Bipartite anchoring of SCREAM enforces stomatal initiation by coupling MAP kinases to SPEECHLESS

Aarthi Putarjunan1,2, Jim Ruble3, Ashutosh Srivastava4, Chunzhao Zhao5,6, Amanda L. Rychel2, Alex K. Hofstetter1, Xiaobo Tang3, Jian-Kang Zhu5,6, Florence Tama4,7,8, Ning Zheng1,3* and Keiko U. Torii1,2,4*

Cell fate in eukaryotes is controlled by mitogen-activated protein kinases (MAPKs) that translate external cues into cellular responses. In plants, two MAPKs—MPK3 and MPK6—regulate diverse processes of development, environmental response and immunity. However, the mechanism that bridges these shared signalling components with a specific target remains unresolved. Focusing on the development of stomata—epidermal valves that are essential for gas exchange and transpiration—here, we report that the basic helix-loop-helix protein SCREAM functions as a scaffold that recruits MPK3/6 to downregulate SPEECHLESS, a transcription factor that initiates stomatal cell lineages. SCREAM directly binds to MPK3/6 through an evolutionarily conserved, yet unconventional, bipartite motif. Mutations in this motif abrogate association, phosphorylation and degradation of SCREAM, unmask hidden non-redundancies between MPK3 and MPK6, and result in uncontrolled stomatal differentiation. Structural analyses of MPK6 with a resolution of 2.75 Å showed bipartite binding of SCLAM to MPK6 that is distinct from an upstream MAPKK. Our findings elucidate, at the atomic resolution, the mechanism that directly links extrinsic signals to transcriptional reprogramming during the establishment of stomatal cell fate, and highlight a unique substrate-binding mode adopted by plant MAPKs.

Organized differentiation of functional tissue types is a critical step towards ensuring the survival and overall fitness of multicellular organisms. Fundamental to these processes are MAPK cascades—which consist of MAP3K (MAPKKK or MEKK), MAP2K (MAPKK or MEK), and MAPK—that phosphorylate and activate downstream targets to regulate cell proliferation, differentiation and polarity1. In plants, the MAPK cascade is known to influence development, environmental response and immunity2-7. Among the 20 known MAPKs in Arabidopsis thaliana, MPK3 and MPK6 play a predominant role in diverse developmental programs, including embryo patterning, inflorescence architecture, floral abscission, anther and ovule development, and stomatal patterning8-10. It is therefore imperative to understand how these MAPKs recognize their target substrates and specifically activate individual developmental programs. Despite the recently reported partial crystal structure of MPK611, the structural basis for its substrate association remains unclear.

The development of stomata is an excellent system for understanding how external signals are interpreted for the specification of cell fate12-13. The EPIDERMAL PATTERNING FACTOR family of upstream peptide signals are secreted from stomatal precursors and detected by the ERECTA-family receptor-like kinases of their neighbouring cells. This activates the downstream MAPK cascade that is composed of YODA (YDA) MAPKK, two redundant MAPKKs (MKK4 and MKK5) and two redundant MAPKs (MPK3 and MPK6), culminating in the prevention of stomatal differentiation by phosphorylation and inhibition of the basic helix-loop-helix (bHLH) transcription factor, SPEECHLESS (SPCH)14-15. Recent studies have shown that BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) recruits YDA and MPK3/6 to the cortical polarity site16. This leads to a reduction in SPCH accumulation and eventual loss of stomatal fate in one of the two daughter cells of stomatal precursors17. However, unlike the mpk3 mpk6 double mutant, which produces an epidermis solely composed of stomata18, the basl null mutant produces a nearly normal epidermis with occasional paired stomata due to misspecification of asymmetric cell divisions19. Thus, the action of BASL cannot explain the mechanism by which the MAPK cascade enforces the decision to initiate stomatal differentiation.

In search of a factor that recruits MAPKs to the nucleus to downregulate SPCH, we revisited the gain-of-function mutant of SCLAM (SCRM; also known as ICE1), scrm-D, which confers constitutive stomatal differentiation16. SCRM and its parologue SCRM2 function as partner bHLH proteins for SPCH as well as for the later-acting stomatal bHLH proteins, MUTE and FAMA21-23. The scrm-D protein has an R-to-H amino-acid substitution (R236H) within its conserved KRAAM motif24; however, nothing is known about the exact function of this motif.

Here we report that the bHLH protein SCRM physically bridges MAPKs and SPCH and plays a direct role in enforcing entry into...
the stomatal lineage. A bipartite module with the unique KRAAM motif, in conjunction with an upstream conventional MAPK docking motif, mediates specific interactions with MPK3 and MPK6 in the stomata-development pathway. Precise dissection of the binding interface revealed the distinct SCRM-binding properties of MPK3 and MPK6, and further resolved cryptic functional non-redundancies between these two redundant MAPKs. The structural analysis of MPK6 at a resolution of 2.75 Å, together with ab initio modelling of the MPK6–SCRM protein–peptide complex, identified the exact amino-acid residues that serve as the binding interface. Our work provides a mechanistic basis for the function of SCRM as an integrator of upstream repressive cues and downstream activators during stomatal development, and highlights the unique recruitment mechanism of the plant MAPK signalling cascade.

Results
ScRM functions as a scaffold to recruit MAPK to SPCH. MPK3/6-mediated phosphorylation of SPCH inhibits entry of cells into the stomatal cell lineage (Fig. 1a). However, it remains unknown whether SPCH directly associates with MPK3 and MPK6. To address this question, we first performed yeast two-hybrid (Y2H) assays using a truncated form of SPCH without its N-terminal domain (ΔN heterodimerizes with SCRM (Fig. 1b)). This suggests that the interaction between SPCH and MPK3/6 is either too weak or too transient to be detected, requires the SPCH N-terminal domain or that it otherwise requires a scaffolding partner.

To test these hypotheses, we designed a three-way bimolecular fluorescent complementation (BiFC) assay. First, the full-length SPCH protein was fused to the N-terminal half of yellow fluorescent protein (YFP; SPCH–YFPn) and MPK3–YFPc, MPK6–YFPc along with 35S::FLAG–SCRM. Scale bars, 25 μm (left two columns). The right two columns are magnified images of a representative nucleus (scale bars, 10 μm). SPCH interacts with MPK3 and MPK6 in the presence, but not the absence, of SCRM. The experiment was repeated independently three times with similar results. BF, bright field.

were detected between SPCHΔN and MPK3 or MPK6, whereas SPCHΔN heterodimerizes with SCRM (Fig. 1b). This suggests that the interaction between SPCH and MPK3/6 is either too weak or too transient to be detected, requires the SPCH N-terminal domain or that it otherwise requires a scaffolding partner.

