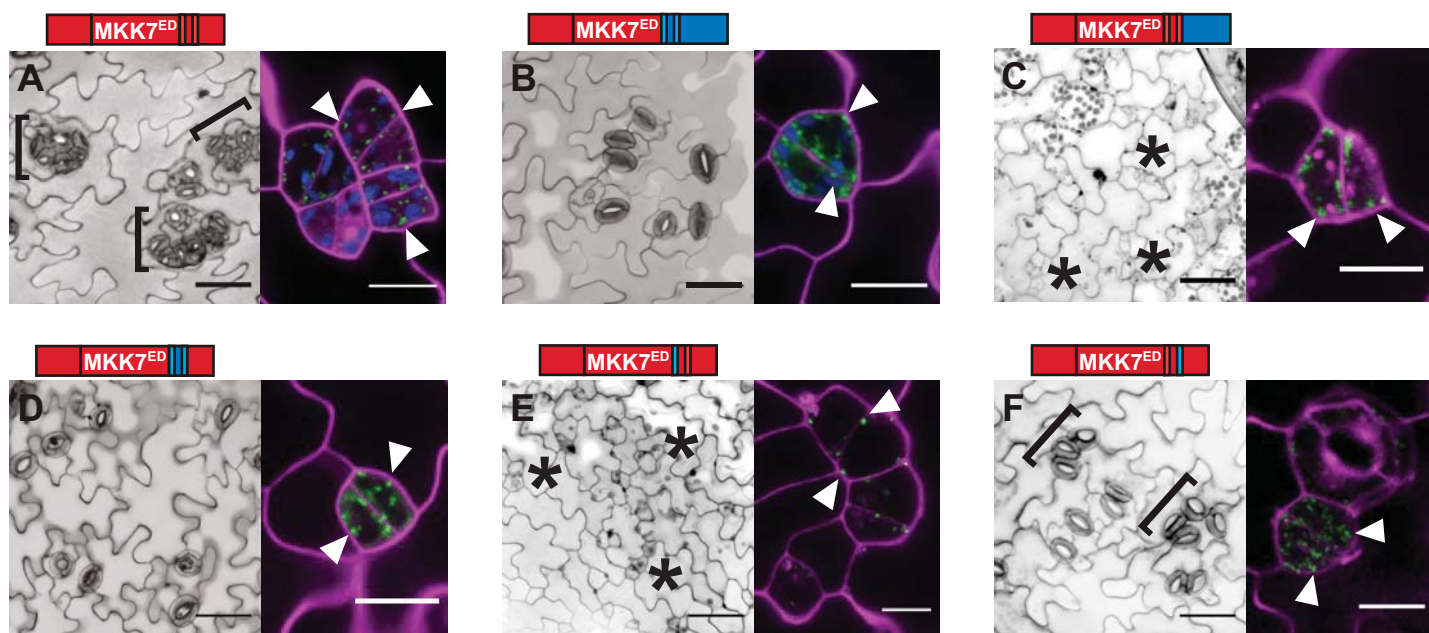
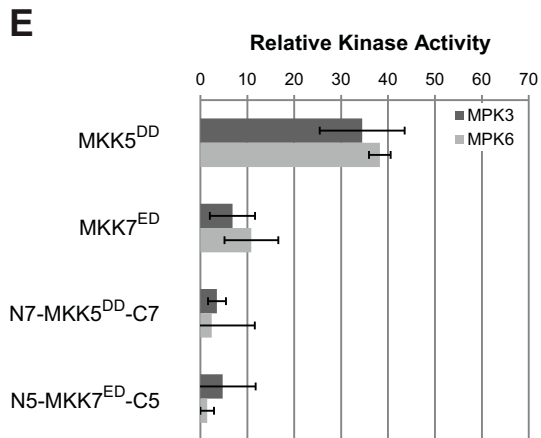
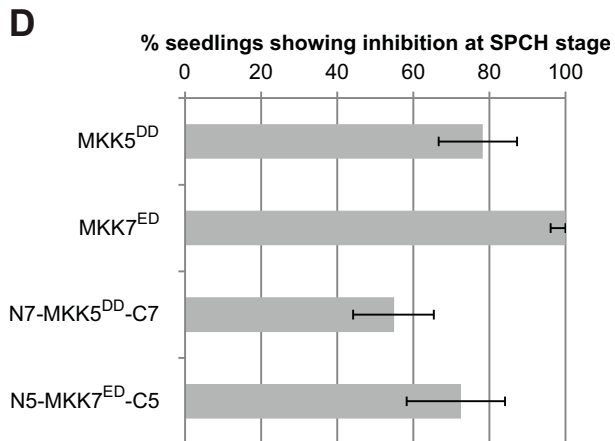
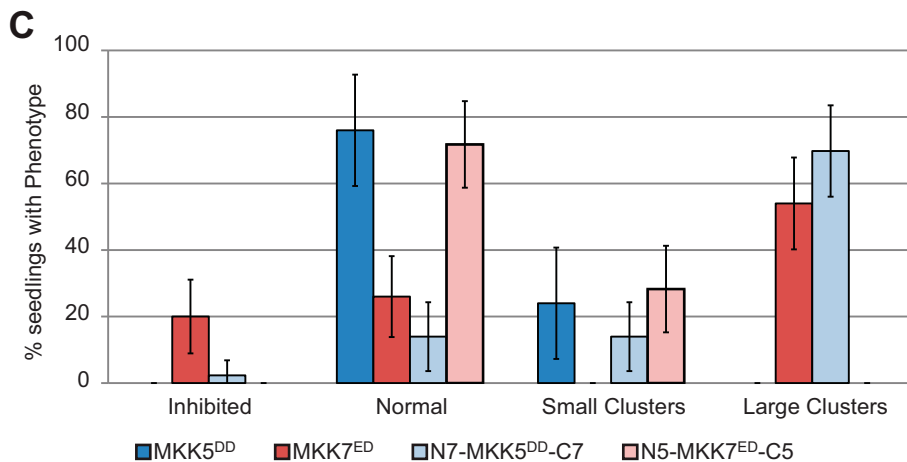
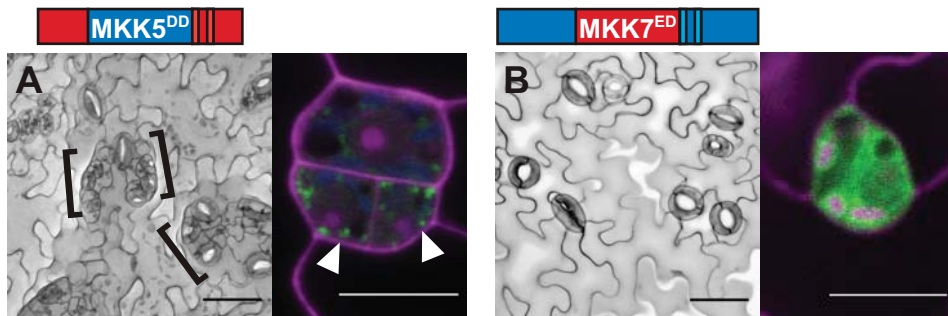


