

# Dual control of nuclear EIN3 by bifurcate MAPK cascades in C<sub>2</sub>H<sub>4</sub> signalling

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**A principal question in MAP kinase (MAPK/MPK) cascade signalling is how similar components dictate different specificity in the information-processing machineries from yeast to humans and plants. In *Arabidopsis*, how MPK3/6 modulates distinct outputs in diverse signal transduction pathways remains elusive. By combining systematic cellular and genetic screens, here we uncover a previously unexpected MKK9–MPK3/MPK6 cascade promoting ethylene-insensitive 3 (EIN3)-mediated transcription in ethylene signalling. The *mkk9* mutant exhibits a broad spectrum of moderate ethylene-insensitive phenotypes, and translocated MKK9 governs nuclear signalling downstream of receptors. Breaking a linear model and conventional MAPK signalling, ethylene inactivates the negative regulator constitutive triple response 1 (CTR1, a Raf-like MAPK kinase kinase (MAPKKK)) to activate the positive MKK9–MPK3/6 cascade. The bifurcate and antagonistic CTR1 and MKK9 pathways are both critical in determining ethylene-signalling specificity through two MAPK phosphorylation sites with opposite effects on EIN3 stability. The results suggest a new paradigm for linking intertwined MAPK cascades to control quantitative responses and specificity in signalling networks.**

Ethylene (C<sub>2</sub>H<sub>4</sub>) was the first example of a gaseous signalling molecule in biological systems, discovered more than a century ago<sup>1</sup>. As a major plant hormone, it controls essential physiological processes, including: germination; root, shoot and flower development; stress, defence and glucose responses; fruit ripening; and senescence<sup>1–5</sup>. Extensive genetic analysis of ethylene signal transduction in *Arabidopsis* has established a linear pathway connecting five receptors<sup>2–7</sup> to a single negative regulator, CTR1 (ref. 8), and two key downstream positive components, EIN2 (ref. 9) and EIN3 (ref. 10). CTR1 encodes a putative Raf-like MAPKKK and interacts with the ethylene response 1 (ETR1) receptor, but its biochemical activity and molecular actions are unclear<sup>8,11,12</sup>. EIN3 and its closely related EIN3-LIKE1 (EIL1) are plant-specific nuclear transcription factors that initiate downstream transcriptional cascades for ethylene responses<sup>10,13–15</sup>. The identification of ethylene receptor, CTR1, EIN2 and EIN3 orthologues in diverse plant species<sup>3</sup> suggests the evolutionary conservation of ethylene signalling. However, it remains unknown how CTR1 functions as a MAPKKK to regulate downstream positive signalling components in the nucleus.

EIN3 interacts with two F-box proteins (EBF1 and EBF2) and is degraded by the 26S proteasome<sup>5,16–19</sup>. Ubiquitin/proteasome-dependent protein degradation mediated by specific F-box proteins of the conserved SCF (SKP1/cullin/F-box protein) E3 ubiquitin ligase complexes has emerged as a universal mechanism in response to multiple plant hormones, including auxin, gibberellin, abscisic acid, jasmonate and ethylene<sup>20,21</sup>. Understanding the distinct upstream signalling pathways that quantitatively control EIN3 and other transcription regulators through F-box protein-mediated degradation is a major interest in biology.

MAPK cascades are pivotal signalling modules controlling diverse signal transduction pathways in eukaryotes. A main question in MAPK cascade signalling has been how similar components control different biological responses<sup>22–27</sup>. In yeast and mammals, the specificity for distinct signalling pathways with shared components is largely determined by scaffolding proteins and specific MAPKs<sup>25–27</sup>. In plants, more complex roles of MAPKs have emerged. For instance,

diverse hormones, stresses, microbial elicitors and developmental processes all activate the same conserved MPK3 and MPK6 in *Arabidopsis* and their orthologues in other plant species<sup>22–24,28,29</sup>. In ethylene responses, genetic and biochemical data present contradicting negative and positive roles of MAPK signalling<sup>8,30–32</sup>. It is unclear whether and how MAPK cascades are involved in the current linear genetic framework of the ethylene signal transduction pathway<sup>3–5</sup>. Adding to the complexity, *Arabidopsis* MPK6 is also shown to phosphorylate and stabilize ethylene biosynthetic enzymes, 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS2/6), to promote ethylene synthesis<sup>33</sup>. Plant genomes encode the most extended families of putative MAPK cascade genes in eukaryotes<sup>22,24</sup>. Combining cellular and genetic screens with genomic information, we have initiated systematic and quantitative analyses of *Arabidopsis* MAPK cascades. Here we report the unexpected discovery of an MKK9–MPK3/6 cascade that positively modulates nuclear EIN3 stability. Remarkably, CTR1 acts as an unconventional MAPKKK, blocking MKK9–MPK3/MPK6 activation and simultaneously enhancing EIN3 degradation by distinct MPK phosphorylation. Our results illuminate new molecular mechanisms for the control of transcription-factor stability by intertwined MAPK cascades to achieve quantitative signalling specificity in eukaryotes.

## Screens for MPKs and MKKs in ethylene signalling

To assess critically the role and molecular identity of MAPK cascades in ethylene signalling, we have initiated an integrative approach by combining molecular, genomic, biochemical and genetic tools. To increase the sensitivity of the MAPK screen, we used *ctr1-1* (*ctr1*) protoplasts with no endogenous CTR1 activity in the background<sup>15,30,31,34–36</sup>. Unexpectedly, constitutively active CTR1 (CTR1a) clearly repressed MPK1, MPK3 and MPK6 activities without altering protein expression based on an immunocomplex MAPK assay<sup>34–36</sup> (Fig. 1a). The result raised a possibility that the putative CTR1 MAPKKK could negatively regulate downstream MAPK activities, which could play a positive role in ethylene signalling<sup>22,30,31</sup>. Because little *MPK1* but abundant *MPK3* and *MPK6* transcripts were detected

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in protoplasts and leaves (Supplementary Fig. 1a), MPK3 and MPK6 are probably the physiological MAPKs in ethylene signalling.

