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# A MAPK Cascade Downstream of ERECTA Receptor-Like Protein Kinase Regulates *Arabidopsis* Inflorescence Architecture by Promoting Localized Cell Proliferation[C][W]

**Xiangzong Meng,[a] Huachun Wang,[a,1] Yunxia He,[a] Yidong Liu,[a] John C. Walker,[b] Keiko U. Torii,[c] and Shuqun Zhang[a,2]**

[a] Division of Biochemistry, Interdisciplinary Plant Group, Bond Life Sciences Center, University of Missouri, Columbia, Missouri 65211
[b] Division of Biological Sciences, Interdisciplinary Plant Group, Bond Life Sciences Center, University of Missouri, Columbia, Missouri 65211
[c] Howard Hughes Medical Institute and Department of Biology, University of Washington, Seattle, Washington 98195

**Spatiotemporal-specific cell proliferation and cell differentiation are critical to the formation of normal tissues, organs, and organisms. The highly coordinated cell differentiation and proliferation events illustrate the importance of cell–cell communication during growth and development. In *Arabidopsis thaliana*, ERECTA (ER), a receptor-like protein kinase, plays important roles in promoting localized cell proliferation, which determines inflorescence architecture, organ shape, and size. However, the downstream signaling components remain unidentified. Here, we report a mitogen-activated protein kinase (MAPK; or MPK) cascade that functions downstream of ER in regulating localized cell proliferation. Similar to an *er* mutant, loss of function of *MPK3*/*MPK6* or their upstream MAPK kinases (MAPKKs; or MKKs), *MKK4*/*MKK5*, resulted in shortened pedicels and clustered inflorescences. Epistasis analysis demonstrated that the gain of function of *MKK4* and *MKK5* transgenes could rescue the loss-of-function *er* mutant phenotype at both morphological and cellular levels, suggesting that the MPK3/MPK6 cascade functions downstream of the ER receptor. Furthermore, YODA (YDA), a MAPKK kinase, was shown to be upstream of MKK4/MKK5 and downstream of ER in regulating inflorescence architecture based on both gain- and loss-of-function data. Taken together, these results suggest that the YDA-MKK4/MKK5-MPK3/MPK6 cascade functions downstream of the ER receptor in regulating localized cell proliferation, which further shapes the morphology of plant organs.**

## INTRODUCTION

Plant growth and development require constant communication between different cells, tissues, and organs. In eukaryotes, cell–cell communication often involves cell surface receptors/sensors, which trigger protein phosphorylation, a universal signaling mechanism involved in almost all fundamental cellular processes (Cohen, 2000; Manning et al., 2002). Plant receptor-like protein kinases (RLKs) form the largest group of protein kinases in the protein kinase gene family. For instance, *Arabidopsis thaliana* has more than 600 different RLKs (Shiu and Bleecker, 2001). The large number of plant RLKs and the fact that a single RLK can sometimes recognize multiple stimuli allow plants to use RLKs to sense a diverse array of extracellular signals (reviewed in Yin et al., 2002; Morris and Walker, 2003; Diévart and Clark, 2004; Morillo and Tax, 2006; De Smet et al., 2009). At present, we have functional information on only a few RLKs. They are involved in sensing/recognizing plant hormones, peptide ligands, and pathogen-derived molecules (Becraft, 2002; Diévart and Clark, 2004; Vert et al., 2005; Matsubayashi and Sakagami, 2006; De Smet et al., 2009; Uchida et al., 2012).

*Arabidopsis ERECTA* (*ER*) regulates several growth/developmental processes including inflorescence architecture, stomatal formation and patterning, and ovule development (Shpak et al., 2003; van Zanten et al., 2009; Pillitteri and Torii, 2012). *ER* has two closely related homologs, *ER-LIKE 1* (*ERL1*) and *ERL2*, which are partially redundant with ER (Shpak et al., 2004; Lee et al., 2012). The role of the ER family receptors in the stomatal development pathway has been well studied (Shpak et al., 2005; Pillitteri and Torii, 2012). After perceiving the secreted peptide ligands, EPF1 and EPF2, the ER family receptors act sequentially to specify stomatal patterning (Hara et al., 2007; Hunt and Gray, 2009; Lee et al., 2012). ER also promotes inflorescence growth by regulating localized cell proliferation. Loss of function of the *ER* gene results in shortened pedicels and clustered inflorescences due to reduced cell proliferation in pedicels (Shpak et al., 2003, 2004; Woodward et al., 2005). Recently, two secreted peptides, EPFL4 and EPFL6, were identified as redundant upstream components of ER-mediated inflorescence growth, possibly as the ligands in the signaling process (Abrash et al., 2011; Uchida et al., 2012). However, the signaling

[1] Current address: Monsanto Company, 700 Chesterfield Parkway West, Chesterfield, MO 63017.
[2] Address correspondence to zhangsh@missouri.edu.
The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Shuqun Zhang (zhangsh@missouri.edu).
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pathways downstream of ER in regulating inflorescence architecture remain unknown at this stage.

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules in eukaryotes. They function downstream of sensors/receptors and convert signals generated at the sensors/receptors into cellular responses (Widmann et al., 1999; Chang and Karin, 2001; Schwartz and Madhani, 2004). Each MAPK cascade is composed of three kinases with MAPK at the bottom tier. Phosphorylation activation of MAPKs is performed by MAPK kinases (MAPKKs), which are in turn activated by MAPKK kinases (MAPKKKs) through phosphorylation. Plant MAPK cascades play important roles in regulating plant growth, development, and responses to stress/defense stimuli (reviewed in Pedley and Martin, 2005; Zhang, 2008; Pitzschke et al., 2009; Rodriguez et al., 2010; Tena et al., 2011). In *Arabidopsis*, there are 20 MAPKs, 10 MAPKKs, and ~60 MAPKKKs (Ichimura et al., 2002; Hamel et al., 2006; Andreasson and Ellis, 2010). Our research has been focused on MPK3 and MPK6, two *Arabidopsis* stress-responsive MAPKs, and their tobacco (*Nicotiana tabacum*) orthologs wounding-induced protein kinase (WIPK), salicylic acid-induced protein kinase (SIPK), and Ntf4 (Zhang and Klessig, 1997, 1998; Ren et al., 2002; Liu and Zhang, 2004; Ren et al., 2006, 2008). In the process of acquiring loss-of-function evidence to support the functions of MPK3 and MPK6 in plant defense/stress signaling, we found that these two MAPKs also play essential roles in several developmental processes, including stomatal patterning, floral organ abscission, and ovule development (Wang et al., 2007; Cho et al., 2008; Wang et al., 2008).

In this report, we provide gain- and loss-of-function genetic evidence to support a MAPK cascade, composed of YODA (YDA), MKK4/MKK5, and MPK3/MPK6, as a key downstream component of the ER receptor in regulating inflorescence architecture. Loss-of-function mutants of *MPK3*/*MPK6* and *MKK4*/*MKK5* phenocopy the *er* mutant, whereas the gain-of-function *MKK4* and *MKK5* transgenes can rescue the *er* mutant at both morphological and cellular levels, demonstrating that the MKK4/MKK5-MPK3/MPK6 module functions downstream of the ER receptor. Furthermore, YDA was shown to be upstream of MKK4/MKK5 and downstream of ER in this signaling pathway. Our previous study demonstrated a key role of the YDA-MKK4/MKK5-MPK3/MPK6 cascade in coordinated cell differentiation leading to proper stomatal formation and patterning (Wang et al., 2007). This study reveals another function of this MAPK cascade downstream of the ER receptor in regulating coordinated local cell proliferation, which shapes the morphology of plant organs.