To test these hypotheses, we designed a three-way bimolecular fluorescent complementation (BiFC) assay. First, the full-length SPCH protein was fused to the N-terminal half of yellow fluorescent protein (YFP; SPCH–YFPn) and MPK3/6 were fused to the complementary half of YFP (MPK3/6–YFPc). On coexpression of SPCH–YFPn and MPK3–YFPc or MPK6–YFPc in Nicotiana benthamiana leaves, no YFP signal was observed, indicating that SPCH does not interact directly with MPK3/6 (Fig. 1c). A subsequent immunoblot analysis detected the accumulation of SPCH protein (Supplementary Fig. 1), thus refuting the possibility that the lack of YFP signal was due to an absence of SPCH protein accumulation. SPCH forms a heterodimer with SCRM to initiate stomatal differentiation (Fig. 1a). Next, we introduced non-fluorescently tagged SCRM (FLAG–SCRM driven by the CaMV35S promoter) along with SPCH–YFPn and MPK3–YFPc or MPK6–YFPc. SPCH and MPK3/6 were able to reconstitute strong YFP signals in the nuclei of N. benthamiana only in the presence of SCRM (Fig. 1c). We thus conclude that SCRM functions as a scaffold to couple MPK3 and MPK6 with SPCH.
The evolutionarily conserved SCRM KiDoK motif defines a direct MPK3/6 interaction surface. To elucidate how SCRM scaffolding of stomatal lineage cells, we revisited the SCRM gain-of-function mutant, *scrm-D*. The stomata-only epidermis in *scrm-D* mutants is essentially identical to that observed after the loss of MPK3 and MPK6 (*mpk3 mpk6*). Sequence analyses of SCRM and its orthologues revealed the presence of a putative MAPK docking site immediately upstream of the KRAAM motif, which is mutated to KHAAM in *scrm-D* (Fig. 2a, Supplementary Fig. 2a). To understand the role of the conserved region that encompasses the MAPK docking and KRAAM (KiDoK) motif, we performed an unbiased Y2H screen against an *Arabidopsis* seedling cDNA library in which we used the KiDoK motif as bait (see Methods). From 158 million interactions tested, only two proteins—MPK3 and MPK6—were identified with confidence scores high enough to be designated as real interactors (Supplementary Table 1). Further targeted Y2H assays validated the interaction of the SCRM KiDoK motif with MPK3 and MPK6, but not with a distantly related AtMAPK homologue, MPK4, that has no major role in stomatal development (Fig. 2b, Supplementary Fig. 2b). Notably, the *scrm-D* mutation in the KiDoK motif completely abolished the ability of the KiDoK motif to interact with both MPK3 and MPK6 (Fig. 2b). Subsequently, we performed BIFC assays in *N. benthamiana* (Fig. 2c). Strong YFP signals were found in the nuclei of leaves coexpressing SCRM–YFPn and MPK3–YFPc or MPK6–YFPc. By contrast, no signal was detected in the pairwise combination of *scrm-D–YFPn and MPK3–YFPc or MPK6–YFPc*. These results indicate that the *scrm-D* (R236H) substitution abolishes the direct interaction with MPK3/6.

The KiDoK motif of SCRM is highly conserved among vascular and non-vascular land plants (Supplementary Fig. 2). To explore whether the KiDoK motif of SCRM constitutes an interaction module for MPK3/6 in diverse land plant lineages, we performed in vitro direct-binding and quantitative kinetic assays of the KiDoK motif peptides from dicots (*Arabidopsis* SCRM and SCRM2, and tomato (*Solanum lycopersicum*) ICE1), monocots (rice (*Oryza sativa*) ICE1 and Brachypodium distachyon SCRM) and a non-vascular plant (*Physcomitrella patens* PpICE) with purified AtMPK3 and AtMPK6 proteins using bio-layer interferometry (BLI; Fig. 2d,e). *BdSCRM2* lacks the KiDoK motif altogether, and was therefore not used for the analyses (see Discussion). All orthologous KiDoK motif peptides showed tight binding to MPK3, with dissociation constant (*Kd*) values at nanomolar levels, whereas all except PpICE1 exhibited tight binding to MPK6 (Fig. 2d,e). Again, *scrm-D* did not show any appreciable affinity to MPK3 or MPK6. Our results establish that the SCRM KiDoK motif is an evolutionarily conserved direct-interaction surface for MPK3/6.

Functional analysis of the SCRM KiDoK motif elucidates non-redundancies of MPK3 and MPK6. To investigate the functional importance of the SCRM KiDoK motif, we performed site-directed mutagenesis of the K235 and/or R236 residues in the KRAAM motif to a non-charged residue, alanine (KRAAM was mutated to KAAAAM, AAAAM, ARAAM and AHAAM), as well as deleted the docking motif from both SCRM (SCRM-ΔDocking) and *scrm-D* (scrm-D-ΔDocking). We then performed interaction analyses of these mutant variants with MPK3/6 using Y2H assays. Whereas the various alanine substitutions in the KRAAM motif abolished interaction with both MPK3 and MPK6, the SCRM-ΔDocking motif abolished interaction with only MPK6, and not with MPK3 (Fig. 3a).

To examine the phenotypic consequence of these mutations in vivo, we introduced each mutant construct, driven by the endogenous SCRM promoter, into the *Arabidopsis* scrm-null-mutant. Similar to *scrm-D*, all alanine substitutions conferred severe stomatal clustering (Fig. 3b,c, Supplementary Fig. 3), emphasizing that the loss of MPK3/6 association due to the altered KRAAM motif triggers constitutive stomatal differentiation. Detailed quantitative analysis of the index of stomata + stomatal precursors (SPI; calculated as ((number of stomata + stomatal precursor cells)/number of epidermal cells)×100) revealed that SCRM with an alanine substitution that retains the R236 residue (ARAAM) is less effective at triggering constitutive stomatal differentiation (Fig. 3c). Similarly, *SCRMpro::SCRM-ΔDocking* confers a much weaker phenotype than *SCRMpro::scrm-D-ΔDocking* (Fig. 3b,c). These results highlight the importance of the R236 residue in the KRAAM motif for enforcing proper stomatal development.

To further dissect the specific association of SCRM with MPK3 and MPK6, we truncated the KiDoK peptide into one containing only the KRAAM motif and a second containing only the docking motif. In vitro quantitative binding assays using BLI showed that, although the KRAAM motif binds to both MPK3 and MPK6 (*Kd* = 2.8 ± 0.7 μM and *Kd* = 2.4 ± 0.4 μM, respectively), the docking motif associates with only MPK6, and not with MPK3 (*Kd* = 2.3 ± 0.5 μM and not detected, respectively; Fig. 3d).

It is well known that MPK3 and MPK6 redundantly repress stomatal development—neither *mpk3* nor *mpk6* single-mutant *Arabidopsis* confers the stomatal clustering phenotype, although they exhibit a slightly higher SPI than the wild type (Fig. 3e). Our finding reveals a unique mode of MPK3/6 association with SCRM: MPK6 requires both the docking and the KRAAM motifs, whereas MPK3 requires only the latter (Fig. 3a,d). If these different binding modes of MPK3 and MPK6 with SCRM reflect their in vivo ability to inhibit stomatal development, it could be used to unmask the hidden non-redundancy of MPK3 and MPK6. We first hypothesized that expression of *SCRMpro::SCRM-ΔDocking* in *mpk3* mutant *Arabidopsis*—which express functional MPK6—confers exaggerated stomatal differentiation because MPK6 requires the docking motif to anchor onto SCRM. By contrast, expression of *SCRMpro::SCRM-ΔDocking* in *mpk6* mutant *Arabidopsis*—which express functional MPK3—will not enhance the phenotype of *SCRMpro::SCRM-ΔDocking* when expressed in the wild-type MPK3/MPK6 background because MPK3 does not seem to require the docking motif to anchor onto SCRM. To test this hypothesis, we introduced *SCRMpro::SCRM-ΔDocking* into the *scrm* background (Fig. 3f, Supplementary Figs. 4a and 6). These striking phenotypic differences between *mpk3* and *mpk6* seedlings expressing *SCRM-ΔDocking* in the *scrm* background emphasize the notion that MPK3 and MPK6 feature a major difference in their binding modes (common-docking (CD) domain, see below) that are responsible for recognizing the conventional MAPK docking motif within the SCRM KiDoK sequence. Thus, by detaching the docking and KRAAM motifs, we can reveal functional non-redundancies between MPK3 and MPK6 in repressing stomatal cell fate.