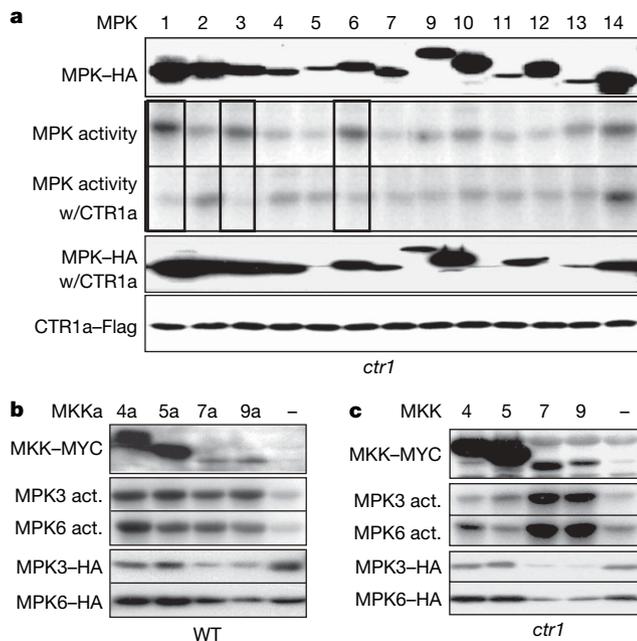
We next attempted to identify the MAPKKs (MKKs) that could activate MPK3/6 in ethylene signalling. There are ten putative *MKK* genes in the *Arabidopsis* genome<sup>22,24</sup>, and all except *MKK8* and *MKK10* are expressed in mesophyll cells. We performed a primary screen to identify constitutively active MKK (MKKa) that could activate co-expressed MPK3/6 in wild-type (WT) protoplasts. The MKKa constructs were generated by site-directed mutagenesis to convert threonine/serine to aspartic acid/glutamic acid mimicking phosphorylation<sup>36</sup> and activation by upstream MAPKKs. In addition to MKK4a and MKK5a<sup>36</sup>, MKK7a and MKK9a activated MPK3/6 based on an immunocomplex MAPK assay (Fig. 1b). Interestingly, overexpressing WT MKK7 and MKK9 specifically and preferentially activated the epitope-tagged MPK3/6 (Fig. 1c) or endogenous MAPKs corresponding to the size of MPK3/6 (Supplementary Fig. 1b) in *ctr1* protoplasts lacking the key negative regulator CTR1. Wild-type MKK4 and MKK5 did not activate MPK3/6 in WT or *ctr1* protoplasts (Fig. 1c and Supplementary Fig. 1c). Although MKK4/5 and MKK7/9 could all activate MPK3/6, they appeared to have differential functions in MAPK signalling networks. The CTR1 MAPKKK could act unconventionally as a direct or indirect negative regulator of the MKK7/9–MPK3/6 cascade.

### MKK9 activates EIN3-mediated transcription in *ctr1*

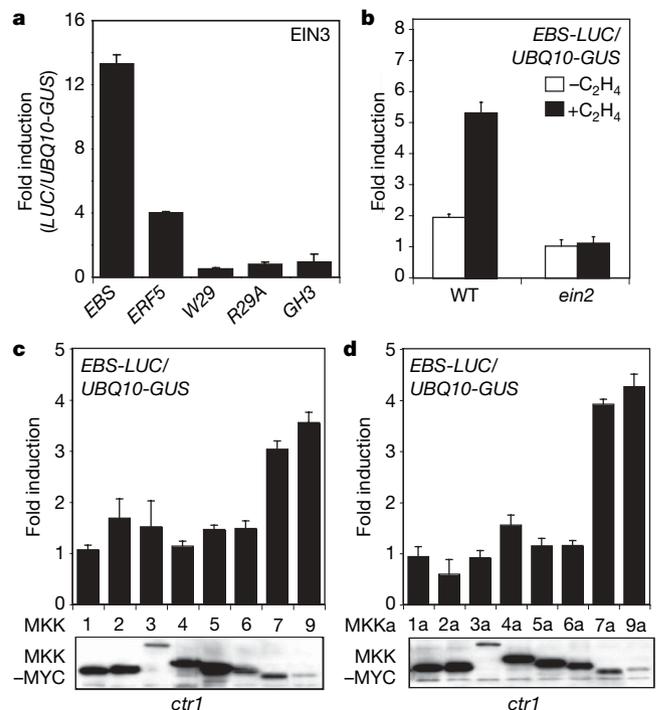
To connect this novel and positive MAPK cascade to the key and specific nuclear transcription factor EIN3 in ethylene signalling, we conducted a second MKK screen using an early ethylene-responsive luciferase (LUC) reporter directly targeted by EIN3 for transcription activation<sup>15</sup>. The reporter carrying four synthetic copies of the defined EIN3 binding site (EBS)<sup>10,13,15</sup> was highly and specifically activated by EIN3 co-expression within 3 h in the transient assay (Fig. 2a). The short timeframe ensured the activation of only the earliest and direct EIN3 target genes and avoided secondary

responses. The promoter of an early ethylene response gene *ERF5*, but not other promoters inducible by flg22 (*WRKY29*)<sup>36</sup>, abscisic acid (*RD29A*)<sup>35</sup> and auxin (*GH3*)<sup>34,35</sup>, was also activated by co-expressed EIN3, confirming the specificity of the assay (Fig. 2a). Furthermore, *EBS-LUC* responded to ethylene in WT but not in ethylene-insensitive *ein2* protoplasts lacking EIN3 (ref. 17) (Fig. 2b). Correlated with the CTR1a repression of MPK3/6 activities (Fig. 1a), CTR1a but not inactive InCTR1<sup>K579M</sup> reduced *EBS-LUC* activity in *ctr1* protoplasts (Supplementary Fig. 1d).

We performed a systematic screen based on quantitative *EBS-LUC* response to identify WT MKKs that could enhance ethylene signalling in *ctr1* protoplasts. In agreement with the specific MPK3/6 activation in *ctr1* protoplasts (Fig. 1c), expression of WT MKK7 or MKK9 uniquely enhanced *EBS-LUC* activity (Fig. 2c). The constitutively active MKK7a or MKK9a displayed even greater ability to activate *EBS-LUC* (Fig. 2d). *Arabidopsis* MKK4a/5a and tobacco NtMEK<sup>DD</sup> activate MPK6, which phosphorylates and enhances cytosolic ACS2/6 stability to elevate ethylene levels<sup>33</sup>. The differential MKK4a/5a–MPK3/6 and MKK7a/9a–MPK3/6 effect on the early ethylene reporter gene activation in *ctr1* suggests signalling specificity downstream of ethylene perception, probably in the nucleus. Because the *ctr1* mutant phenotype is not as severe as *etr1 ers1* or *ebf1 ebf2* double mutants<sup>16,18,19,37,38</sup> and can further respond to ethylene<sup>39</sup>, this positive MAPK cascade may represent an additional regulatory pathway between the receptors and the F-box proteins. Although MKK7 and MKK9 are highly homologous and showed similar activities in the MAPK and reporter activation, the steady-state *MKK9* transcript level was much higher in protoplasts and leaves (Supplementary Fig. 1e). Thus, MKK9 is likely to play a predominant role in ethylene signalling in mesophyll cells. Consistently, the activated MKK9 but not the MKK9 kinase mutant immunoprecipitated from protoplasts could directly phosphorylate *in vitro* GST–MPK3/6



**Figure 1 | Cell-based genetic screens for specific MPKs and MKKs in ethylene signalling.** **a**, Specific MAPKs are activated in the *ctr1* protoplasts but inactivated by CTR1a. MAPK activity and protein expression (MPK–HA or CTR1a–Flag) are shown. The experiments were repeated twice. **b**, Constitutively active MKK4a, MKK5a, MKK7a and MKK9a activate MPK3 and MPK6 in WT protoplasts. **c**, Transient expression of WT MKK7 or MKK9 preferentially increases MPK3 and MPK6 activity (act.) in *ctr1* protoplasts. Empty vector (–) was used as a DNA transfection control. Protein expression (MKK–MYC or MPK–HA) is shown.