## RESULTS

### Loss-of-Function *mpk3* and *mpk6* Mutant Plants Phenocopy *er* Mutants

In the process of generating loss-of-function mutants to explore the roles of *MPK3* and *MPK6* in plant defense signaling, we found that *mpk6* single mutant plants showed a weak *er* phenotype with a more clustered inflorescence in comparison to wild-type plants (Columbia-0 [Col-0]) (Figure 1A), whereas *mpk3* single mutant plants did not display this phenotype. As with *er-105*, an *ER* null mutant allele in the Col-0 background (Shpak et al., 2003), the clustered inflorescence of the *mpk6* single mutant was associated with shortened pedicels (Figures 1B and 1C). Consistent with the gene redundancy and dosage effects of *MPK3* and *MPK6* (Wang et al., 2007, 2008), *mpk3*$^{+/-}$ *mpk6*$^{-/-}$ plants exhibited a more compact inflorescence, which was closer to that of the *er-105* mutant. Because the *mpk3 mpk6* double mutant is embryo lethal, we examined another loss-of-function *MPK3*/*MPK6* system, in which a dominant-negative *MPK6*$^{KR}$ mutant protein was expressed in the *mpk3* mutant background (Cho et al., 2008). As shown in Figure 1, *MPK6*$^{KR}$ *mpk3* plants also displayed a clustered inflorescence with shortened pedicels, whereas *MPK6*$^{KR}$ plants did not show alterations in inflorescence morphology. These results indicate that MPK3 and MPK6 have overlapping functions in regulating inflorescence architecture. In this process, MPK6 plays a more important role than MPK3 because mutations in *MPK6*, but not *MPK3*, conferred a more clustered inflorescence.

We also observed a more severely clustered flower phenotype in the *er-105 mpk6* double mutant (Figure 2), an indication that these two components might function in the same pathway in

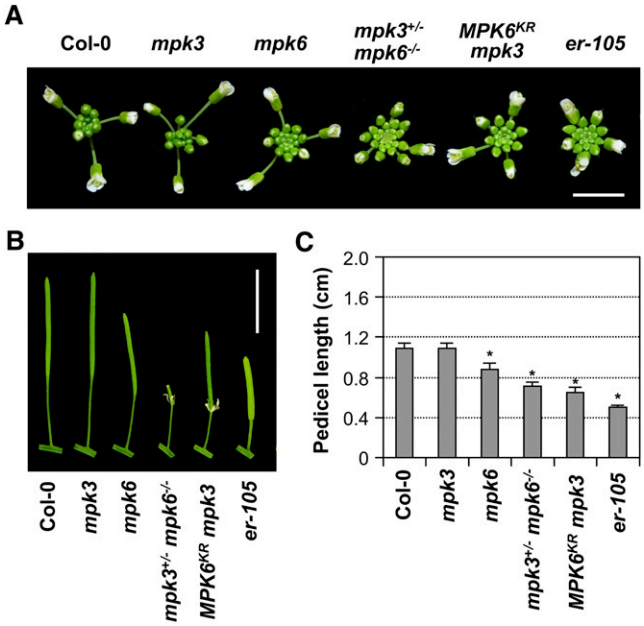


**Figure 1.** Loss-of-Function *MPK3* and *MPK6* Mutant Plants Phenocopy the *er* Mutant in Inflorescence Architecture and Pedicel Length.

**(A)** Top view of Col-0, *mpk3*, *mpk6*, *mpk3*$^{+/-}$ *mpk6*$^{-/-}$, *MPK6*$^{KR}$ *mpk3*, and *er-105* inflorescence. Bar = 0.5 cm.

**(B)** Pedicels of mature siliques of Col-0, *mpk3*, *mpk6*, *mpk3*$^{+/-}$ *mpk6*$^{-/-}$, *MPK6*$^{KR}$ *mpk3*, and *er-105* plants. Bar = 1 cm.

**(C)** Lengths of mature pedicels on the main stems of 7-week-old plants. Bars represent average values + SD ($n$ = 30 to 40 pedicels per genotype). Asterisks above the columns indicate significant differences compared with Col-0 ($P < 0.0001$, Student's $t$ test).

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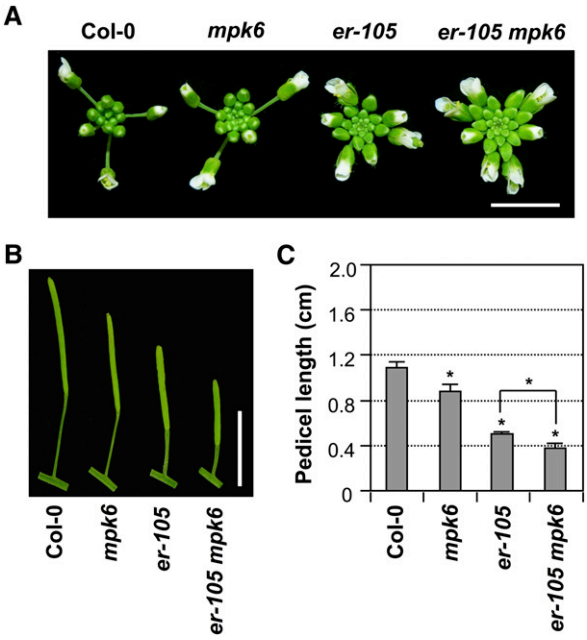


**Figure 2.** Additive Effect of *MPK6* and *ER* in Regulating Inflorescence Architecture and Pedicel Elongation.

**(A)** Inflorescence stem apices of Col-0, *mpk6*, *er-105*, and *er-105 mpk6* plants. Bar = 0.5 cm.

**(B)** Mature siliques and attached pedicels of respective genotypes. Bar = 1 cm.

**(C)** Lengths of mature pedicels on the main stems of ~7-week-old plants. Bars represent average values + SD ($n$ = 30 to 40 pedicels per genotype). Asterisks above the columns indicate significant differences compared with Col-0 or in the marked comparisons ($P < 0.0001$, Student's $t$ test).

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regulating the inflorescence architecture. *ER* has two additional paralogs, *ERL1* and *ERL2*, and the higher order mutants showed more severe phenotypes (Shpak et al., 2004). In the sensitized single *er* mutant background, mutation of additional components such as *MPK6* in the same pathway would result in a more severe phenotype.

### Gain of Function of *GVG*-Nt-*MEK2$^{DD}$* Can Rescue the *er* Mutant Phenotype

Previously, we successfully used *GVG-Nt-MEK2$^{DD}$* transgenic plants (hereafter abbreviated as *DD*) as a conditional gain-of-function MPK3/MPK6 system (Ren et al., 2002, 2008; Wang et al., 2007). Nt-*MEK2* is the tobacco ortholog of *Arabidopsis MKK4* and *MKK5*. In *DD* plants, a constitutively active Nt-*MEK2* variant (Nt-*MEK2$^{DD}$*) driven by a steroid-inducible promoter can activate the endogenous MPK3/MPK6 after dexamethasone treatment (Ren et al., 2002, 2008). We observed that *DD* plants had much longer pedicels than wild-type plants (Figures 3A and 3B), which is opposite to that of the loss-of-function *MPK3/MPK6* plants (Figure 1). This gain-of-function phenotype in *DD* plants is a result of basal level expression of *DD* transgene since steroid application was not required for the phenotype. In the *mpk6* mutant background, the *DD*-mediated gain-of-function phenotype was completely reversed, whereas mutation of *MPK3* had much less effect (Figures 3A and 3B), suggesting again that MPK6 plays a predominant role in this process. These results indicate that gain-of-function activation of MPK3/MPK6 could promote pedicel elongation and affect inflorescence architecture.

To determine if *MPK3/MPK6* function downstream of *ER* in regulating inflorescence structure, we analyzed potential epistatic interactions by crossing the *DD* transgene into the *er-105* background. As shown in Figures 3C to 3E, the shortened pedicel and clustered inflorescence phenotypes of *er-105* were completely reversed in *DD er-105* plants, suggesting that the activation of MPK3/MPK6 by *DD* is sufficient to rescue the loss of *ER* function. Previous studies showed that *ER* regulates inflorescence growth by promoting cell proliferation. The defect of pedicel elongation in *er-105* is due to reduced cell proliferation, notably in the cortex, accompanied by compensatory cell expansion (Shpak et al., 2004; Woodward et al., 2005; Uchida et al., 2012). We observed the same cell proliferation phenotype in the *er-105* mutant (Figures 3G to 3I). Interestingly, the cell size of the pedicel cortex of *DD* plants was similar to that of wild-type plants (Figures 3G and 3H), and the elongated pedicel of *DD* plants was associated with an increase in the cell number (Figure 3I). In *DD er-105* plants, cell numbers in a longitudinal cortical file were significantly increased in comparison with *er-105*, with a concomitant increase in the final pedicel length. These results demonstrate that the activation of MPK3/MPK6 cascade promotes the cell proliferation in pedicels, which results in pedicel elongation and a less clustered inflorescence. More importantly, the rescue of the *er* mutant by a gain-of-function *DD* transgene suggests that the MPK3/MPK6 cascade functions downstream of ER in controlling the same biological process (i.e., localized cell proliferation), which in turn determines inflorescence architecture. Consistent with this conclusion, promoter–β-glucuronidase (GUS) reporter analyses indicated that *ER*, *MPK3*, and *MPK6* are coexpressed in growing inflorescence stems and pedicels (Figure 3F).