Structural analyses of the SCRM KiDoK–MAPK interaction module. The KRAAM motif is unique to SCRM orthologues and has otherwise not been found to be involved in binding MAPK in plants or animals. To gain structural insight into its function, we first independently determined the crystal structure of MPK6 (residues 32–395, MPK6ΔN1) at a resolution of 2.75 Å (Supplementary Figs. 4a and 6; see Methods). MPK6 crystallized with two molecules in the asymmetric unit and displayed packing and main chain alignment that closely resembled the partial structure previously determined at a resolution of 3.2Å (Supplementary Figs. 4a and 6). MPK6 forms a bilobed structure divided between the N and
Fig. 2 | The evolutionarily conserved KiDoK motif of SCRM defines a direct MPK3/6 interaction surface. a, A diagram of the SCRM protein. The predicted kinase docking motif is indicated in pink, the KRAAM motif is indicated in cyan, the bHLH domain is indicated in blue and the C-terminal domain is indicated in yellow. The amino-acid sequence of the kinase docking motif, KRAAM motif and the R236H mutation in scrm-D (green) are shown at the top. A cartoon representation of the wild-type and the scrm-D epidermis is shown below. b, Y2H assays in which the DNA-binding domain alone (DB), SCRM KiDoK and scrm-D KiDoK were used as bait, and the activation domain alone, MPK3 and MPK6 were used as prey. Yeast were spotted in 10-fold serial dilutions on appropriate selection media. The experiment was repeated independently three times with similar results. c, BiFC assays showing three-week-old N. benthamiana leaves that were agroinfiltrated with pairwise combinations of SCRM–YFPn or scrm-D–YFPn and MPK3–YFPc or MPK6–YFPc. Scale bars, 25 μm (left two columns). The right two panels show magnified images of a representative nucleus. Scale bars, 10 μm. The experiment was repeated independently three times with similar results. d, e, Quantitative analyses of SCRM KiDoK from various plant species with MPK3 (d) and MPK6 (e) using BLI. In vitro binding response curves for GST–MPK3 and biotinylated peptides from the KiDoK motif of AtSCRM, AtSCRM2, BdSCRM, OsICE1, SlICE1, PpICE1 and Atscrm-D at six different concentrations (1,000 nM, 333.33 nM, 111.11 nM, 37.04 nM, 12.34 nM and 4.11 nM) are shown. The response curves for GST–MPK6 with the same biotynylated peptides were obtained by performing the experiment at lower concentrations (150 nM, 75 nM, 37.5 nM, 18.75 nM, 9.375 nM and 4.6875 nM) to enable a better fit for calculating $K_d$ values. Data are mean ± s.d., representative of three independent experiments. $K_d$ values are indicated on the right. ND, not detected; NF, not fittable.
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In fact, the structure of MPK6 reveals the highly conserved AtMEKs), inactivators (MKPs) and substrates27. In most MAPKs, these exposed, negatively charged residues (aspartic acid or glutamic acid) are involved in mediating peptide–substrate binding28.

Mammalian MAPKs possess a docking groove in the C-terminal lobe that contains a highly conserved CD domain mostly composed of negatively charged residues that bind to activators (MAPKKs and MEKs), inactivators (MKPs) and substrates27. In most MAPKs, these exposed, negatively charged residues (aspartic acid or glutamic acid) are involved in mediating peptide–substrate binding28.

We found that the CD domain is mostly conserved between the MPK3/6 and mammalian ERK/p38 family (Supplementary Fig. 7). In fact, the structure of AtMPK6 reveals the highly conserved D353 and D356 residues exposed from H18 within the CD domain (Supplementary Fig. 4).

To probe the possible role of the CD domain of MPK6 in SCRM binding, we performed Y2H assays with CD domain mutations (D353N and D356N (MPK6-CDDm)) that disrupt the two conserved aspartic acid residues. MPK6–CDm was unable to associate with both SCRM and scrm-D KiDoK motif (Fig. 4a), indicating

Fig. 3 | MPK3 and MPK6 exhibit different binding modes to the SCRM KiDoK motif to repress stomatal cell fate. a, Y2H assays of SCRM KiDoK motif substitution and deletion mutants and MPK3 or MPK6. The DNA-binding domain (DB) alone, SCRM KiDoK, scrm-D KiDoK, the KAAAM, ARAAM, AHAAM and ARAAM versions of the SCRM KiDoK motif, SCRM–ΔDocking and scrm–ΔDocking were used as bait. The activation domain fused to MPK3 and MPK6 was used as prey. The SCRM–ΔDocking motif abolished interaction with MPK6, but not with MPK3. The experiment was repeated independently three times with similar results. b, Confocal imaging of cotyledon abaxial epidermis of seven-day-old wild-type (WT) and transgenic Arabidopsis seedlings expressing the SCRM KiDoK motif substitutions and deletions (scrn–D, SCRMP:SCRMP–KAAAM, SCRMP:SCRMP–ARAAM, SCRMP:SCRMP–AHAAM, and SCRMP:SCRMP–AAAAM in the scrm background, and SCRMP:FLAG–SCRM–ΔDocking and SCRMP:FLAG–scrm–ΔDocking in the scrm2 background). Scale bars, 20 μm. Each representative confocal image was obtained after imaging at least two independent frames from six seedlings for each genotype. c, Quantitative analysis of the SPI of the cotyledon abaxial epidermis from seven-day-old wild-type (WT) and transgenic Arabidopsis seedlings expressing the SCRM KiDoK motif substitutions and deletions (scrm–D, scrm–ΔDocking, and mpk3 and mpk6 backgrounds along with scrm and mpk3 single mutants. Abaxial epidermis from seven-day-old plants was imaged. Scale bars, 20 μm. Each representative confocal image was obtained after imaging at least two independent frames from six seedlings for each genotype. d, Quantitative analysis of the SPI of the cotyledon abaxial epidermis from seven-day-old Arabidopsis seedlings of scrm2, scrm2 mpk3 and scrm2 mpk6 genotypes that express SCRM–ΔDocking, and of mpk3 and mpk6 genotypes that do not express SCRM–ΔDocking. For each genotype, one image per seedling was analysed (n = 6). One-way analysis of variance (ANOVA) followed by Tukey’s honest significant difference (HSD) test was performed to compare all other genotypes and classify their phenotypes into four categories (a–d). Results from the Tukey’s HSD test are listed in Supplementary Table 5. d, Quantitative analysis of interactions between the KRAAM motif and the kinase docking motif of SCRM with GST–MPK3 and GST–MPK6 using BLI. In vitro binding response curves for purified GST–MPK3/6 and biotinylated KRAAM motif peptide and docking motif peptide at six different concentrations (10,000 nM, 3,333.33 nM, 1,111.11 nM, 370.4 nM, 123.4 nM and 41.1 nM) are shown. Data are mean ± s.d., representative of three independent experiments. K_i values are indicated on the right. e, Representative stomatal phenotypes of SCRMP:FLAG–SCRM–ΔDocking in scrm2, scrm2 mpk3 and scrm2 mpk6 backgrounds along with mpk3 and mpk6 single mutants. Abaxial epidermis from seven-day-old plants was imaged. Scale bars, 20 μm. Each representative confocal image was obtained after imaging at least two independent frames from six seedlings for each genotype. f, Quantitative analysis of the SPI of the cotyledon abaxial epidermis from seven-day-old Arabidopsis seedlings of scrm2, scrm2 mpk3 and scrm2 mpk6 genotypes that express SCRM–ΔDocking, and of mpk3 and mpk6 genotypes that do not express SCRM–ΔDocking. For each genotype, one image per seedling was analysed (n = 6). One-way ANOVA followed by Tukey’s HSD test was performed to compare all other genotypes and classify their phenotypes into three categories (a–c). Results from the Tukey’s HSD test are listed in Supplementary Table 5. For the boxplots in c and f, the horizontal bar indicates the median value. The upper and lower hinges of each box indicate the top and the bottom quartile of the reported values, respectively. The whiskers correspond to 1.5x the interquartile range. Black points indicate outliers. Pink points indicate values of individual datapoints. The different letters above each box indicate statistically significant differences. ND, not detected.
that MPK6 binds to SCRM via its conserved CD domain, possibly using a mechanism similar to that in mammalian MAPKs. These results were further supported by in vitro BLI measurements, which showed that MPK6-CD\textsuperscript{D\textsubscript{mut}} failed to show appreciable affinity towards SCRM KiDoK (K\textsubscript{D} value: not fittable) compared with the wild-type MPK6 protein (K\textsubscript{D} = 47 ± 6 nM; Fig. 4b). Taken together, the structure–function analyses of MPK6 and the SCRM KiDoK motif reveal a conserved mechanism of substrate binding between
Deciphering the MPK6–SCRM binding interface. To understand the structural basis of how the single-residue substitution within the SCRM-KRAAM motif abolishes the association with MAPKs, we initially sought to resolve the crystal structure of MPK6–SCRM KiDoK complex, which was unfortunately recalcitrant to crystallization. As an alternative approach, we resorted to ab initio modelling of the MPK6–SCRM protein–peptide complex and performed docking simulations of SCRM KiDoK peptide onto our AtMPK6 crystal structure (Protein Data Bank (PDB) 6DTL; Fig. 4c–f). Linear binding motifs that interact with the kinases are often devoid of tertiary structure and form a well-defined structure only in the presence of its binding partner25. In light of this, flexible docking was performed whereby the SCRM KiDoK peptide was allowed to fold near the CD domain and docking groove with multiple constraints imposed using information from previous studies11,26 as well as experiments defined in this study (see Methods). The SCRM docking motif was restrained to the D-motif binding site—which comprises the CD domain and the hydrophobic docking groove11—and the KRAAM motif was restrained to be in the vicinity of the MPK6 structure. The R213 residue of the SCRM docking motif was restrained to remain near the negatively charged CD domain residues D353 and D356 (Fig. 4d). A comparison of previously determined ERK2-substrate peptide complexes (ERK2–pepMNK1, PDB 2Y9Q and ERK2–pepRSK1, PDB 3TEI)28 with our MPK6 crystal structure indicated a possible interaction between the positively charged residue K215 of the SCRM docking motif and E120 of MPK6 (Fig. 4e). Here the resolved structures of human ERK2–pepMNK1 and ERK2–pepRSK1 complexes show that ERK2 E81 and the homologous MPK6 E120 come in direct contact with R447 and R725 of pepMNK1 and pepRSK1, respectively (Supplementary Fig. 5).