**Figure 2 | Specific MKKs modulate EIN3-dependent transcription.** **a**, EIN3 specifically induces *EBS-LUC* and *ERF5-LUC* activities in WT protoplasts. **b**,  $C_2H_4$  (10 p.p.m.) induces *EBS-LUC* activity in WT but not in *ein2-1* protoplasts. **c**, WT MKK7 or MKK9 specifically enhances *EBS-LUC* activities in *ctr1* protoplasts. **d**, Constitutively active MKK7a or MKK9a further enhances *EBS-LUC* activities in *ctr1* protoplasts. Protein expression (MKK–MYC) is shown. Error bars, s.d. ( $n = 3$ ). All experiments were repeated three times with similar results.

produced from *Escherichia coli* without autophosphorylation<sup>36</sup> (Supplementary Fig. 2).

### *mkk9* exhibits diverse ethylene-insensitive phenotypes

To obtain genetic evidence to independently evaluate the surprising and specific positive role of MKK9 in ethylene signalling, we performed quantitative ethylene response screens of *mkk* loss-of-function mutants collected from various transferred DNA (T-DNA) insertion resources<sup>40,41</sup> (Supplementary Figs 3–5 and Supplementary Table 1). The WT and various mutant plants were grown under the same condition and harvested at the same time to minimize plant-to-plant variations. As shown in Fig. 3a, only the etiolated *mkk9* seedlings displayed ethylene insensitivity in hypocotyl similar to *ein3* in the presence of 0.5–1  $\mu$ M of ACC<sup>3–5,10,14,19</sup>. The ethylene-insensitive phenotype was similar in two distinct *mkk9* alleles from different sources (Supplementary Fig. 5). We further examined the specific role of MKK9 in ethylene signalling by comparing the phenotypes of *mkk9-1* and *ein3* in five additional assays<sup>10,15,42,43</sup>. Both *mkk9* and *ein3* displayed similar hypersensitivity

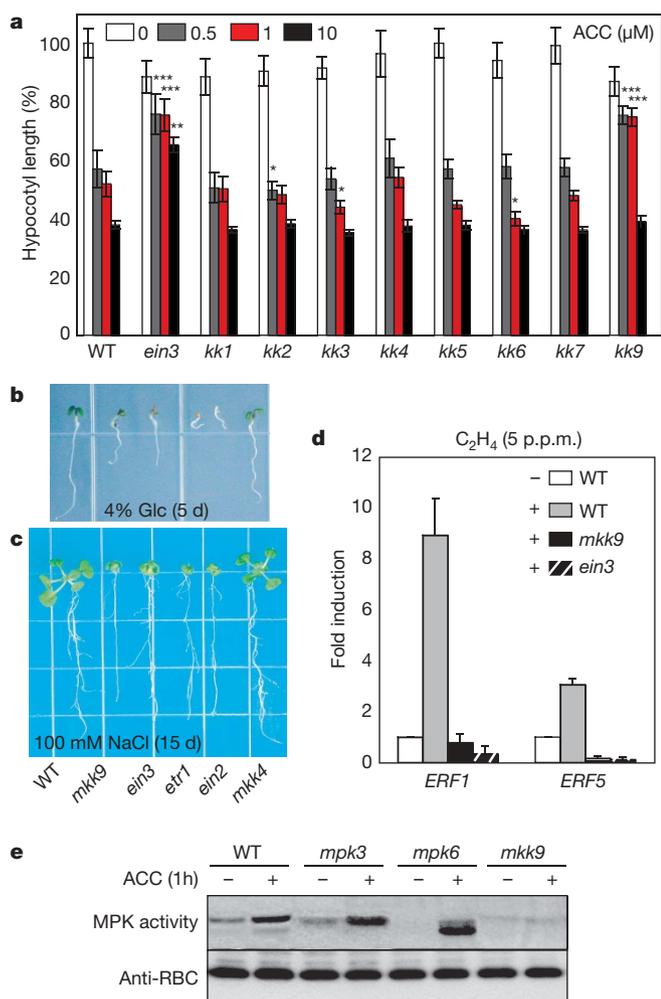
to glucose and salt (Fig. 3b, c). The link between ethylene and glucose or salt sensitivity was further supported by the hypersensitive phenotypes of other stronger ethylene-insensitive mutants, such as *etr1* and *ein2* (refs 42, 43). Both *mkk9* and *ein3* leaves showed little chlorophyll degradation induced by ACC in the dark<sup>10</sup> (Supplementary Fig. 6a), and were relatively resistant to ethylene inhibition of leaf and petiole elongation under light<sup>10</sup> (Supplementary Fig. 6b). Importantly, ethylene activation of immediate early marker genes, *ERF1* and *ERF5*, was abolished in *mkk9* and *ein3* leaves (Fig. 3d). These marker genes are direct EIN3 targets<sup>13,15</sup> (Fig. 2a) and are specific indicators of early ethylene signalling. The inability of *mkk9* to respond to exogenous ethylene in early transcription activation ruled out the possibility that the *mkk9* phenotypes were due to lower endogenous ethylene biosynthesis. The results also provided important evidence to uncouple the MAPK regulation of ethylene signalling from ethylene synthesis.

To assess whether the ethylene insensitivity of *mkk9* is due to the loss of the MKK9-dependent MPK3/6 activation in ethylene signalling, ACC activation of MAPK activity was measured in detached leaves after assay optimization (Supplementary Fig. 7a). Two endogenous MAPKs were activated in WT but not in *mkk9* after ACC feeding through the petiole to minimize mechanical stress during the treatment (Fig. 3e and Supplementary Fig. 7a). Maximal MAPK activities were detected at 1 h because of the time required for ACC uptake and conversion to ethylene *in vivo*. We used the loss-of-function *mpk3* and *mpk6* single mutants<sup>33</sup> to verify that the ACC- and MKK9-dependent activation of MAPKs were indeed MPK3 and MPK6 (Fig. 3e). Our results are consistent with previous independent studies showing that ethylene or ACC activated MAPK<sup>30,31</sup>. Interestingly, MPK6 activation by ACC was elevated in *mpk3*, whereas the activation of MPK3 was greatly enhanced in *mpk6*. The results suggest that MPK3 and MPK6 play redundant roles and can compensate for the loss of each other in ethylene signalling (Fig. 3e). Using the virus-induced gene silencing (VIGS) method<sup>44</sup> to bypass embryo lethality<sup>29</sup>, we showed that the inductions of *ERF1* and *ERF5* were significantly diminished in the *mpk3 mpk6* double mutants (Supplementary Fig. 7b). The combined genetic analyses of *mkk9* and *mpk3 mpk6* mutant plants provide compelling *in vivo* evidence to support their specific roles in ethylene signalling.