### Constitutively Active MKK4 and MKK5, but Not Other MAPKKs, Rescued the *er* Mutant Phenotype

*Arabidopsis* MKK4 and MKK5 were shown to be functionally interchangeable with tobacco Nt-MEK2 in several biological pathways (Ren et al., 2002, 2008; Wang et al., 2007; Han et al., 2010). Several other MAPKKs, including MKK1, MKK2, MKK7, and MKK9, were also reported as upstream kinases of MPK3/MPK6 (Teige et al., 2004; Mészáros et al., 2006; Xing et al., 2008; Xu et al., 2008; Lampard et al., 2009; Popescu et al., 2009). To determine the endogenous MAPKKs downstream of ER in regulating inflorescence architecture in the ER signaling pathway, we conducted experiments by expressing constitutively active MAPKK variants (MAPKK$^{DD}$) under the control of the *ER* promoter (Figure 4A) in the *er-105* background. Besides MKK4 and MKK5, we also tested MKK1, MKK2, MKK7, and MKK9. The use of the *ER* promoter is intended to provide spatiotemporal activation of MPK3/MPK6 related to ER functions.

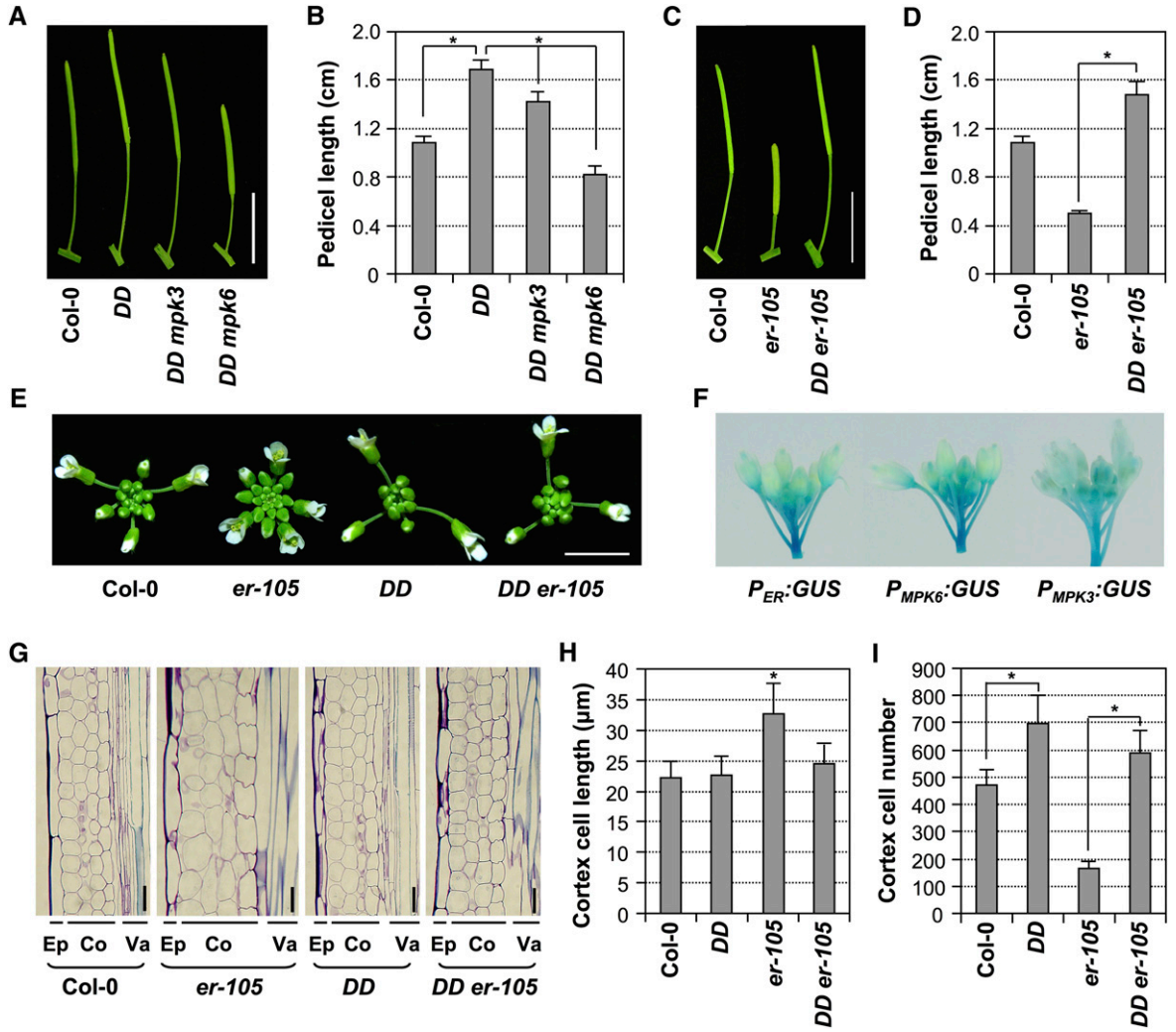



**Figure 3.** Expression of Constitutively Active Nt-*MEK2*$^{DD}$ (*DD*) Rescued the Developmental Defects of the *er-105* Inflorescence by Promoting Cell Proliferation.

**(A)** Elongated pedicel length of *DD* plants is dependent on downstream *MPK3* and *MPK6*. Mature siliques and attached pedicels of Col-0, *DD*, *DD mpk3*, and *DD mpk6* plants. Bar = 1 cm.

**(B)** Lengths of mature pedicels on the main stem of ~7-week-old plants (*n* = 30 to 40 pedicels per genotype).

**(C)** Mature siliques and attached pedicels of Col-0, *er-105*, and *DD er-105* plants. Bar = 1 cm.

**(D)** Lengths of mature pedicels on the main stem of ~7-week-old Col-0, *er-105*, and *DD er-105* plants (*n* = 30 to 40 pedicels per genotype).

**(E)** Inflorescence stem apices of Col-0, *er-105*, *DD* and *DD er-105* plants. Bar = 0.5 cm.

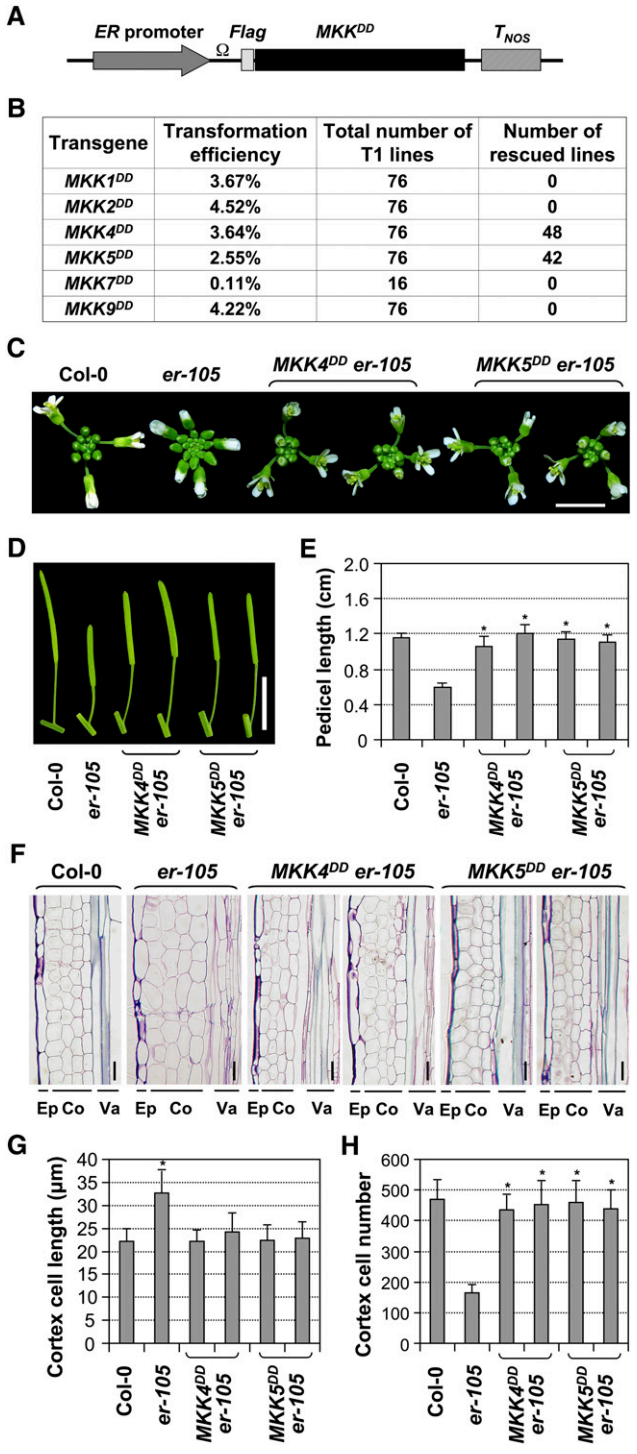
**(F)** $P_{ER}$:*GUS*, $P_{MPK3}$:*GUS*, and $P_{MPK6}$:*GUS* expression patterns in inflorescences.