Next, we performed functional analyses to validate the two top-scoring final models. One model predicted amino acids E163 and E164 of MPK6 as possible sites that interact with R236 of SCRM. This was rejected by the experimental validation because site-directed mutations within these MPK6 residues to eliminate negative charges (E163N and E164N) showed no effects on SCRM interaction (Supplementary Fig. 7). The other model predicted amino acid D148 of MPK6 as a potential interaction partner of the R236 residue within the KRAAM motif, which was fully supported by further experimental verifications (see below).

Bipartite recruitment of MPK6 by SCRM using a conventional docking site and a SCRM-specific motif. The MPK6–SCRM interaction model predicted the bipartite binding mode of SCRM to MPK6—the docking motif that associates with the CD domain of MPK6, the KRAAM motif that lies at the neck of MPK6 N- and C-terminal lobes with MPK6 D148 as a site that interacts directly with SCRM R236, the key residue within the KRAAM motif (R236 is mutated to H in scrm-D; Fig. 4). Importantly, the MPK6 D148 site is not conserved among mammalian MAPKs (Supplementary Fig. 7b). To investigate the importance of MPK6 D148 and SCRM R236 as a binding interface, we first performed site-directed mutagenesis. The D148N substitution in MPK6 compromised its interaction with the SCRM KiDoK motif substantially in Y2H and in vitro quantitative binding kinetics assays that used BLI (Fig. 4g,i, Supplementary Fig. 7a), indicating that the MPK6 D148 is indeed critical for binding with SCRM through the R236 residue within the KRAAM motif.

Next, to address whether the polar interactions between MPK6 D148 and SCRM R236 is sufficient for MPK6–SCRM association, we swapped these residues to D148R and R236D, respectively. As shown in Fig. 4h, MPK6(D148R) and SCRM(R236D) were able to maintain interaction, albeit to a lesser extent than their wild-type counterparts. This suggests that MPK6 D148 and SCRM R236 serve as a critical interaction interface even when they are placed out of context from their native protein environments.

To gain further insight into the bipartite binding mode, we performed surface conservation mapping of the AtMPK6–SCRM complex and H. sapiens ERK1, ERK2, ERK5, p38α, p38β, p38γ and p38δ. Whereas AtMPK6 residues that bind the SCRM docking motif (that is, D353, D356 and E120) are highly conserved in mammalian MAPKs, the D148 contact site is far less conserved, thus reflecting the uniqueness of the binding of the SCRM-KRAAM motif to MPK6 (Fig. 4g,h,j). Finally, to decipher the specific role of MPK6 D148 during association with SCRM-KRAAM motif, we examined whether the MPK6(D148N) substitution—which disrupts association with SCRM—has any effects on the ability of MPK6 to recruit the upstream MAPKK, MKK526. Indeed, MPK6(D148N) retained interaction with MKK5 at a similar level to wild-type MPK6 (Fig. 4g). MPK6 with an additional substitution of E120N also retained the ability to associate with MKK5 but showed compromised binding to SCRM KiDoK (Fig. 4g,j), further emphasizing the intricate nature of MPK6–SCRM binding. Taken together, our structural and ab initio modelling of the MPK6–SCRM KiDoK complex and further experimental verifications elucidate a unique, bipartite binding mode of SCRM with MPK6—one that uses both conserved substrate binding sites that are similar to animal MAPKs, and contact sites that are highly unique to SCRM, the combination of which results in the specialized direction of cell fate during stomata development.

Binding of SCRM KiDoK and MPK3/6 is critical for SCRM phosphorylation and stability. To examine whether MPK3/6 can modify SCRM but leave scrm-D unaffected, we first performed an in vitro kinase assay using purified, recombinant constitutively active MAPKK (MKK520), MPK3, MPK6, SCRM and scrm-D (see Methods). In the presence of MPK3 and MPK6 that is active MAPKK (MKK5DD), MPK3, MPK6, SCRM and scrm-D were able to modify SCRM but leave scrm-D unaffected, we first performed an in vitro kinase assay using purified, recombinant constitutively active MAPKK (MKK520), MPK3, MPK6, SCRM and scrm-D (see Methods). In the presence of MPK3 and MPK6 that is active MAPKK (MKK5DD), we detected strong phosphorylation of SCRM but not of scrm-D. To study the in vivo consequences of the MPK3/6-mediated SCRM phosphorylation, we first generated double transgenic Arabidopsis seedlings that expressed a functional, epitope-tagged SCRM as well as scrm-D driven by the endogenous promoter (SCRMpro::FLAG-SCRM and SCRMpro::FLAG-scrm-D) and an inducible constitutively active MAPKK, DEX::FLAG-NΔMEK220, which has been widely used to activate Arabidopsis MPK3/6 in vivo20 (Fig. 5b). On dexamethasone (DEX) induction, the SCRM protein readily degrades, whereas after treatment with the proteasome inhibitor MG132 in the presence of DEX induction, the protein accumulation level of FLAG–SCRM is restored in the SCRMpro::FLAG–SCRM seedlings (Fig. 5b), indicating that SCRM is subjected to proteasome-mediated degradation following induction of MAPK-mediated phosphorylation. By contrast, the scrm-D protein remained stable regardless of the NΔMEK220 induction or treatment with a proteasome inhibitor (Fig. 5b).

Next, we examined the in vivo stability of SCRM and scrm-D proteins during seedling epidermal development using double transgenic seedlings that expressed functional green fluorescent protein (GFP)-fused SCRM and scrm-D along with DEX::FLAG-NΔMEK220 (Fig. 5c). Whereas NΔMEK220 induction triggered loss of GFP signals from the epidermis of SCRMpro::GFP–SCRM 24h after treatment with DEX (Fig. 5c,d), the GFP signals from SCRMpro::GFP–scrm-D seedlings persist even 24h after the activation of MAPKs in vivo (Fig. 5c,d). We further examined the in vivo
stability of the SPCH–GFP protein—a heterodimeric partner of SCRM that is also targeted by MPK3/6\(^{17,23}\) following induction of NtMEK2\(^{20}\). Similar to SCRM::GFP-SCRM, SPCHpro::SPCH-GFP signals diminished by 6h and were lost by 24h after DEX treatment (Fig. 5c,d). On the basis of these results, we conclude that the direct association of SCRM and MPK3/6 through the KiDoK motif is critical for subsequent phosphorylation and degradation of SCRM as well as SPCH, thereby specifying proper cell fate during stomatal differentiation (Fig. 6).