### MKK9a promotes constitutive ethylene signalling

To substantiate the role of MKK9 in ethylene signalling, we generated transgenic *Arabidopsis* plants expressing the constitutively active MKK9a in WT or different ethylene-insensitive mutants. Transgenic lines were first screened for equal MKK9a transgene expression in the WT, *etr1* and *ein2* background (Supplementary Fig. 8a) without overt cell death before the phenotypic analyses. MKK9a transgenic plants showed constitutive ethylene phenotypes in the WT background (Fig. 4a, b). Similar ethylene phenotypes have been observed in multiple receptor mutants<sup>7,37,38</sup> or the double mutant *ebf1 ebf2* accumulating EIN3 (refs 16, 18, 19). Moreover, *ERF1* and *ERF5* were highly activated by MKK9a in the transgenic *Arabidopsis* (Fig. 4c).

To place MKK9a in the genetically established ethylene-signalling pathway<sup>2–5</sup>, we analysed the effects of MKK9a in the ethylene-insensitive *etr1* and *ein2* mutants. The MKK9a phenotypes, including etiolated seedling responses and gene activation, were not blocked by *etr1* with little ethylene perception (Fig. 4c, d). The results were consistent with the insensitivity of MKK9a seedling phenotypes to an ethylene receptor antagonist Ag<sup>+</sup> (ref. 17) (Fig. 4a), suggesting that MKK9a acted downstream of the ethylene receptors but not simply enhancing ethylene synthesis. In contrast, the MKK9a phenotypes were diminished in the *ein2* mutant (Fig. 4c, d). A straightforward genetic model would place MKK9 upstream of EIN2, analogous to the common interpretation of the *ctr1 ein2* double mutant<sup>2–5</sup>. However, it is equally possible that the reduced effects of MKK9a could be explained by the absence of EIN3 as the MKK9 cascade



**Figure 3 | The loss-of-function *mkk9* mutant displays diverse ethylene-insensitive phenotypes.** **a**, The *mkk9* and *ein3* seedlings exhibit ethylene insensitivity in hypocotyl elongation at 0.5–1  $\mu$ M ACC. Error bars, s.d. ( $n = 20$ ). Asterisks indicate differences between WT and mutant with statistical significance at \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  ( $t$ -test). **b**, **c**, The seedlings of *mkk9* and other ethylene-insensitive mutants show glucose (**b**) and salt (**c**) hypersensitivity. **d**, Immediate early gene induction by C<sub>2</sub>H<sub>4</sub> (5 p.p.m.) is defective in *mkk9* and *ein3* leaves measured by qRT-PCR. Error bars, s.d. ( $n = 3$ ). **e**, Endogenous MAPKs activated by ACC in WT, *mpk3* and *mpk6* leaves. Leaves were treated with 200  $\mu$ M ACC for 1 h<sup>34–36</sup>. Rubisco (anti-RBC), loading control.

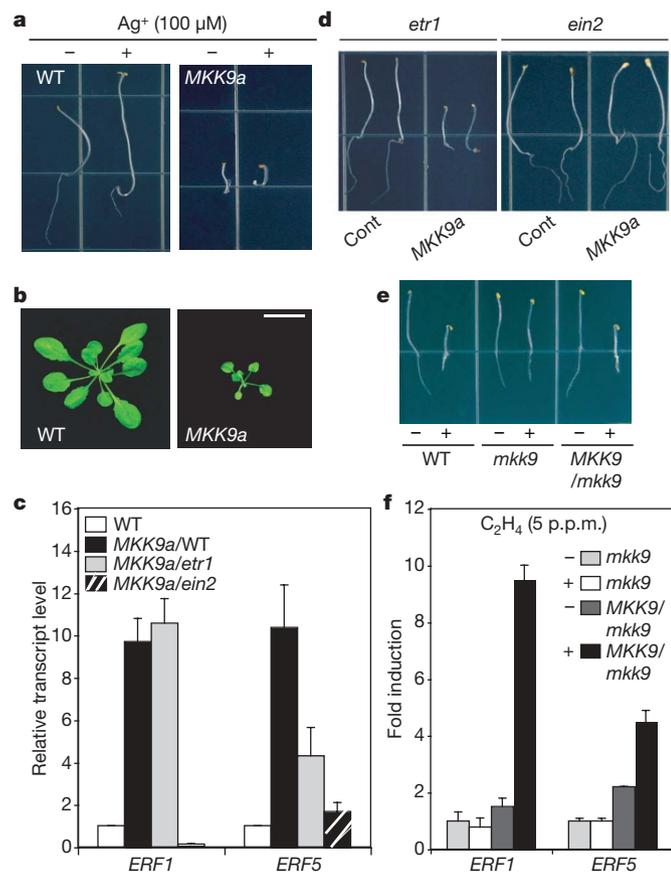
target in the *ein2* mutant<sup>17</sup>, in which EIN3 overexpression could overcome its defect<sup>10</sup>. The gain-of-function analyses of MKK9a in transgenic plants provided additional *in vivo* evidence to support its role in ethylene signalling. The *mkk9* mutant was complemented (Fig. 4e, f) with WT MKK9 (Supplementary Fig. 8b). Although the etiolated *ctr1* and the *ctr1 mkk9* double mutant seedlings looked similar, the elongated light-grown *ctr1* seedling phenotype<sup>9</sup> was diminished in the *ctr1 mkk9* double mutant in the absence or presence of ACC (Supplementary Fig. 9), supporting the idea that MKK9 acted downstream of CTR1.