**(G)** Longitudinal sections of mature pedicels from Col-0, *er-105*, *DD*, and *DD er-105* plants. Co, cortex; Ep, epidermis; Va, vasculature. Bar = 25 μm.

**(H)** Quantitative analysis of cortex cell length (*n* = 15 to 20 pedicels per genotype).

**(I)** Cell numbers in the longitudinal cortex file of a mature pedicel (*n* = 15 to 20 pedicels per genotype). Bars represent average values + SD. Asterisks above the columns indicate significant differences in the marked comparisons or compared with Col-0 ($P < 0.0001$, Student's *t* test).

T1 transgenic plants in *er-105* were screened for the rescue of *er* mutant phenotypes. As summarized in Figure 4B, the typical *er* phenotypes were reversed in a large percentage (40 to 50%) of $P_{ER}$:*MKK4*$^{DD}$ and $P_{ER}$:*MKK5*$^{DD}$ T1 plants, which have longer pedicels and less clustered inflorescences. By contrast, none of the $P_{ER}$:*MKK1*$^{DD}$ and $P_{ER}$:*MKK2*$^{DD}$ T1 plants rescued the *er* phenotypes. A large percentage of $P_{ER}$:*MKK9*$^{DD}$ T1 plants showed defective growth and development associated with localized cell death (see Supplemental Figure 1 online), and no rescued lines were obtained. A very low transformation rate was obtained with the $P_{ER}$:*MKK7*$^{DD}$ construct, and none of the transgenic lines showed rescue of *er* phenotypes. The low transformation rate might be a result of lethality since even basal level expression of *MKK7*$^{DD}$ in the steroid-inducible pTA7002-*MKK7*$^{DD}$ transgenic plants can cause an HR-like lethal phenotype (see Supplemental Figure 2 online). Taken together, our




| Transgene | Transformation efficiency | Total number of T1 lines | Number of rescued lines |
|---|---|---|---|
| *MKK1$^{DD}$* | 3.67% | 76 | 0 |
| *MKK2$^{DD}$* | 4.52% | 76 | 0 |
| *MKK4$^{DD}$* | 3.64% | 76 | 48 |
| *MKK5$^{DD}$* | 2.55% | 76 | 42 |
| *MKK7$^{DD}$* | 0.11% | 16 | 0 |
| *MKK9$^{DD}$* | 4.22% | 76 | 0 |

**Figure 4.** Rescue of the *er* Inflorescence Phenotype by *ER* Promoter–Driven Expression of Constitutively Active *MKK4* and *MKK5*, but Not *MKK1*, *MKK2*, *MKK7*, and *MKK9*.

**(A)** Schematic diagram of *ER* promoter–driven expression constructs for constitutively active *MKKs*. Ω, TMV omega translation enhancer; *MKK$^{DD}$*, constitutively active *MKK* mutants; $T_{NOS}$, NOS terminator.

**(B)** Transformation efficiencies of different *MKK$^{DD}$* constructs in the *er-105* background and frequencies of rescued T1 lines.

data indicate that MKK4 and MKK5, but not MKK1, MKK2, MKK7, or MKK9, function downstream of ER in regulating inflorescence architecture. We further performed more detailed characterization of selected $P_{ER}$:*MKK4$^{DD}$* and $P_{ER}$:*MKK5$^{DD}$* lines in the T2 generation. As shown in Figure 4C, expression of the constitutively active *MKK4* and *MKK5* rescued the clustered inflorescence phenotype of *er-105*, a result of elongated pedicels (Figure 4D). Quantitative measurement showed that both *MKK4$^{DD}$* and *MKK5$^{DD}$* completely rescued the shortened pedicel phenotype of *er-105* (Figure 4E).

Longitudinal sectioning of pedicel tissues revealed that the cortex cells of $P_{ER}$:*MKK4$^{DD}$* and $P_{ER}$:*MKK5$^{DD}$* transgenic lines resembled those of wild-type plants, in contrast with the large and expanded cortex cells in *er-105* (Figures 4F and 4G). In addition, $P_{ER}$:*MKK4$^{DD}$* and $P_{ER}$:*MKK5$^{DD}$* transgenic plants had comparable cell numbers in the cortical cell files as wild-type plants, whereas the cortex cell number was dramatically reduced in *er-105* (Figure 4H). Our data suggest that gain-of-function MKK4/MKK5 could rescue the inflorescence developmental defects of *er-105* at both morphological and cellular levels. In addition to the rescue of *er* phenotypes, *ER* promoter–driven expression of *MKK4$^{DD}$* or *MKK5$^{DD}$* in Col-0 also gave a gain-of-function phenotype with much longer pedicels than wild-type plants (Figures 5A to 5C). The longer pedicels of the gain-of-function transgenic lines were correlated with increased cortex cell numbers (Figure 5D), whereas cortex cell sizes were not significantly different between the transgenic lines and wild-type plants (Figure 5E). These results provide both genetic and cellular evidence that MKK4/MKK5 are downstream of ER in regulating pedicel elongation and inflorescence architecture.

## Loss-of-Function *MKK4*/*MKK5* Plants Phenocopy the *er* Mutant

Gain-of-function data suggest a positive role for MKK4/MKK5 in regulating inflorescence architecture downstream of ER. To provide loss-of-function evidence, we used a tandem *mkk4*/*mkk5* RNA interference (RNAi) construct to silence both genes

**(C)** Inflorescence stem apices of Col-0, *er-105*, and two representative lines each of T2 $P_{ER}$:*MKK4$^{DD}$* and $P_{ER}$:*MKK5$^{DD}$* transgenic plants in the *er-105* background. Bar = 0.5 cm.

**(D)** Mature siliques and attached pedicels of the indicated genotypes. Bar = 1 cm.

**(E)** Lengths of mature pedicels on the main stem of ~7-week-old plants ($n$ = 30 to 40 pedicels per genotype).

**(F)** Longitudinal sections of mature pedicels from Col-0, *er-105*, and two representative lines of T2 $P_{ER}$:*MKK4$^{DD}$* and $P_{ER}$:*MKK5$^{DD}$* transgenic plants in the *er-105* background. Co, cortex; Ep, epidermis; Va, vasculature. Bars = 25 μm.

**(G)** Quantitative analysis of cortex cell length ($n$ = 15 to 20 pedicels per genotype).

**(H)** Number of cells in the longitudinal cortex file of a mature pedicel of the indicated genotypes ($n$ = 15 to 20 pedicels per genotype). Bars represent average values + SD. Asterisks above the columns indicate significant differences compared with Col-0 **(G)** or *er-105* (**[E]** and **[H]**) ($P < 0.0001$, Student's $t$ test).