**Discussion**

Our study elucidates—at the atomic resolution—the mechanism by which SCRM integrates upstream MAPK cascade-repressors and the downstream transcription factor SPCH to enforce the initiation of stomatal cell lineages on the developing plant epidermis (Fig. 6). Both the kinase docking and KRAAM motifs of SCRM are necessary for MPK6 binding, whereas the former motif seems to be dispensable for association with MPK3 (Fig. 3). The sequence conservation of the SCRM KiDoK motif among SCRM orthologues (Supplementary Fig. 2a) suggests their nuanced regulation by MAPKs. The high sequence conservation of the KRAAM motif within the SCRM proteins from both vascular and non-vascular plants correlates with their tight binding to AtMPK3, whereas the relatively low sequence conservation of the kinase docking motif correlates with their disparate binding to AtMPK6 (Fig. 2d,e). This is most evident in *P. patens* SCRM (PpICE1), which has a highly conserved KRAAM (KRAAS) motif but a poorly conserved kinase docking motif—PpICE1 associates strongly with AtMPK3 but very poorly with AtMPK6. These findings imply an elaboration of a bipartite binding mode of SCRM with MAPKs during the evolution and diversification of land plants. The cryptic functional non-redundancies between MPK3 and MPK6, unravelled through mutational analysis of the KiDoK motif (Fig. 3e,i), further hint at the hidden, unique properties associated with these two MAPKs. Among the SCRM orthologues, BdSCRM2 lacks the entire KiDoK region altogether, yet it can substitute for BdICE1 to produce normal stomata in *Brachypodium*\(^{41}\). As expected from the amino-acid sequence, BdSCRM2 does not interact with AtMPK3/6 (Supplementary Fig. 2c), suggesting that BdSCRM2 is not under direct regulation by the MAPK cascade. Therefore, grass species may have not only rewired the core stomatal bHLH transcription factors\(^{41}\), but also reshaped the architecture of the repressive signalling pathway feeding into the stomatal differentiation program.

The inability of the scrm-D protein to associate with MPK3/6 prevents its phosphorylation and subsequent degradation (Figs. 5 and 6). Thus, the KiDoK motif of SCRM essentially functions as a degron, controlling the stability of the SCRM protein. Over the years, a number of components that facilitate the degradation of SCRM in diverse biological contexts have been identified. For example, during freezing acclimation, the E3 ligases HOS1 and SIZ1 mediate the ubiquitination and SUMOylation of SCRM/ICE1 and its subsequent degradation\(^{5,34}\). Furthermore, the OST1 kinase enhances SCRM/ICE1 stability by suppressing HOS1-mediated degradation of SCRM in the cold\(^{34}\). Recently, another E3 ligase, COP1, has been shown to suppress stomatal development in the dark by ubiquitinating and degrading SCRM\(^{41}\). This COP1-mediated SCRM degradation can be abrogated by light. As such, identification of E3 ligases that regulates the SPCH–SCRM bHLH heterodimer module during normal stomatal development is an important future direction.

SCRM controls developmental and environmental processes outside of stomatal differentiation, likely by associating with specialized bHLHs\(^{38-40}\). For example, SCRM forms a heterodimer with ZHOUPI, a specialized bHLH protein involved in endosperm and seed development\(^{41}\). Therefore, our discovery that SCRM acts as a scaffold to recruit MPK3/6 to its partner bHLH, SPCH, could be a universal mechanism for MPK3/6-mediated regulation of transcriptional reprogramming in diverse contexts. Recent studies have shown that phosphorylation of SCRM by MPK3/6 is critical for acclimatization of plants to freezing conditions\(^{1,2,3}\). It is unknown whether SCRM forms heterodimers with specialized bHLHs and regulates their phosphorylation as a scaffold for the cold-tolerance pathway.

The proposed bipartite binding mode of the SCRM KiDoK motif interaction with MPK6 explains the complexity of binding specificity between MPK3/6 and SCRM. Although mammalian MAPKs are known to interact with their substrates using a conserved kinase interaction motif [KR]X\(_{4-6}\)[LI]X[LI]\(^{42}\), this type of motif has been identified in plants only in APC21, an *Arabidopsis* type 2C Ser/Thr phosphatase that negatively regulates MPK4 and MPK6 and modulates innate immunity, jasmonic acid and ethylene levels\(^{42}\). It seems rather unlikely that diverse MAPK substrates use similar kinase interaction motifs to interact with MAPKs that are known to simultaneously influence a variety of developmental programs.

Our quantitative binding assays using BLI revealed that individual docking and KRAAM peptides exhibit lower affinity (K\(_d\) values in the micromolar range) to MPK6, whereas the longer KiDoK

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**Fig. 5 | Direct MPK3/6 association is required for the phosphorylation and degradation of SCRM.** a. An in vitro phosphorylation assay of SCRM and scrm-D. Purified recombinant SCRM, scrm-D, MKK5\(^{20}\). MPK3 and MPK6 were subjected to in vitro phosphorylation followed by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) analysis. The top image shows an autoradiograph and the bottom image shows Coomassie brilliant blue staining (CBB). Asterisks indicate non-specific signals. The experiment was repeated independently three times with similar results. b. Induced NtMEK2\(^{20}\) overexpression triggers in vivo protein degradation of SCRM, but not scrm-D. Transgenic *Arabidopsis* seedlings carrying DEX::FLAG-NtMEK2\(^{20}\) with SCRMpro::FLAG-SCRM or with SCRMpro::FLAG-scrm-D were grown in the presence of 0.5\(\mu\)M DEX or mock. At 4d after germination, the seedlings were treated with the proteasomal inhibitor MG132 (50\(\mu\)M) or mock for 6h. The total proteins were separated by SDS–PAGE (bottom; CBB) and subjected to immunoblot with anti-FLAG antibody (top). The experiment was repeated independently three times with similar results. c. Induced NtMEK2\(^{20}\) overexpression triggers loss of SPCH–GFP and GFP but not scrm-D–GFP. Confocal microscopy of cotyledon abaxial epidermis from two-day-old double transgenic *Arabidopsis* seedlings that carried inducible NtMEK2\(^{20}\) (DEX::FLAG-NtMEK2\(^{20}\)) with SPCHpro::SPCH-GFP, SCRMpro::GFP-SCRM or SCRMpro::GFP-scrm-D and were grown in the presence of 0.05\(\mu\)M DEX (+DEX) or ethanol (mock). Imaging was performed after induction of NtMEK2\(^{20}\) for 24 h. Scale bar, 20\(\mu\)m. Each representative confocal image was obtained after imaging at least two independent frames from three seedlings for each genotype. d. Quantitative analysis of nuclear GFP signals of seedling cotyledon epidermis from two-day-old seedlings expressing SPChpro::SPCH-GFP, SCRMpro::GFP-SCRM or SCRMpro::GFP-scrm-D after NtMEK2\(^{20}\) induction by 0.05\(\mu\)M DEX at the times indicated. Each dot represents the total intensity value of the GFP signal from each nucleus expressing the respective fusion proteins. Serial z-stack projection images were used for quantitative analysis (see Methods) to ensure complete coverage of the nuclei that express GFP. The mean value at each time point is connected by a line to visualize the change in the intensity dynamics over time. Purple datapoints indicate the initial values at time-point 0h. The cyan datapoints indicate the mock treatment. The magenta datapoints indicate induction of NtMEK2\(^{20}\). Experiments were repeated three times; n=6 seedlings for each genotype for time-point 0h; n=3 seedlings for each genotype per treatment condition for subsequent time points. A total of 2,623, 2,000 and 6,518 nuclei were analysed for SPChpro::SPCH-GFP, SCRMpro::GFP-SCRM and SCRMpro::GFP-scrm-D, respectively.
motif shows a tight binding (Kₐ values in the nanomolar range; Figs. 2e and 3d). This is probably due to cooperative binding of these two motifs to MPK6. Interestingly, MPK3 exhibits weak binding to the KRAAM peptide but no binding to the docking peptide (Fig. 3d). However, the KiDoK peptide associates with MPK3 just as tightly as MPK6 (Fig. 2d,e). These findings suggest that, although the docking motif per se is not sufficient for binding to MPK3 with an affinity detectable by BLI, it might help the KRAAM motif to bind more tightly to MPK3 and vice versa. Future structural analysis of the MPK3–KiDoK peptide complex will help elucidate the interface and the underlying mechanism.