### Dual phosphorylation modulates EIN3 stability

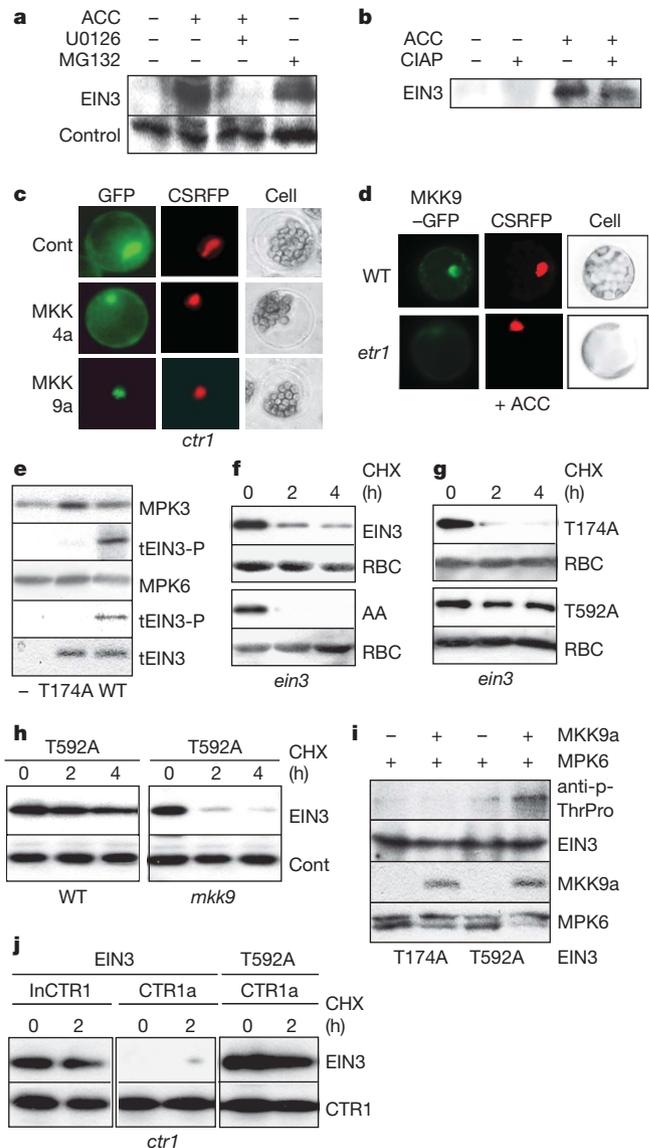
We next examined how MKK9–MPK3/6 cascades might regulate EIN3 in ethylene signalling<sup>15–18</sup>. Interestingly, an MKK-specific inhibitor, U0126, blocked EIN3 accumulation induced by ACC (Fig. 5a), and phosphatase caused EIN3 band shift, suggesting EIN3 phosphorylation in seedlings (Fig. 5b). Constantly, the constitutively active MKK9a–GFP but not MKK4a–GFP preferentially localized in the nucleus (Fig. 5c), whereas MPK6 distributed in both the cytoplasm and nucleus (Supplementary Fig. 10a). Furthermore, the WT MKK9–GFP was translocated into the nucleus in response to ACC in WT but not in *etr1* protoplasts (Fig. 5d), even though total MKK9–GFP protein levels were similar in WT and *etr1* (Supplementary Fig. 10b). Significantly, the immunoprecipitated MPK3 or MPK6 activated by MKK9a in protoplasts could directly

phosphorylate EIN3 *in vitro* (Fig. 5e). These results suggest that direct protein phosphorylation by MKK9–MPK3/6 in the nucleus may be a key step for EIN3 protein stabilization and ethylene signalling.

To map the MAPK phosphorylation site(s) in EIN3 directly, we combined computational analyses and targeted mutagenesis, which were followed by comprehensive cellular and transgenic analyses *in vivo*. Two robust and comprehensive motif search algorithms, Eukaryotic Linear Motif (ELM)<sup>45</sup> and Scansite 2.0 (ref. 46), were used to search for putative MAPK phosphorylation sites and the conserved



**Figure 4 | Constitutively active MKK9a confers ethylene signalling.** **a**, MKK9a confers constitutive ethylene responses. Ag<sup>+</sup> (100 μM). **b**, Adult 28-day-old MKK9a transgenic plants exhibit a constitutive ethylene phenotype. Scale bar, 10 mm. **c**, MKK9a promotes immediate early ethylene gene expression measured by qRT–PCR. Error bars, s.d. (*n* = 3). **d**, MKK9a action is blocked in *ein2* but not in *etr1* seedlings. Control (Cont) *etr1* or *ein2* seedlings do not carry MKK9a. **e**, The *mkk9* etiolated seedlings are complemented by MKK9 (MKK9/*mkk9*). **f**, Induction of ethylene response genes measured by qRT–PCR is restored in the MKK9-complemented lines (MKK9/*mkk9*). Error bars, s.d. (*n* = 3).



**Figure 5 | Dual phosphorylation regulates EIN3 stability.** **a**, EIN3 regulation in seedlings. Tubulin (control). **b**, EIN3 phosphorylated *in vivo*. The EIN3 protein mobility shifts without (–) or with (+) phosphatase (CIAP). **c**, MKK9a–GFP is localized in the nucleus. Nuclear RFP marker<sup>36</sup> (CSRFP). MKK4a–GFP is localized in both the cytoplasm and nucleus as GFP (cont). **d**, MKK9–GFP is translocated into the nucleus by ACC treatment in WT but not in *etr1*. **e**, MPK3 and MPK6 directly phosphorylate T174 in a truncated EIN3 protein *in vitro*. tEIN3 (ref. 15), amino acids 141–352. MPK3/6 (anti-HA) and tEIN3 (Coomassie blue staining) protein levels are shown. **f**, The EIN3<sup>AA</sup> mutant shows enhanced protein degradation. RBC as control. **g**, Opposite effects of two phosphorylation sites on EIN3 stability. h, Enhanced EIN3<sup>T592A</sup> stability requires MKK9. **i**, T174 in EIN3 is phosphorylated *in vivo* by MKK9a-activated MPK6. Immunoprecipitated EIN3 was examined with a p-Thr-Pro-specific antibody. **j**, Active CTR1a promotes EIN3 degradation through T592 phosphorylation. Inactive InCTR1 is a control.

docking sites for MAPK in EIN3. There were two predictable MAPK phosphorylation sites (174T175P and 592T593P), each coupled with a putative conserved-docking motif nearby, conserved in EIN3 and EIL1 (refs 14, 19). Remarkably, the predicted MAPK conserved-docking motif (amino acids 244–252) near 174T was mutated in the *ein3-3* mutant (K244D) that diminished EIN3 function<sup>10</sup>. In an *in vitro* assay, the T174A mutation prevented EIN3 phosphorylation by activated MPK3/6 (Fig. 5e).