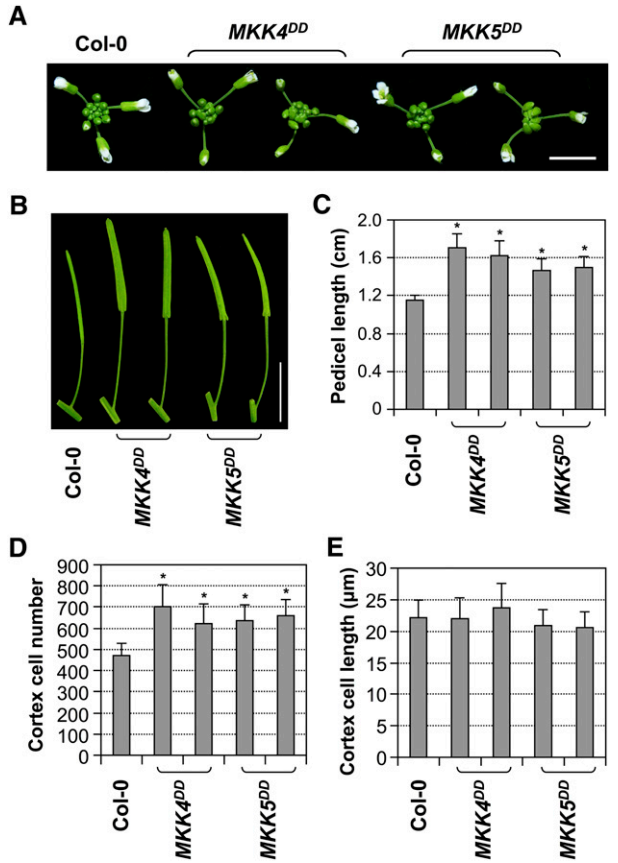



**Figure 5.** *ER* Promoter–Driven Expression of Constitutively Active *MKK4* or *MKK5* Resulted in a Gain-of-Function Phenotype with Elongated Pedicels and Enhanced Cell Proliferation.

**(A)** Inflorescence stem apices of Col-0 and two representative T2 lines each of $P_{ER}$:*MKK4*$^{DD}$ and $P_{ER}$:*MKK5*$^{DD}$ transgenic plants in Col-0 background. Bar = 0.5 cm.

**(B)** Mature siliques and attached pedicels of the indicated genotypes. Bar = 1 cm.

**(C)** Lengths of mature pedicels on the main stem of ~7-week-old plants ($n$ = 30 to 40 pedicels per genotype).

**(D)** Quantitative analysis of cortex cell length ($n$ = 15 to 20 pedicels per genotype).

**(E)** Number of cells in the longitudinal cortex file of a mature pedicel of the indicated genotypes ($n$ = 15 to 20 pedicels per genotype).

Bars represent average values + SD. Asterisks above the columns indicate significant differences compared with Col-0 ($P < 0.0001$, Student's $t$ test).

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because loss-of-function alleles of *MKK4* and *MKK5* are not available in public depositories. Previously, we used this RNAi strategy to demonstrate the function of MKK4/MKK5 as the upstream kinase of MPK3/MPK6 in regulating the stomatal development and floral organ abscission (Wang et al., 2007; Cho et al., 2008). The majority of T1 transgenic plants had severe stomata developmental defects (Wang et al., 2007) and were unable to survive beyond the seedling stage. Among the transgenic plants that survived, we found that the lines with abscission defects also displayed a clustered inflorescence phenotype nearly identical to that of *er-105* (Figures 6A and 6B). There were also lines that had a weak *er* phenotype, but without abscission defects (Figure 6). The pedicels of these *mkk4/mkk5 RNAi* lines were much shorter than those of wild-type plants, reminiscent of *er-105* and opposite of the gain-of-function *MKK4*$^{DD}$ and *MKK5*$^{DD}$ transgenic plants (Figures 6B and 6C). These loss-of-function data further support that MKK4/MKK5 function downstream of ER in regulating pedicel elongation and inflorescence architecture. Based on data from loss-of-function and epistasis analyses, we conclude that MKK4/MKK5 are downstream of ER and upstream of MPK3/MPK6 in regulating localized cell proliferation, which further influences pedicel elongation and inflorescence architecture. MKK4 and MKK5 have been previously reported to be upstream of MPK3/MPK6 in both defense- and development-related processes (Ren et al., 2002, 2008; Wang et al., 2007; Han et al., 2010).

## YDA Is the Upstream MAPKKK of MKK4/MKK5 in the ER Signaling Pathway

We previously showed that YDA, a MEKK1 type of *Arabidopsis* MAPKKK, is upstream of MKK4/MKK5 in regulating stomatal development and patterning (Wang et al., 2007). To determine whether YDA is also upstream of the MKK4/MKK5-MPK3/MPK6 module in regulating inflorescence architecture, we characterized transgenic plants expressing the constitutively active *YDA* mutant (Δ*N-YDA*) driven by its endogenous promoter (Lukowitz et al., 2004). Similar to the gain-of-function *DD*, *MKK4*$^{DD}$, and *MKK5*$^{DD}$ transgenic plants, Δ*N-YDA* plants also displayed elongated pedicels (Figures 7B and 7C). Opposite to the gain-of-function Δ*N-YDA* plants, the loss-of-function *yda* mutant had extremely short pedicels, resulting in a very tightly compact inflorescence, along with other phenotypes such as defective floral organs and sterile plants (Figure 7). We then crossed the gain-of-function *DD* transgene into the *yda* mutant for epistatic interaction analysis. As shown in Figure 7, *DD* partially rescued the *yda* mutant phenotype. Compared with the tightly compact inflorescence of the *yda* mutant with almost no pedicels, *DD yda* plants showed significantly extended inflorescences with much longer pedicels. However, *DD* failed to rescue the sterile phenotype of *yda* (Figure 7B). This epistasis analysis suggests that YDA is upstream of MKK4/MKK5 in regulating pedicel elongation and inflorescence architecture.

To further clarify the genetic relationship between *YDA* and *ER*, we transformed the constitutively active Δ*N-YDA* transgene driven by the native promoter into *er-105*. As shown in Figure 8, expression of Δ*N-YDA* partially rescued the *er* mutant phenotypes and conferred significantly elongated inflorescences and pedicels in comparison to those of *er-105*, suggesting that YDA is a downstream component of ER signaling. The incomplete rescue of the *er-105* mutant phenotype by Δ*N-YDA* suggests the presence of a YDA-independent pathway(s), possibly mediated by other MAPKKKs, in regulating inflorescence architecture. Alternatively, the incomplete rescue of the *er-105* mutant phenotype by Δ*N-YDA* could be a consequence of the lack of N terminus of the gain-of-function *YDA* transgene. Deletion of the large N-terminal domain makes Δ*N-YDA* constitutively active, yet in the meantime, may make it less efficient in

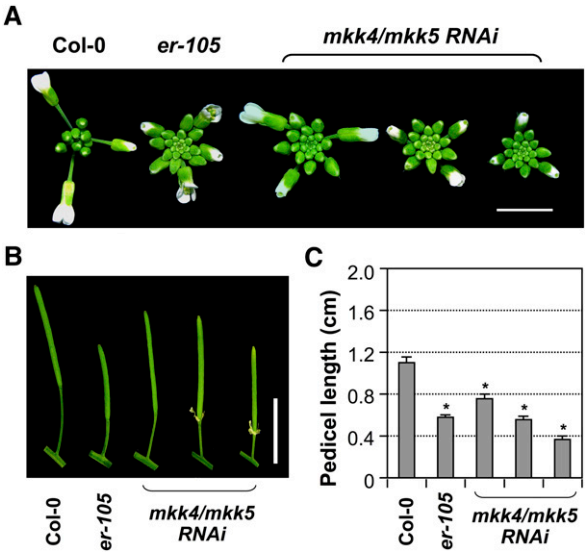


**Figure 6.** Tandem RNAi Suppression of *MKK4* and *MKK5* Resulted in a Clustered Inflorescence and Shortened Pedicels, Phenocopying the *er-105* Mutant.

**(A)** Inflorescence stem apices of Col-0, *er-105*, and three representative T1 *mkk4/mkk5 RNAi* transgenic plants. Bar = 0.5 cm.
**(B)** Mature siliques and attached pedicels of the indicated genotypes. Bar = 1 cm.
**(C)** Lengths of mature pedicels on the main stem of ~7-week-old plants ($n$ = 10 to 15 pedicels per genotype). Bars represent average values + SD. Asterisks above the columns indicate significant differences compared with Col-0 ($P < 0.0001$, Student's $t$ test).
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performing other functions, such as interacting with other components in the signaling pathway, rendering it less effective in rescuing the *er-105* phenotypes. By contrast, gain-of-function MAPKKs were generated by making Ser/Thr-to-Asp point mutations in their activation loop (Yang et al., 2001), which should not alter the functionalities of other domains. Nonetheless, the Δ*N-YDA* transgene is sufficient to confer a gain-of-function phenotype in the wild type (Figures 7B and 7C). In summary, our loss- and gain-of-function genetic data suggest that YDA, MKK4/MKK5, and MPK3/MPK6 function together in a MAPK cascade downstream of the ER receptor in regulating cell proliferation during pedicel growth/development, which determines the inflorescence architecture in *Arabidopsis*.