The highly conserved KRAAM motif is found only in SCRM and SCRM2 and its plant orthologues, reiterating the notion that distinct binding motifs in MAPK substrates are used to elicit unique developmental responses. Furthermore, it is also known that putative kinase interaction motifs in MAPK substrates rarely overlap with other highly conserved domains (for example, the bHLH domain), indicating that diverse MAPK substrates might not compete for the same binding site on MAPKs to specify a unique developmental outcome. Our structural and functional analyses of the MPK6–SCRM KiDoK complex indicate that the simultaneous association of the docking and the KRAAM motifs of SCRM with MPK6 is required to dictate cell fate specification during stomatal development. Whereas it is evident from the surface conservation mapping of AtMPK6 and mammalian MAPK orthologues that the D148 residue of AtMPK6 is not as conserved with mammalian MAPK orthologues (Fig. 4), it is perfectly conserved among Arabidopsis MAPKs (Supplementary Fig. 7d). Conversely, a sequence conservation analysis among CD domain residues of all AtMAPK family members revealed the contact sites to be highly diverse (Supplementary Fig. 7e). These findings explain why the bipartite binding mode is required for SCRM to uniquely interact with MPK6—but not with other MPKs such as MPK4 (Supplementary Fig. 2b)—to specify cell fate during stomatal development.

The scaffolding action of SCRM, as discovered through our structural and functional analyses, has shed light on its post-translational regulation during stomata development. Although wild-type SCRM enables MAPK to interact with SPCH and, consequently, regulate its activity through phosphorylation, scrm-D can no longer serve as a scaffold to facilitate this interaction owing to its R236H substitution, which abolishes interaction with D148 of MPK6 (Fig. 6). A direct ramification is the enhanced stability of SPCH in the scrm-D background, conferring constitutive stomatal differentiation (Fig. 6).

It has been shown that BASL acts as a scaffold protein by directly binding to YDA, and localizes MPK3/6 activity to the cellular cortex within the stomatal lineage cells11,15. While this manuscript was under review, another stomatal-lineage polarity protein—POLAR44—was reported to function as a scaffold to recruit GSK3–BIN2 that suppresses the MAPK cascade so that SPCH levels remain high in the meristemoid mother cells to promote division45. POLAR was under review, another stomatal-lineage polarity protein—POLAR44—was reported to function as a scaffold to recruit GSK3–BIN2 that suppresses the MAPK cascade so that SPCH levels remain high in the meristemoid mother cells to promote division45. Collectively accounting for these discoveries, we propose that multiple actions of different scaffold proteins converge onto the regulation of SPCH activity and accumulation at specific developmental stages—SCRM acting during the initial decision process, POLAR promoting asymmetric division and BASL differentiating the fate of two daughter cells. By serving as a direct scaffolding partner for
SPCH and MPK3/6, SCRM bridges the gap in our understanding of how multiple opposing signalling cues get integrated simultaneously into the highly regulated stomatal differentiation pathway to seamlessly deliver systematic cell-state transitions during plant development.

Methods

Plant materials and growth conditions. All Arabidopsis plants used in this study are from the Columbia (Col-0) background, scrm, scrm2, scrm-D, SCRMpro-GFP, SCRMpro-GFP-crm-D, mpk3, mpk6 and MEK2DD have been reported previously10,21. Transgenic plants expressing the SCRM-KRAAM motif substitutions (SCRMpro::SCRM-KAAAAM, SCRMpro::SCRM-AARAAM, SCRMpro::SCRM-AHAAAM, SCRMpro::SCRM-AAAAAM) as well as the SCRM docking motif deletions (SCRMpro::FLAG-SCRM-Docking and SCRMpro::FLAG-scrm-D::Docking) in scrm and scrm2 backgrounds were generated in this study. Plants were grown at 21°C with a long-day light cycle (16 h light and 8 h dark). The primers used for genotyping and construct generation are listed in Supplementary Table 3.

Plasmid construction and generation of transgenic plants. For the complete list of plasmids generated in this study, see Supplementary Table 3. To generate the MPK6 ΔN terminus for-recombinant fusion protein expression, the coding sequence of MPK6 (85–1188 bp) was amplified using Phusion polymerase and cloned into pGEX-4T-1 using the BamHI and NotI restriction sites. For Y2H assays, the coding sequence of the gene of interest was fused to either the DNA-binding domain of the pGBK7 vector or the activation domain of the pGADT7 vector using restriction sites EcoRI and BamHI. For site-directed mutagenesis, complementary primers with corresponding mutation sites were designed for PCR amplification of the template plasmid using PrimeStar polymerase. The PCR products were then treated with DpnI to digest the parental methylated DNA. The digested PCR products were purified using a Qiagen PCR purification kit and transformed into DH5α chemically competent cells. The mutated transformants were confirmed by sequencing. For BiFC assays, split YFP constructs were generated by cloning the gene of interest into either the 3SS::SPYNE-GW vector21, which contains the N-terminal of EFYF (YPn, 174 amino acids) or 3SS::SPYCE-GW, which contains the C-terminal of EFYP (YPc, 64 amino acids), using the LR Gateway recombination cloning method. The constructs were then transformed into the Agrobacterium strain GV3101 and co-transformed along with the silencing suppressor plasmid p19 into N. benthamiana leaves. To generate transgenic plants, constructs were transformed into the Agrobacterium strain GV3101, and transgenic plants were subsequently generated using the floral dipping method20. The alanein restriction site constructs were transformed into a null allele of SCRM (scrm::1xci-d) using a Ti vector. The scrm::1xci-d construct was transformed into the scrm::1xci-d double mutant background to uncover a subtle phenotypic difference that might be masked by the presence of functional SCRM2 alleles. For this study, we isolated the following numbers of T1 lines (after confirming the phenotypes by confocal microscopy): SCRMpro::SCRM-KAAAAM, 8 lines; SCRMpro::SCRM-AARAAM, 12 lines; SCRMpro::SCRM-AAAAAM, 23 lines; SCRMpro::SCRM-AHAAAM, 6 lines; SCRMpro::FLAG-SCRM-Docking, 17 lines; and SCRMpro::FLAG-scrm-D::Docking, 14 lines. We self-pollinated two to three independent lines for each construct to T1 generation, and used representative homozgyous lines for characterization.

Y2H screen. Y2H screening was performed by Hybrigenics Services (http://www.hybrigenics-services.com). The coding sequences for A. thaliana SCRM (encoding amino acids 207–297, NM_113586.4) were PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA (orientation LexA-bait). The construct was checked by sequencing the entire insert and used as a bait to screen a random-primed cDNA library in A. thaliana one-week-old seedlings that was constructed into pPl6, pB27 and pPl6 were derived from the original pBPl116 and pGADGH12 plasmids, respectively. We screened 158 million interactions using a mating array with YHGX3 (Y187 ade2-1 Xpl-Jaxa kanMX Japlox MATa) and L40 Gal4 (MATa) yeast strains as previously described. Fifty positive clones were selected on a medium lacking tryptophan, leucine and histidine. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5’ and 3’ junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI) using a fully automated procedure. A confidence score (predicted biological score) was attributed to each interaction as previously described; see Supplementary Table 1 for the full list.