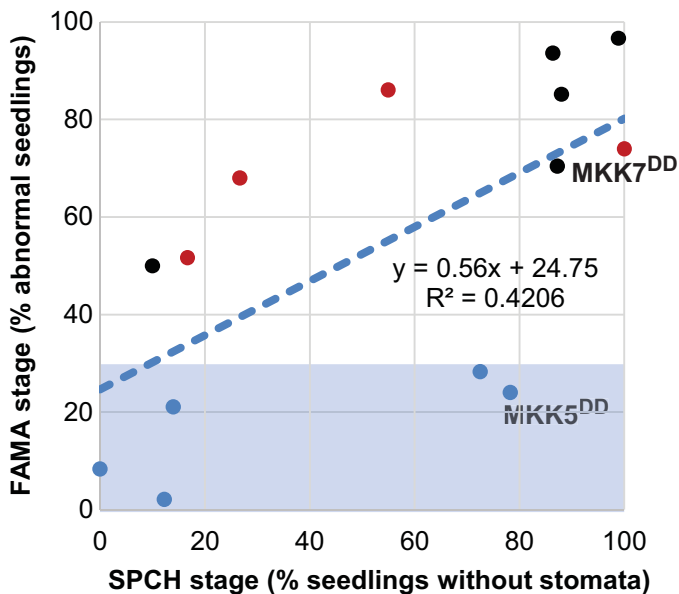


Figure 6



# Figure 7

## A



## B

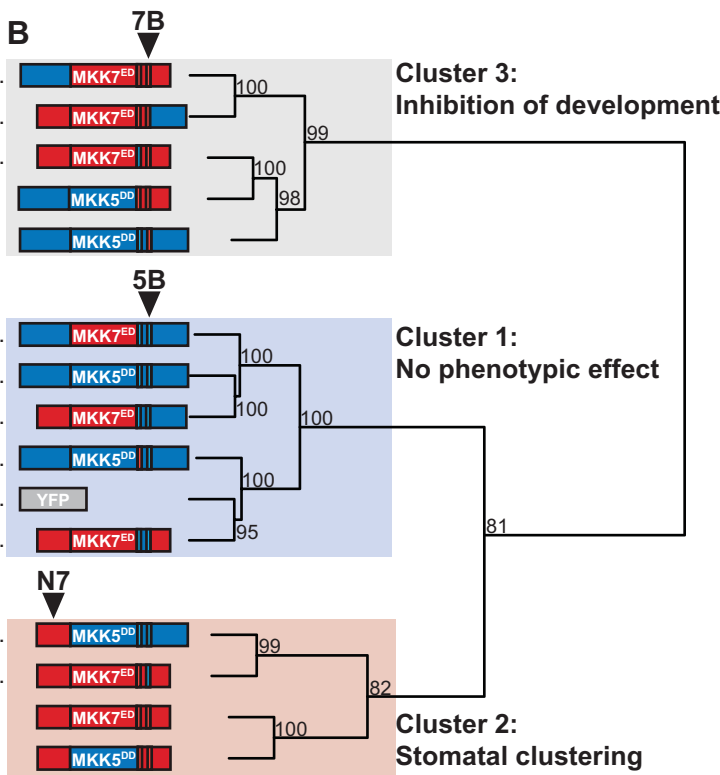
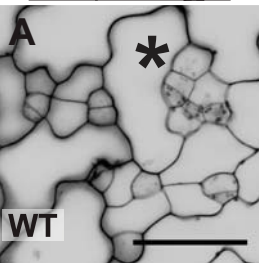
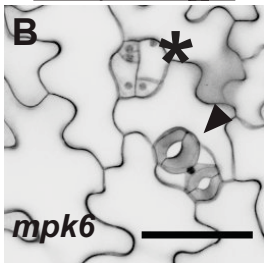