### Constitutively Active MKK4 and MKK5 Also Rescue the Short Petiole and Dwarf Phenotypes of the *er* Mutant

In addition to short pedicels and the resulting clustered inflorescence phenotype, the *er* mutant also has short leaf petioles, which causes clustered rosettes. Moreover, *er* displays a significant reduction in overall height (i.e., a dwarf phenotype). These organ growth defects of *er* were shown to be a result of reduced cell proliferation as well (Shpak et al., 2004; Woodward et al., 2005; Uchida et al., 2012). In characterizing $MKK4^{DD}$ and $MKK5^{DD}$ transgenic plants, we found that *ER* promoter–driven expression of $MKK4^{DD}$ or $MKK5^{DD}$ also rescued other growth defects of *er-105*, in addition to the shortened pedicels. As shown in Figure 9A, the short petiole phenotype of *er-105* was reversed by both $MKK4^{DD}$ and $MKK5^{DD}$. In addition, the dwarf phenotype of *er-105* was almost completely rescued by $MKK4^{DD}$, whereas $MKK5^{DD}$ partially rescued this mutant phenotype (Figures 9B and 9C). These results suggest that the YDA-MKK4/MKK5-MPK3/MPK6 cascade may function downstream of ER in regulating developmental processes in multiple organs through promoting cell proliferation.

## DISCUSSION

Cell differentiation and spatiotemporal-specific cell proliferation are critical to plant growth and development. ER is among one of the first few RLKs implicated in the regulation of plant growth and development (Torii et al., 1996; van Zanten et al., 2009). Loss of function of *ER* results in a clustered inflorescence and rosette, a result of defective localized cell proliferation (Shpak et al., 2003, 2004; Woodward et al., 2005). Analysis of high-order mutants of *ER*, *ERL1*, and *ERL2* also revealed that they play a critical role in cell differentiation, such as stomatal

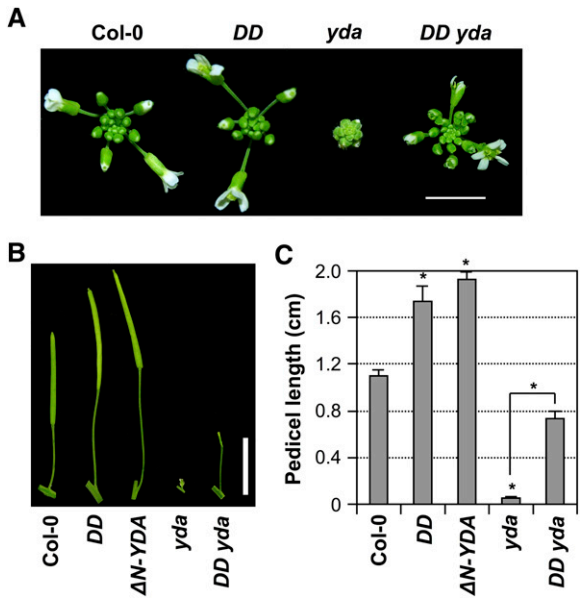


**Figure 7.** YDA Is Upstream of MKK4/MKK5-MPK3/MPK6 in Regulating Inflorescence Architecture.

**(A)** Inflorescence stem apices of Col-0, *DD*, *yda*, and *DD yda* plants illustrating the extremely clustered inflorescence of *yda* and the partial rescue of this mutant phenotype in *DD yda* plants. Bar = 0.5 cm.
**(B)** Mature siliques and attached pedicels of Col-0, *DD*, Δ*N-YDA*, *yda*, and *DD yda* plants, showing the very long pedicel of the Δ*N-YDA* plant, the extremely short pedicel of the *yda* mutant, and the partially rescued pedicel of the *DD yda* plant. Bar = 1 cm.
**(C)** Lengths of mature pedicels on the main stem of ~7-week-old plants ($n$ = 30 to 40 pedicels per genotype). Bars represent average values + SD. Asterisks above the columns indicate significant differences compared with Col-0 or in the marked comparison ($P < 0.0001$, Student's $t$ test).
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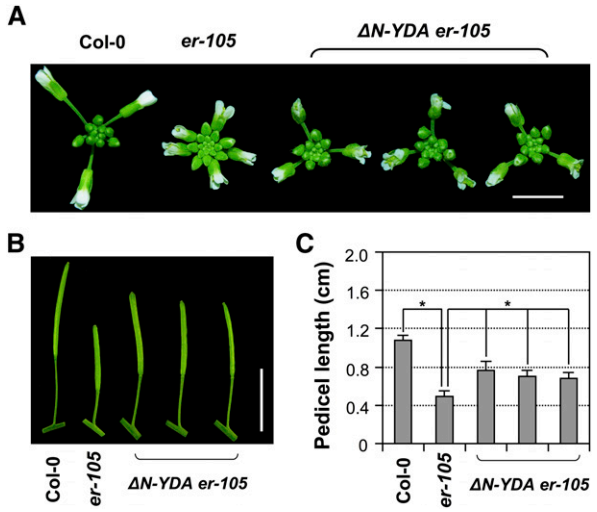


**Figure 8.** Expression of Constitutively Active Δ*N-YDA* Partially Rescued the Inflorescence Developmental Defect of *er-105*.

**(A)** Inflorescence stem apices of Col-0, *er-105*, and three representative T1 lines of Δ*N-YDA* transgenic plants in the *er-105* background. Bar = 0.5 cm.
**(B)** Mature siliques and attached pedicels of the indicated genotypes. Bar = 1 cm.
**(C)** Lengths of mature pedicels on the main stem of ~7-week-old plants ($n$ = 10 to 15 pedicels per genotype). Bars represent average values + SD. Asterisks above the brackets indicate significant differences between the sample group(s) and *er-105* ($P < 0.0001$, Student's $t$ test).
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differentiation (Shpak et al., 2005). Several transcription factors have been shown to be downstream of *ER* and *ERL*s in regulating stomatal formation and patterning (Pillitteri and Torii, 2012). However, the signaling process downstream of ER in regulating cell proliferation is largely unknown. This research reveals that a MAPK cascade functions downstream of ER in regulating coordinated cell proliferation, which plays a critical role in shaping organ size and structure during plant growth and development.

### A Complete MAPK Cascade Downstream of ER in Regulating Inflorescence Architecture by Promoting Cell Proliferation

Loss-of-function mutations of MPK3/MPK6 or their upstream MKK4/MKK5 resulted in an *er*-like inflorescence phenotype (Figures 1 and 6), while expression of constitutively active *MKK4*, *MKK5*, or their tobacco ortholog, Nt-*MEK2*, led to the rescue of the clustered inflorescence phenotype of *er* mutants and the gain-of-function inflorescence phenotype in wild-type plants by promoting cell proliferation (Figures 3 to 5). These data suggest that the MKK4/MKK5-MPK3/MPK6 module functions downstream of ER in regulating inflorescence architecture. Moreover, gain- and loss-of-function genetic data support YDA as the upstream MAPKKK of MKK4/MKK5 in the ER signaling pathway (Figures 7 and 8). Together with the recently identified ER ligands EPFL4 and EPFL6 (Abrash et al., 2011; Uchida et al., 2012), we proposed a signaling pathway that controls inflorescence architecture in *Arabidopsis* from ligands, to receptors, to downstream effectors (Figure 10). After perceiving the peptide ligands, EPFL4 and EPFL6, which are specifically expressed in the stem endodermis, ER activates the downstream YDA-MKK4/MKK5-MPK3/MPK6 cascade, which transduces the signal(s) to components further downstream through the phosphorylation of unidentified MAPK substrates. These events eventually lead to spatiotemporal-specific cell proliferation within the pedicel cortex and thereby determine inflorescence