Y2H assay. Bait and prey constructs were cotransformed into the yeast strain AH109 using the yeast transformation kit (Frozen-EZ Yeast Transformation II Kit, Zymo Research). Y2H assays were performed using the Matchmaker 3 Two-Hybrid system (Clontech). The resulting transformants with appropriate positive and negative controls were spotted on SD (–Leu, –Trp) plates to check for growth in the absence of selection. The transformants were then spotted on SD (–Trp, –Leu, –His) selection media containing 1 mM 3-amino-1,2,4-triazole (Sigma, A8056). The positive interactors were then scored on the basis of the stringency of the selection.

BIFC assay. BiFC assays were carried out as previously described with minor modifications. Split YFP constructs were generated for SCRM, scrm-D, MPK3, MPK6 and SPCH by cloning them into either the 3SS::SPYFEY and/or the 3SS::SPYCE Gateway recombination vector.19 The constructs were then transformed into the Agrobacterium tumefaciens strain GV3101. Luria–Bertani (LB; 3 ml) medium was inoculated with the Agrobacterium transformants and incubated overnight with gentle shaking at 28°C. The next morning, cultures were spun down at 4,500 rpm for 10 min and resuspended in infiltration buffer (100 μl of 10 mM MES (pH 5.6) and 150 μM acetylsyringone). Bacterial culture densities were adjusted to a final optical density of 0.6 (OD600) of 1.0, and the cell suspensions were incubated at room temperature for 4 h before infiltration. Equal volumes of cultures carrying the corresponding complementary pair of BiFC constructs (YFPn and YFPc) along with a silencing suppressor plasmid–p19 (a gift from D. Baulcombe)—were then coinfiltrated into 3–4-week-old N. benthamiana leaves. The infiltrated leaves were imaged using confocal microscopy two days after infiltration.

Confocal microscopy. Confocal imaging of Arabidopsis seedlings was performed using a Zeiss LSM700 confocal microscope. Cell peripheries were visualized by staining with propidium iodide (Molecular Probes) and imaged by using the following settings: excitation 515 nm, emission 623–642 nm. Confocal imaging for N. Benthamiana leaves was performed using a Leica SP5x confocal microscope simultaneously capturing YFP (using the white-light laser; excitation at 518 nm and emission at 540 nm for YFP) and bright-field channels. The confocal images were linearly adjusted uniformly for brightness and contrast using Photoshop CS6 (Adobe).

Quantitative analysis and statistics. Quantitative analyses of SPCH–GFP, SCRM–GFP and scrm–ΔGFP signal intensities were performed using Imaris v8.1.3 as previously described.22 Specifically, serial z-stack confocal images covering the entire nuclei were subjected to surface rendering in the green channel to capture SPCH–GFP, SCRM–GFP, SCRM–GFP and scrm–ΔGFP expressing nuclei in the three-dimensional space (for each time point). A cut-off value of 0.7 was set for sphericity, which effectively removed objects with non-specific signals. To ensure unbiased, highly stringent analysis of the GFP intensities, signals were restricted such that only those that originated from stomatal precursor cells were used for the analysis, and any GFP signals of SCRM–GFP and scrm–ΔGFP detected from mature guard cells (which had essentially formed by bypassing the effect of NMEK2Δ treatment) were eliminated from the analyses. The GFP signal intensity sum and s.d. for each nucleus was subsequently calculated. Graphs were generated using R ggplot2 as previously described.1 All statistical analyses were performed using R v.3.3.1. See Supplementary Table 5 for exact P values for statistical analysis.

Proteasome inhibitor treatment. Arabidopsis seedlings carrying DEX-inducible NIMEK2Δ (DEX::FLAG-NIMEK2Δ)20 with SCRMpro::GFP-SCRM and SCRMpro::GFP-crm-D were grown in sterile Petri dishes containing water for 4 d, after which 30 μM MG132 (or an equal volume of ethanol for the mock control) and 2 μM SCRM DEX (or an equal volume of ethanol) were added and incubated for 6 h. The tissue was then collected and flash-frozen in liquid nitrogen. Total protein was extracted using extraction buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 100 μM phenylmethylsulfonyl fluoride (PMSF) and 1:100 protease inhibitor cocktail. Total protein (30 μg) was loaded onto an 8% SDS–PAGE gel, after which the proteins were transferred from gel to membrane and probed using an anti-FLAG antibody (Sigma).

Recombinant protein expression and purification. A. thaliana MPK6 encompassing residues 29–305 (MPK6ΔN) was cloned and expressed in Esherichia coli with an N-terminal GST tag and a thrombin cleavage sequence. Initial purification from cell lysate was performed using a glutathione agarose column. After loading and washing the column, the GST tag was removed using an on-column thrombin cleavage reaction to elute MPK6ΔN. Further purification was performed using anion-exchange and gel-filtration columns. The final protein elution buffer consisted of 20 mM Tris pH 8.0, 200 mM NaCl and 5 mM dibromoPFP (DTT).

Crystallization conditions and data collection. MPK6ΔN crystals were grown at 4°C using hanging-drop vapour diffusion. Protein (1.5 μl) was mixed with 1.5 μl mother liquor (0.1 M sodium citrate pH 6.5, 16% PEG 8000) and suspended from a 1:1:1 ratio of ethanol for the reservoir solution. The reservoir solution was replaced at 2% per day. The protein elution buffer consisted of 20 mM Tris pH 8.0, 200 mM NaCl and 5 mM dibromoPFP (DTT).

Crystal structure determination and refinement. The MPK6ΔN structure was solved by molecular replacement using the Phaser-MR program23 in the Phoenix software suite v1.10.1–2155 and PDB 5CI6 as a search model. An initial MPK6ΔN structure was created with AutoBuild and subsequently refined over
successive cycles with Coot v.0.8.6.1 (ref. 19) and phenix.refine20. All structure figures were created using PyMOL v.2.2.0 (https://pymol.org). See Supplementary Table 2 for statistics from crystallographic analysis.

Modelling and docking of SCR peptide to MPK6 crystal structure. The non-terminal missing regions of the MPK6 structure (chain B) were modelled using Modeller v.9.17 (ref. 21). The modelled MPK6 structure was used as an input for Rosetta flexpdpck22 along with the 35-residue-long amino-acid sequence of the SCR peptide for ab initio flexible docking. The initial structure of peptide sequence was modelled using the ab initio modelling protocol of Rosetta software suite v.3 (refs. 23,24). For the initial docking model, constraints were imposed to keep the Cα atoms of the SCR amino acids R121 and K215 within 10 Å of Cα atoms of the MPK6 CD domain conserved residues D333 and D356. Furthermore, residues L217, K218, E221 and L223 of SCR were restrained to be within 10 Å of the Cα atoms of MPK6 residues Y168, N200, H155, L197, H115a and L116 of MPK6. These constraints were determined from previous studies on MAPK–peptide interactions25,26. Finally, the Cα atoms of the SCR-KRAM motif (residues 235–239) were restrained to remain within 10 Å of the MPK6 molecule. For the final models, side chain constraints were used to restrain the SCR residue R213 near MPK6 residues D353 and D356. The side chain of SCR residue K215 was restrained to interact with amino acid E120 of MPK6. Additionally, the side chain of SCR residue R236 was restrained to interact with amino acid D148 of MPK6. The rest of the constraints were similar to the initial run with a shorter distance cutoff of 8 Å.

Surface conservation mapping. FASTA alignments of A. thaliana MPK6 and H. sapiens ERK1, ERK2, ERK5, p38α, p38β and p38γ were generated using Qian Gen Bioinformatics CLC Sequence Viewer v8.0.0. To map these onto our MPK6, p38α and p38γ were generated using δγδ150 nM, 75 nM, 37.5 nM, 18.75 nM, 9.375 nM and 4.6875 nM. All preformed binding buffer used in these experiments contained 1× PBS supplemented with 0.1% BSA. The concentrations of the GST–MPK3 as the analyte in the binding buffer were 1.000 nM, 333.33 nM, 111.11 nM, 37.03 nM, 12.34 nM and 4.11 nM. The concentrations of the GST–MPK6 as the analyte in the binding buffer were 150 nM, 75 nM, 37.5 nM, 18.75 nM, 9.375 nM and 4.6875 nM. All preformed complexes remained stable as suggested by the constant signal during the washing step after loading. There was no binding of the analytes to the unloading probes as shown by the control wells. Binding kinetics to all six concentrations of the analytes were measured simultaneously using default parameters on the instrument. The data were analysed using the Octet data analysis software. The association and dissociation curves were fit with the 1:1 homogeneous ligand model. The kobs (observed rate constant) values were used to calculate Kd with steady-state analysis of the direct binding. See Supplementary Fig. 8 for raw sensorogram data. See Supplementary Table 4 for the exact values for each experiment.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The PDB accession number for the MPK6αN structure reported in this paper is 6DTL. All data generated and/or analysed during the current study are available from the corresponding authors on reasonable request.