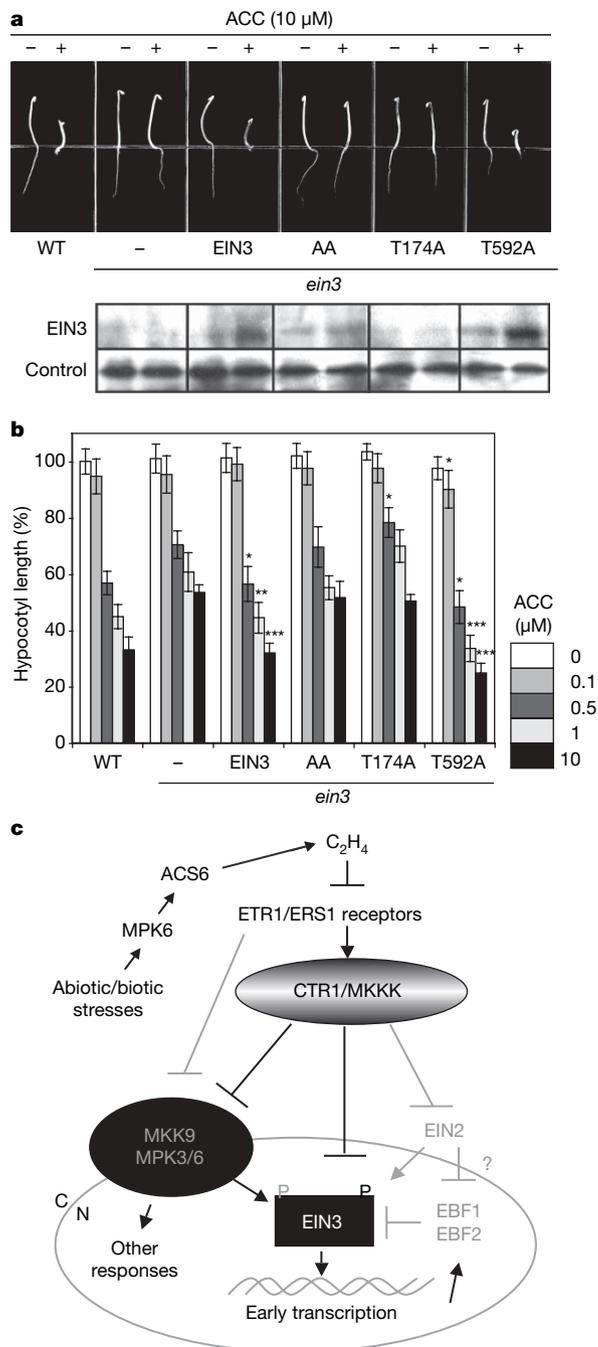
To determine the *in vivo* function of T174 and T592 in ethylene signalling, we generated the EIN3<sup>T174A/T592A</sup> (EIN3<sup>AA</sup>) mutant, and the stability of EIN3 and EIN3<sup>AA</sup> proteins was monitored after cycloheximide (CHX) treatment in the transfected *ein3* protoplasts. The non-phosphorylatable EIN3<sup>AA</sup> mutant always degraded faster than the WT EIN3 protein (Fig. 5f). Unexpectedly, when the individual phosphorylation site mutants (EIN3<sup>T174A</sup> or EIN3<sup>T592A</sup>) were tested with the same assay, we discovered their opposite effects on EIN3 stability. Compared with EIN3 (Fig. 5f), the T174A mutation enhanced EIN3 degradation whereas the T592A mutation enhanced EIN3 stability (Fig. 5g). It was clear that the endogenous MKK9 was required to stabilize EIN3<sup>T592A</sup> (Fig. 5h) in WT protoplasts with some level of constitutive ethylene signalling (Fig. 2b) that could phosphorylate T174. We also showed that the activated MPK6 phosphorylated EIN3<sup>T592A</sup> but not EIN3<sup>T174A</sup> *in vivo* by a specific antibody that recognized a phospho-Thr-Pro motif in protoplasts (Fig. 5i). Finally, CTR1a abolished EIN3 but not EIN3<sup>T592A</sup>, supporting a direct link between T592 phosphorylation and degradation by the CTR1 pathway (Fig. 5j). As CTR1–GFP did not accumulate in the nucleus, the results indicated that the CTR1 pathway probably acted through MAPKs (Fig. 1a) to phosphorylate T592 and promotes EIN3 degradation in the nucleus. We proposed a model that the MKK9 cascade phosphorylates T174 to promote EIN3 stability, whereas T592 is phosphorylated by a MAPK pathway mediated by CTR1 to promote EIN3 degradation.

To test our model in plants further, we generated and examined *ein3* transgenic plants complemented with WT EIN3, EIN3<sup>AA</sup>, EIN3<sup>T174A</sup> and EIN3<sup>T592A</sup>. For informative comparisons and to avoid the dominant effect of EIN3 overexpression<sup>10</sup>, we selected multiple transgenic lines for each construct with similar levels of EIN3 expression as the endogenous EIN3 in WT (Supplementary Fig. 11). Consistent with the results obtained using the cellular assays, EIN3<sup>AA</sup> and EIN3<sup>T174A</sup> transgenic lines were insensitive to saturating ACC (Fig. 6a). On the contrary, the EIN3<sup>T592A</sup> lines exhibited ACC hypersensitivity. EIN3 protein accumulation patterns reflected the transgenic phenotypes (Fig. 6a). Detailed quantification of ACC dose responses confirmed that the transgenic EIN3<sup>T174A</sup> lines were more insensitive to ACC and the transgenic EIN3<sup>T592A</sup> lines were hypersensitive to ACC (Fig. 6b). The fact that the EIN3<sup>T592A</sup> lines only displayed weak constitutive ethylene phenotypes in the absence of ACC strongly supports the model that EIN3 phosphorylation on T174 by MKK9–MPK3/6 is activated by ethylene signalling and is critical for its stabilization even in the absence of T592 phosphorylation, which enhanced EIN3 degradation (Fig. 5g, j). Both the inhibition of CTR1 and activation of MKK9 are required for ethylene signalling specificity. The data explain why *mkk9* exhibited moderate ethylene-insensitive phenotypes (Fig. 3) and escaped classical mutant screens, as only one of the bifurcate pathways (Fig. 6c) was inactive. The stronger constitutive ethylene signalling phenotypes in *ctr1* are partly attributed to the activation or de-repression of the MKK9–MPK3/6 cascade revealed in *ctr1* protoplasts (Figs 1 and 2).