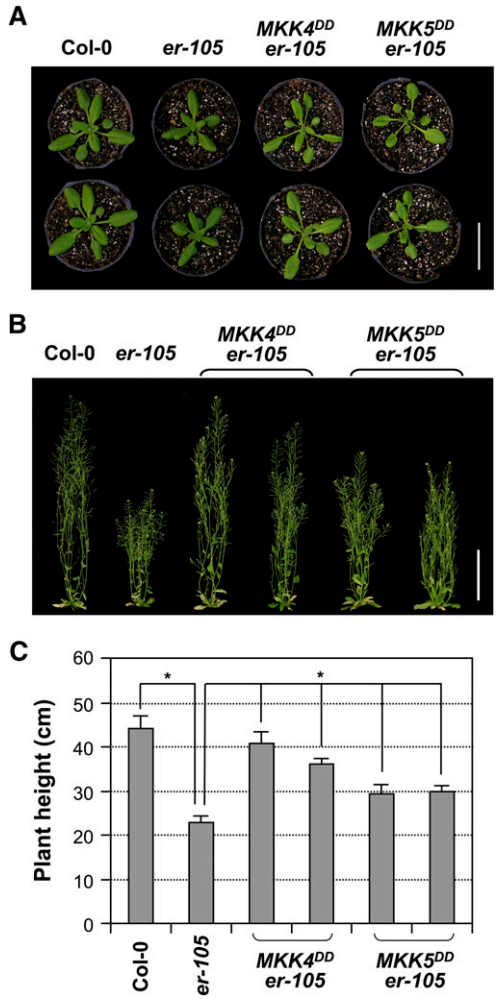


**Figure 9.** Rescue of the Short Petiole and Dwarf Phenotypes of *er-105* by *ER* Promoter–Driven Expression of Constitutively Active *MKK4* and *MKK5*.

**(A)** Four-week-old plants of Col-0, *er-105*, and two representative T2 lines of $P_{ER}$:$MKK4^{DD}$ and $P_{ER}$:$MKK5^{DD}$ transgenic plants in the *er-105* background. Bar = 3 cm.
**(B)** Eight-week-old plants of the genotypes shown in **(A)**. Bar = 10 cm.
**(C)** Plant heights of 8-week-old plants of the genotypes shown in **(A)**. Bars represent average values + SD ($n$ = 10 to 15 plants per genotype). Asterisks above the brackets indicate significant differences between the sample group(s) and *er-105* ($P < 0.0001$, Student's $t$ test).
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architecture. In this MAPK cascade, MPK6 is likely to play a more important role than MPK3 downstream of ER because mutation of *MPK6* in either wild-type or *DD* plants resulted in more severe effects on pedicel elongation and inflorescence architecture (Figures 1 and 3). In addition, the additive effect of *er* and *mpk6* mutations in the *er-105 mpk6* double mutant (Figure 2) suggests that MPK6 might function downstream of multiple ER family receptors in regulating inflorescence architecture.

### MPK3/MPK6 Cascade Functions Downstream of ER Receptors in Regulating Multiple Biological Processes

MPK3/MPK6 and ER/ERL1/ERL2 have been implicated in regulating similar biological functions in *Arabidopsis*. Previously, *mpk3*$^{+/-}$ *mpk6*$^{-/-}$ and *er erl1 erl2*$^{+/-}$ plants were shown to have an ovule development defect due to reduced cell proliferation in the integuments, suggesting that the MPK3/MPK6 cascade might function downstream of ER family receptors in regulating ovule integument development (Pillitteri et al., 2007b; Wang et al., 2008). In this report, we demonstrate that the MPK3/MPK6 cascade functions downstream of ER in regulating inflorescence architecture by promoting cell proliferation in pedicels. We also found that gain-of-function *MKK4*$^{DD}$ and *MKK5*$^{DD}$ could rescue other organ growth defects of the *er* mutant, including short leaf petioles and reduced plant height, all of which are largely due to reduced cell proliferation (Shpak et al., 2004; Woodward et al., 2005; Uchida et al., 2012). Taken together, the MPK3/MPK6 cascade plays a pivotal role downstream of ER receptors in regulating localized cell proliferation during plant growth and development, which shapes the morphology of plant organs.

In addition to their roles in cell proliferation, ER, ERL1, and ERL2 RLKs also regulate cell differentiation by repressing asymmetric cell divisions during guard cell differentiation and anther development (Shpak et al., 2005; Hord et al., 2008). These functions are also shared with the MPK3/MPK6 cascade (Wang et al., 2007; Hord et al., 2008). Loss of function of *YDA*, *MPK3*/*MPK6*, or *MKK4*/*MKK5* disrupts the coordinated cell fate specification of stomata versus pavement cells, resulting in the formation of clustered stomata, similar to that of *er erl1 erl2* triple mutant plants (Bergmann et al., 2004; Shpak et al., 2005; Wang et al., 2007). Although there is no direct evidence linking the two components in the literature, several studies have suggested that the MPK3/MPK6 cascade may function downstream of ER/ERL1/ERL2 receptors in regulating stomatal development and patterning (Wang et al., 2007; Kim et al., 2012; Pillitteri and Torii, 2012).

### Signaling Strength and Signaling Specificity of the ER Signaling Pathways

ER/ERL1/ERL2 and MPK3/MPK6 bear similarity in their gene dosage–dependent cellular function. Mutation of *ERL1* and/or *ERL2* genes further reduces the pedicel length of the *er* mutant, resulting in a much more clustered inflorescence (Shpak et al., 2004). Similarly, *mpk3*$^{+/-}$ *mpk6*$^{-/-}$ plants showed a more severely clustered inflorescence than the *mpk6* single mutant. In addition, *ERL2* is haplo-insufficient in maintaining normal cell proliferation of the integuments when *ER* and *ERL1* are mutated (Pillitteri et al., 2007b). Similarly, *MPK3* is haplo-insufficient in the *mpk6* mutant background, resulting in a sterile phenotype almost identical to that of *er erl1 erl2*$^{+/-}$ plants (Wang et al., 2008).

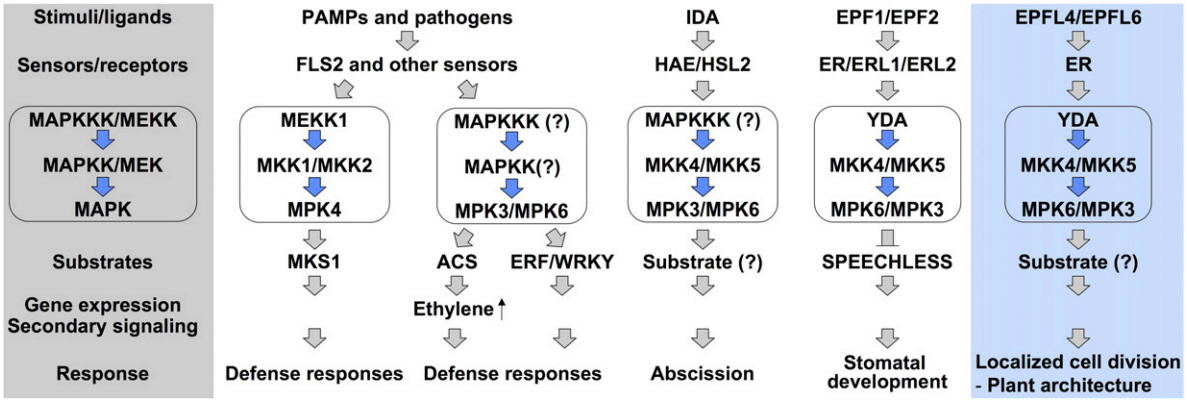


**Figure 10.** A Model Depicts the Functions of the YDA-MKK4/MKK5-MPK3/MPK6 Cascade Downstream of *ER* in Regulating Cell Proliferation and Plant Organ Development.