Figure 8



SPCH stage



FAMA stage

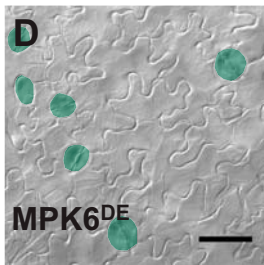
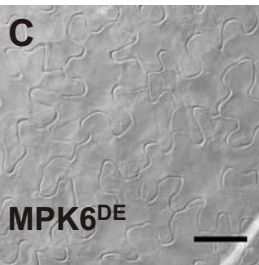
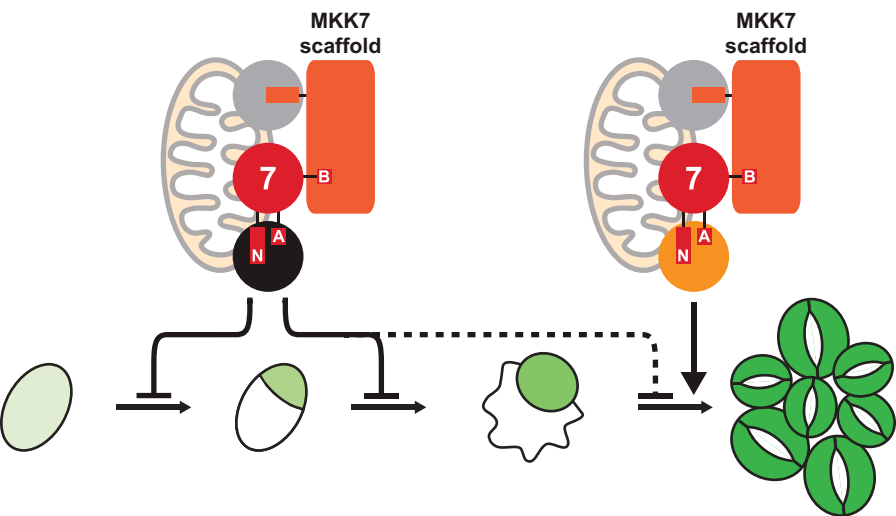


Figure 9

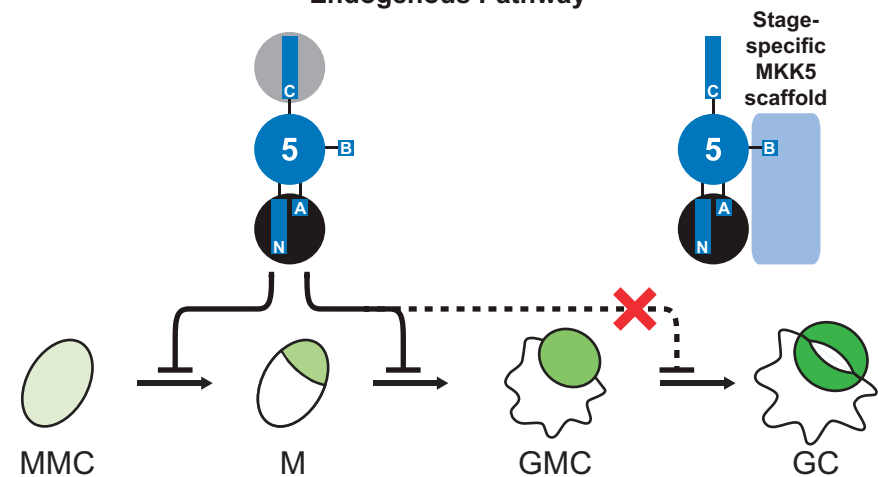
A

Pathways activated by MKK7



B

Endogenous Pathway



DEVELOPMENTAL SIGNALS

DEFENSE AND STRESS RESPONSES