## Discussion

Signalling specificity is fundamental to proper function of regulatory pathways in eukaryotic cells. A common theme in the evolutionarily conserved MAPK cascade signalling is the use of the same components in different signal transduction pathways. Many strategies can maximize the functions of a limited set of MAPK cascade modules for different biological responses and processes, including different

partners in distinct cell types, subcellular compartmentalization, response amplitude and duration, temporal separation, and the use of scaffolding proteins<sup>22,25–27</sup>. In *Arabidopsis*, the confounding effects of MAPKs on both ethylene synthesis and signalling, as well as other stress and defence responses and diverse developmental processes,



**Figure 6 | Analysis of EIN3 mutants in transgenic *ein3* plants.** **a**, Ethylene sensitivity assay. EIN3 protein accumulation in the transgenic lines is shown. Histone (control). **b**, Quantitative analyses of hypocotyl elongation of transgenic lines. Error bar, s.d. ( $n = 20$ ). Asterisks indicate differences between *ein3* and transgenic *ein3* plants with statistical significance at  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$  ( $t$ -test). **c**, Model of the bifurcate MAPK cascades in ethylene signalling. The two EIN3 phosphorylation sites (P) with opposite functions are marked. Without ethylene, CTR1 directly or indirectly inactivates MKK9–MPK3/6 and probably activates downstream MAPKs to phosphorylate T592 to promote EIN3 degradation. Ethylene inactivates CTR1 for MKK9–MPK3/6 activation and T174 phosphorylation to stabilize EIN3. Arrow and blunt ends indicate positive and negative regulations, respectively. ACS, ACC synthase; MKKK, MAPKKK; C, cytoplasm; N, nucleus.

impose great challenges in defining MAPK signalling specificity<sup>22–24,28–36</sup>. We provide compelling evidence for the identification of a novel MKK9–MPK3/6 cascade that phosphorylates and stabilizes EIN3 in ethylene signalling. The MKK9–MPK3/6 cascade targets EIN3 in the nucleus to distinguish temporarily and spatially its positive ethylene signalling function from the MKK4/5–MPK6 activity on ACS6 for ethylene biosynthesis in the cytoplasm<sup>33</sup>. Further biochemical and genetic analyses reveal the surprising opposite functions of the dual EIN3 phosphorylation sites, T174 for stabilization and T592 for degradation. The simultaneous activation of the MKK9 cascade and the inhibition of the CTR1 pathway specify quantitative control of EIN3 levels and EIN3-mediated transcription. CTR1 as a unique MAPKKK appears to control bifurcate and antagonistic MAPK cascades targeted to the same key nuclear transcription factor in ethylene signalling (Fig. 6c). The flexible and quantitative mechanism can facilitate the molecular connections in a complex signal network modulated by hormonal, metabolic and environmental signals to serve an adjustable and adaptive lifestyle characteristic of plants. The involvement of CTR1 in auxin, gibberellin and glucose responses<sup>15,47</sup> suggests that the CTR1 pathway may integrate other signals, and can be involved in phosphorylation and control of other transcription factors in distinct signalling pathways without the MKK9 partnership. It is also possible that the MKK9–MPK3/6 cascade can be modulated by signals other than ethylene and can participate in multiple stress and defence responses independent of CTR1 (Fig. 6c). Similar systematic and integrated approaches used in this study may be applicable for the identification of the subtle and redundant MAPK cascade components presumably acting downstream of CTR1. How CTR1 regulates EIN2 and inhibits MKK9–MPK3/6, whether other MAPKKKs activate MKK9, and whether EBF1 and EBF2 are also regulated by MAPK cascades, remain to be determined (Fig. 6c). There are over 100 putative MAPK cascade genes in *Arabidopsis*, and many are conserved in agriculturally important plants<sup>24,48</sup>. It will be interesting to elucidate how the positive and negative functions of MAPK cascades interact in the signalling networks modulated by a myriad of internal and external signals to govern essential biological processes<sup>22–27</sup>.

## METHODS SUMMARY

**Protoplast transient assays.** Transient expression and MAPK activity assays were performed in *Arabidopsis* mesophyll protoplasts as described<sup>15,35,36,49</sup>. Subcellular localizations of MKK4a–GFP, MKK9a–GFP and ACC-treated MKK9–GFP were observed in transfected protoplasts by fluorescent microscopy<sup>15,36,49</sup>. Protein stability assays were conducted using WT and mutant EIN3 proteins as described<sup>15</sup>. Transfected *ein3* protoplasts were incubated for 4 h before treatment with 10  $\mu$ M CHX to stop *de novo* protein synthesis. EIN3 protein degradation was visualized by immunoblot analysis.

**MPK and MKK screens.** The MKK9–MPK3/6 module was identified by sensitized screens with epitope-tagged MPKs and MKKs by using immunocomplex MAPK activity<sup>35,36</sup> and the ethylene-specific *EBS-LUC* reporter<sup>15</sup> assays in *ctr1* protoplasts<sup>8,49</sup>.

**The *mkk* mutant screens.** The loss-of-function *mkk* mutants were identified by standard methods<sup>40,41</sup> and screened for the ethylene-specific hypocotyl response using etiolated seedlings free of other stresses<sup>10,14</sup>.

**Gene expression analysis.** Quantitative polymerase chain reaction with reverse transcription (qRT–PCR) was used with gene-specific primers for *ERF1* and *ERF5* in WT, mutants and transgenic lines.

**Transgenic plants.** The *mkk9-1* mutant was complemented with a WT MKK9 genomic construct. The gain-of-function *MKK9a* transgenic lines were generated in WT, *etr1-1* and *ein2-1* background and used for epistasis analyses. The WT and various EIN3 mutants were introduced into *ein3* plants. Multiple transgenic lines were selected with equal transgene expression for analyses of ethylene response.

**EIN3 *in vivo* phosphorylation.** We examined EIN3 accumulation and phosphorylation induced by ACC (100  $\mu$ M) with etiolated seedlings in the absence or presence of an MKK inhibitor (U0126, 10  $\mu$ M), a proteasome inhibitor (MG132, 50  $\mu$ M) and/or a general phosphatase (calf intestine alkaline phosphatase) using protein blot analyses with a specific EIN3 antibody<sup>15</sup>. We detected *in vivo* EIN3 phosphorylation by activated MPK6 in protoplasts with protein blot analysis

using a p-Thr-Pro-specific antibody and the EIN3 phosphorylation mutants EIN3<sup>T174A</sup> and EIN3<sup>T592A</sup>.

**Full Methods** and any associated references are available in the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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## METHODS

**Plasmid constructs.** The reporter constructs, *EBS-LUC (EBS)*<sup>15</sup>, *WRKY29-LUC (W29)*<sup>36</sup>, *RD29A-LUC (R29A)*<sup>35</sup> and *GH3-LUC (GH3)*<sup>34</sup>, have been described previously. The 3.9 kilobases (kb) of the *ERF5* (At5g47230) promoter were amplified by PCR and fused to the *LUC* gene to create the *ERF5-LUC* reporter construct. All effector constructs were generated by inserting the complementary DNA (cDNA) between the *35S4PPDK* promoter and the *NOS* terminator in a plant expression vector for protoplast transient assays<sup>15,34–36,49</sup> or a mini-binary vector pCB302 or pBIN19 for transgenic plant analysis<sup>15</sup>. All inserts were verified by DNA sequencing.