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Reporting Summary

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Statistical Parameters

When statistical analyses are reported, confirm that the following items are present in the relevant location (e.g. figure legend, table legend, main text, or Methods section).

| n/a | Confirmed |
|-----|-----------|
| ❑   | ❑ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement |
| ❑   | ❑ An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
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| ❑   | ❑ A description of all covariates tested |
| ❑   | ❑ A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ❑   | ❑ A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
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| ❑   | ❑ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ❑   | ❑ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated |
| ❑   | ❑ Clearly defined error bars |
| ❑   | ❑ State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection |
|-----------------|
| Diffraction data for MPK6&Nt was collected remotely from the Advanced Light Source at Lawrence Berkeley National Laboratory on the BL8.2.1 beamline |

| Data analysis |
|---------------|
| 1. Diffraction data was indexed, integrated and scaled with HKL2000 (Otwinowski and Minor, 1997) |
| 2. MPK6&Nt structure was solved by molecular replacement using the Phaser-MR v2.1 (McCoy et al., 2007) program in the Phenix software suite v1.10.1-2155 (Adams et al., 2010) and PDB Sci6 as a search model. |
| 3. The initial MPK6&Nt structure was created with AutoBuild was refined over successive cycles with Coot v0.8.6.1 (Emsley et al., 2010). |
| 4. All structure figures were created using PyMOL v0.99 (DeLano Scientific LLC (Schrödinger, LLC)) |
| 5. The non-terminal missing regions of the MPK6 structure (chain B) were modeled using Modeler (Sali and Blundell, 1993). |
| 6. Thie modeled MPK6 structure was used as input for Rosetta flexpepdock program (Raveh et al., 2011) along with the 35 residue long amino-acid sequence of the SCRM peptide for ab initio flexible docking. |
| 7. Initial structure of peptide sequence was modeled using the ab-initio modeling protocol of Rosetta Software Suite(Leaver-Fay et al., 2011; Simons et al., 1999). |
| 8. FASTA alignments of A. thaliana MPK6 and H. sapiens ERK1, ERK2, ERK5, p38, p38, p38, and p38 were generated with Qiagen Bioinformatics CLC Sequence Viewer v8.0.0. |
| 9. The alignments were exported into UCSF Chimera v1.11 (Pettersen et al., 2004), developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with support from NIH P41-GM103311. |
| 10. The Chimera MSMS package (Sanner et al., 1996) and POV-Ray v3.6 (Persistence of Vision Pty. Ltd. (2004)) were used to model solvent-excluded molecular surfaces and generate raytraced images. |
| 11. R version 3.3.1 was used to generate Box plots, dot plots and performing statistical analysis. It is available from the website:https://www.r-project.org/ |
| 12. Imaris 8.3.1. was used to quantify nuclear GFP signals. Software is commercially available from BitPlane (https://imarix.onixinst.com/) |
For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
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MPK6 ΔN, PDB ID: 6DTL. X-ray crystallography data collection and refinement statistics supplied as Table S2. Raw data for all BLI experiments supplied as Table S4

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

Life sciences

All studies must disclose on these points even when the disclosure is negative.

Sample size
Sample size was determined based on similar studies in this field.

Replication
For Octet data:
The K.D. value was deemed Not Fittable (N.F.) when any of following conditions were met:
1. The response values of at least 4 of the 6 concentration points used for the protein sample were less than 1nm
2. The intercept value from the equation obtained after curve fitting was negative (Equation of the form: y=m*x+c where m is the slope and c is the intercept, c/m gives the KD Value of a particular sample.)
3. The R2 value is <0.9
The K.D. value was deemed Not Determined (N.D.) when any of following conditions were met:
1. The response value of the highest concentration point is < 0.5nm.
2. The intercept value from the equation obtained after curve fitting was negative (Equation of the form: y=m*x+c where m is the slope and c is the intercept, c/m gives the KD Value of a particular sample.)
3. The R2 value is <0.9
For quantitative analysis of GFP signals, Imaris ver 8.1.3 was used to analyze the maximal projection of extensive Z-stack images with the following conditions:
1. Cut off value for sphericity of 0.7 has been applied to remove non nuclear signals (e.g. background autofluorescence of cell walls and chloroplasts).
2. Unlike SPCH-GFP, GFP-SCRG and GFP-SCRG-0 signals persist even in the differentiated guard cells, which escape the MAPK-mediated inhibition. These cell types were hence masked from the quantitative time-course analysis.

Randomization
Plants for all phenotypic characterizations were randomly chosen among each genotype population.

Blinding
No blinding was performed in this study. This is because blinding requires mixing seeds of different genotypes (mutants and transgenic lines), which could lead to a risk of mislabeling.

Reporting for specific materials, systems and methods
Materials & experimental systems

| Involved in the study | n/a |
|-----------------------|-----|
| Unique biological materials | ☑ |
| Antibodies | ☑ |
| Eukaryotic cell lines | ☑ |
| Palaeontology | ☑ |
| Animals and other organisms | ☑ |
| Human research participants | ☑ |

Methods

| Involved in the study | n/a |
|-----------------------|-----|
| ChIP-seq | ☑ |
| Flow cytometry | ☑ |
| MRI-based neuroimaging | ☑ |

Unique biological materials

Policy information about availability of materials

Obtaining unique materials

All materials used in this study will be made available through the lead contact on the manuscript, Keiko U. Torii (ktorii@u.washington.edu)

Antibodies

Antibodies used

1. Mouse monoclonal anti-HA, Abcam, Cat# ab18181 RRID: AB_444303, Lot#GR291879-2; 1:1000 dilution; clone HA.5
2. Mouse monoclonal anti-Myc, Abcam, Cat# ab32 RRID: AB_303599, Lot#GR206680-B; 1:2000 dilution; clone 9E10
3. Rabbit polyclonal anti-HA, Abcam, Cat# ab9110 RRID: AB_307019, Lot#GR285587-4; 1:5000 dilution
4. Rabbit polyclonal anti-Myc, Abcam, Cat# ab106 RRID: AB_307014, Lot#GR130480-27; 1:200 dilution
5. Mouse monoclonal anti-FLAG, Sigma Aldrich, Cat# F3165 RRID: AB_259529, Lot#GR656236; 1:5000 dilution; clone M2
6. Rabbit polyclonal anti-GFP, Abcam, Cat# ab290 also ENClB1515250 RRID: AB_2533111, Lot#QC215114, 1:10000 dilution; clone C16
7. Mouse monoclonal anti-GFP, Thermo Scientific Fisher, Cat# 33-2600 RRID: AB_2533111, Lot#QC215114, 1:4000 dilution; clone C16
8. Anti-Mouse HRP conjugated secondary antibody, ECL, Cat#NA9310 Ref# 07/2014, 1:2000 dilution
9. Anti-Rabbit HRP conjugated secondary antibody, Cell-Signaling, Cat#7074S Ref# 07/2014, 1:2000 dilution

Validation

1. https://www.abcam.com/ha-tag-antibody-hac5-ab18181-references.html#top-232
2. https://www.abcam.com/myc-tag-antibody-9e10-chip-grade-ab32-references.html#top-0
3. https://www.abcam.com/ha-tag-antibody-chip-grade-ab9110-references.html#top-176
4. https://www.abcam.com/myc-tag-antibody-ab9106-references.html#top-16
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