**Effector constructs.** The coding regions of MPKs, MKKs and MKKKs were tagged at carboxy (C) termini with double HA, double MYC and single Flag epitopes, respectively.

The effectors used in the experiment were MPK1 (At1g10210), MPK2 (At1g59580), MPK3 (At3g45640), MPK4 (At4g01370), MPK5 (At4g11330), MPK6 (At2g43790), MPK7 (At2g18170), MPK9 (At3g18040), MPK10 (At3g59790), MPK11 (At1g01560), MPK12 (At2g46070), MPK13 (At1g07880), MPK14 (At4g36450), MKK1 (At4g26070), MKK2 (At4g29810), MKK3 (At5g40440), MKK4 (At1g51660), MKK5 (At3g21220), MKK6 (At5g56580), MKK7 (At1g18350) and MKK9 (At1g73500)<sup>24</sup>. Constitutively active forms of MKKs were generated by changing T or S in the activation domain of [T/S]XXXXX[S/T] to D or E by PCR-based site-specific mutagenesis<sup>36</sup>. MKK1a (T218E, S224D), MKK2a (T220D, T226E), MKK3a (S235E, T241D), MKK4a (T224D, S230E), MKK5a (T202E, S208E), MKK6a (S221D, T227E), MKK7a (S193E, S199D) and MKK9a (S195E, S201E) were used in the experiments. The active CTR1a and inactive InCTR1 constructs (At5g03730) are the same as previously described<sup>15,36</sup>. The EIN3 WT and mutant (T174A, T592A, and T174A and T592A as AA) constructs were generated similarly.

***Arabidopsis* mesophyll protoplast transient expression assay.** Protoplast isolation and transient expression assays were performed as described<sup>15,34–36,49</sup>. For ethylene treatment, protoplast samples in culture dishes were placed in air-tight transparent containers with or without 10 p.p.m. ethylene (Alltech) for 6 h. Protoplasts were incubated in a minimal volume of medium (0.6 mm in depth) to facilitate ethylene gas perception. All protoplast transient assays were performed with *UBQ10-GUS* as an internal control<sup>15,34–36,49</sup>. All reporter activities were calculated based on *LUC/GUS* ratio and normalized to the values obtained without the treatment or effector expression. Subcellular localizations of activated MKKs were determined by microscopic observation of protoplasts co-transfected with MKK4a–GFP, MKK9a–GFP or GFP (Control) with a nuclear RFP marker<sup>36</sup>. Protoplast experiments were repeated at least three independent times with consistent results and presented as means with standard deviations. Protein expression was examined by immunoblot analysis using commercially available monoclonal antibodies to HA (Roche), MYC (Roche), GFP (Roche), Flag (Sigma), tubulin (Sigma), MPK3 (Sigma), histone H1 (Upstate) and phospho-threonine-proline (Cell Signaling), a polyclonal antibody to EIN3 (anti-EIN3)<sup>15</sup>, RBC (anti-RBC)<sup>15</sup> or MPK6 (anti-MPK6) generated against the synthetic peptide LIYREALAFNPEYQ.

**Protein kinase assays.** MAPK *in-gel* kinase and immunocomplex MAPK assays were performed with myelin basic protein as a general substrate as described<sup>34–36</sup>. Epitope-tagged MAPKs were immunoprecipitated from lysates of the

transfected protoplasts with the corresponding antibodies and analysed with [ $\gamma$ -<sup>32</sup>P]ATP and myelin basic protein<sup>34–36</sup>. Experiments were repeated at least three times with consistent results. The expression of CTR1a was not sufficient to reveal overt activation of co-expressed MAPKs in WT protoplasts, and *ctr1* protoplasts were used for MAPK screens. The MAPK repression by CTR1a required its protein kinase activity, as the kinase-dead inactive InCTR1<sup>K579M</sup> (ref. 15) failed to show similar activity when expressed at the same protein level. MAPK assays were repeated at least five independent times with consistent results, and representative results are presented. As shown previously<sup>30,31</sup>, we confirmed that the same ACC treatment did not activate any endogenous MAPKs in the dominant ethylene receptor *etr1-1* mutant with little ethylene perception (data not shown).

**RNA isolation and transcript measurement.** Total RNA was isolated by the Trizol method (Invitrogen) and 1  $\mu$ g of total RNA was used for cDNA synthesis. Quantitative PCR was performed with iQ SYBR Green dye-added PCR mix according to the manufacturer's instruction (Bio-Rad). *Tubulin4* (At1g04820) or *elongation initiation factor4a (ELF4a)*, At3g13920 transcript was used as an RT-PCR control with gene-specific primers. Experiments were repeated three times with consistent results. The primer sequences are provided in Supplementary Table 1. Each primer set was pre-tested by PCR for a single gene product. Microarray data of *MPKs* and *MKKs* transcript levels in adult leaves were collected by Genevestigator<sup>50</sup>.

**Transgenic and mutant plant analyses.** *Arabidopsis* transgenic plants were generated with WT (Col-0), *etr1 (etr1-1)*, *ein2 (ein2-1)* and *ein3 (ein3-1)* by the floral dipping method as described before<sup>15</sup>. Transgene expression levels were measured by RT-PCR using gene-specific forward primer (*MKK9\_f*) and a transgene specific reverse primer (*Ts\_r*). Multiple independent transgenic lines were generated and analysed for consistency. We analysed the phenotypes of etiolated seedlings with at least two independent lines of T2 or T3 generations. The *mpk3*, *mpk6* and *mkk* mutants were identified from the Salk and Syngenta SAIL T-DNA insertion collections<sup>40,41</sup> (Supplementary Figs 3–5). The primer sequences are provided in Supplementary Table 1. The growth response assays of etiolated seedlings were performed with silver ion (Sigma) or ACC (Sigma) in the complete dark for three or four days. Seeds were germinated on MS agar (1% sucrose) media after four days of cold treatment to ensure uniform germination<sup>17</sup>. For glucose repression assays, seedlings were grown on 4% glucose (Glc) MS medium for five days under constant light (60  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>)<sup>15</sup>. For salt sensitivity assays, seedlings were grown on 100 mM NaCl 0.5  $\times$  MS medium for 15 days under constant light (60  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup>). Total chlorophyll assay was performed with detached mature green leaves (28 days) treated with 1  $\mu$ M ACC for three days in the dark as described<sup>10</sup>. To observe ethylene-induced changes of rosette size, plants were grown for 14 days and then transferred to chambers without or with a daily dose of 5 p.p.m. C<sub>2</sub>H<sub>4</sub> for a week. Transcript levels of immediate early genes were measured by qRT-PCR with RNA extracted from leaves treated with 5 p.p.m. C<sub>2</sub>H<sub>4</sub> for 1 h. Molecular and phenotypic analyses were repeated at least three times with similar results.

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