After sensing tissue/cell-specific signaling molecules (i.e., peptide ligands EPFL4 and EPFL6), ER receptors activate the YDA-MKK4/MKK5-MPK3/MPK6 cascade, either directly or through an additional mediator(s). Activated MPK3 and MPK6 phosphorylate their substrate(s), which eventually leads to tissue-specific cell proliferation and thereby regulates plant growth including pedicel, petiole, and inflorescence elongation. The shadowed area on the left highlights a typical MAPK-mediated signaling pathway downstream of receptors/sensors in eukaryotes. The placement of the YDA-MKK4/MKK5-MPK3/MPK6 cascade downstream of ER/ERLs in stomatal developmental regulation is solely based on correlative evidence. Activation of MPK3/MPK6 in response to pathogen-derived stimuli, such as the sensing of flg22 by FLS2, occurs in most cell/tissue types. By contrast, their activation after sensing of peptide ligands by HAE/HSL2 and ER/ERLs receptors is limited to specific cell types and during specific growth/developmental stages. The functions and signal specificity of MPK3/MPK6 are mostly dependent on the cell/tissue-specific activation of upstream receptors and spatiotemporal expression of downstream MAPK substrates.
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The observed gene dosage effect is likely a result of a reduction in signaling strength in the mutants. Direct measurement of the kinase activity of ER and ERLs is not possible at this stage. Because of the covalent modification of MAPKs and the fact that the active state can be preserved during protein extraction, in vivo MAPK activity can be determined. Previously, we demonstrated that wounding-induced MPK3/MPK6 activation is gene dosage dependent (Wang et al., 2008). Although *mpk3*$^{+/-}$ *mpk6*$^{-/-}$ plants are sterile and show clustered inflorescence and shortened pedicels, they have normal stomatal development. Only the *mpk3 mpk6* double mutant has abnormal stomatal development and patterning (Wang et al., 2007). These findings suggest that signaling strength is very important and that different processes have different signal thresholds.

### How Does MPK3/MPK6 Cascade Achieve Signaling Specificity in Different Biological Processes?

The multifunctional aspect of the MPK3/MPK6 cascade downstream of ER receptors opens up an interesting question of how the signaling specificity of MPK3/MPK6 is maintained in different growth and developmental pathways. Tissue- or cell-specific expression of input signaling molecules, especially the small peptide ligands, may play a critical role in maintaining the signaling specificity of different ER signaling pathways. Recently, several secretory peptides of the EPFL family were shown to be ligands of ER. Of these ER ligands, EPF1 and EPF2, which are expressed specifically within subsets of stomatal lineage cells, play critical roles in stomatal development (Hara et al., 2007; Hunt and Gray, 2009; Lee et al., 2012). EPFL4 and EPFL6, two redundant upstream components of ER-mediated inflorescence growth, showed the highest expression level in inflorescence stems (Abrash et al., 2011; Uchida et al., 2012). Signaling specificity can also be maintained by substrate specificity; the same MAPK can activate different substrates that are differentially expressed. For instance, MPK3/MPK6 was shown to regulate stomatal development through phosphorylation and inactivation of the basic helix-loop-helix transcription factor SPEECHLESS, which is expressed in stomatal lineage cells (Lampard et al., 2008). As a result, the MPK3/MPK6 cascade functions more like a molecular switch. Depending on the upstream and downstream components, it can affect different biological processes.

### MAPK Cascades as Key Signaling Modules Downstream of Plant RLKs

RLKs, the largest kinase subfamily with more than 600 members in the model plant *Arabidopsis*, play diverse functions from growth/development to response to environmental stimuli in plants (Diévart and Clark, 2004; Morillo and Tax, 2006; De Smet et al., 2009). It is likely that they employ various signaling modules to transduce extracellular signals, generated upon perception of ligands, into intracellular responses. One of the best-studied examples is the brassinosteroid (BR)-BRI1 signaling pathway. BR binding to BRI1 triggers the formation of the BRI1-BAK1 receptor complex, which leads to BRI1 activation and phosphorylation of cytoplasmic BR signal kinases, ultimately culminating in activation of the transcription factors BZR1/2 and BR-responsive gene expression (Clouse, 2011). By contrast, signaling events/components downstream of other RLKs are largely unknown. It is interesting that MPK3 and MPK6, two closely related MAPKs, were recently shown to be downstream of several RLKs (Figure 10), from FLS2, a RLK involved in plant sensing of bacterial flagellin (Asai et al., 2002), to HEA/HSL2, two RLKs playing redundant function in *Arabidopsis* floral organ abscission (Cho et al., 2008), to CLV1/CLV2, two RLKs involved in plant stem cell population maintenance (Betsuyaku et al., 2011). In this report, we demonstrate that the ER RLK also employs MPK3/MPK6 to transduce the signal for localized cell proliferation.

In *Arabidopsis*, there are 18 additional MAPKs besides MPK3 and MPK6. The functions of many of them are unknown. It is likely that more MAPKs besides MPK3/MPK6 are also downstream of RLKs and are involved in converting other signals generated by ligand-activated RLK receptors into cellular responses. At this moment, how MAPK cascades carry out their functions downstream of RLKs is an actively researched area. FLS2-mediated MPK3/MPK6 activation mounts a global response that occurs in many, if not all, tissue/cell types. As a result, this process can be easily monitored biochemically using, for example, in-gel kinase activity assays and anti-phospho-specific antibodies. More importantly, several MPK3/MPK6 substrates or putative substrates, including ACS2/ACS6, ERF104, WRKY33, PHOS32, VIP1, and NIA2, have been identified (Liu and Zhang, 2004; Djamei et al., 2007; Merkouropoulos et al., 2008; Bethke et al., 2009; Wang et al., 2010; Mao et al., 2011). These substrates are involved in regulating biotic or abiotic stress responses in *Arabidopsis*. By contrast, the identification of MPK3/MPK6 substrates involved in regulating plant growth and development is much more difficult. The biochemical approach is not as effective since the activation of these two MAPKs occurs only in a few cells at a certain time. SPEECHLESS was identified in a genetic screen for mutants with stomatal developmental defects (MacAlister et al., 2007; Pillitteri et al., 2007a) and was later shown to be a substrate of MPK3/MPK6 (Lampard et al., 2008). Yet, it is challenging to demonstrate in vivo phosphorylation of SPEECHLESS by MPK3/MPK6 using biochemical assays because the expression of SPEECHLESS is limited to stomatal lineage cells at a specific stage of stomatal differentiation.

Using molecular genetic approaches, we demonstrated that the YDA-MKK4/MKK5-MPK3/MPK6 cascade is a key downstream signaling component in ER-mediated cell proliferation in this study. Identifying components further downstream of this MAPK cascade especially the MPK3/MPK6 substrate(s) in the pathway will ultimately reveal the molecular mechanisms underlying the regulation of localized cell proliferation during plant growth/development by this ER-MAPK signaling pathway.

## METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* Col-0 ecotype was used as the wild type. Mutant alleles of *mpk3-1* (SALK_151594), *mpk6-2* (Salk_073907), and *er-105* were reported previously (Torii et al., 1996; Liu and Zhang, 2004). The T-DNA

insertional mutant of *YDA* was obtained from the ABRC (SALK_105078) (Alonso et al., 2003). The transgenic lines of *DD*, *mkk4/mkk5 RNAi*, $P_{MPK3}$:*GUS*, $P_{MPK6}$:*GUS*, and $P_{ER}$:*GUS* were generated previously (Ren et al., 2002; Shpak et al., 2004; Wang et al., 2007, 2008). Generation of *mpk3*$^{+/-}$ *mpk6*$^{-/-}$, *MPK6*$^{KR}$ *mpk3*, *DD mpk3*, *DD mpk6*, and *DD yda* double mutant plants was described previously (Wang et al., 2007, 2008; Cho et al., 2008; Ren et al., 2008). Homozygous *DD er-105* and *er-105 mpk6* F3 plants were generated by genetic crosses. For these crosses, *er* and *mpk6* mutations were followed by PCR genotyping as described previously (Liu and Zhang, 2004; Shpak et al., 2004), and the *DD* transgene was followed by hygromycin resistance (Ren et al., 2002).

Seeds were plated on half-strength Murashige and Skoog medium with 0.7% phytagar and appropriate antibiotics for selection after surface sterilization and imbibing at 4°C for 3 to 5 d. Plates were incubated in a tissue culture chamber at 22°C under continuous light (70 $\mu E/m^{-2}\ s^{-1}$) for 7 d. Seedlings were then transplanted into soil and grow in a greenhouse with a 16-h-light/ 8-h-dark cycle.

### Transgenic Plant Generation

To make *ER* promoter–driven constitutively active MAPKK constructs, we amplified the *ER* promoter region using primer pair forward, 5′-GCAGAAGAGAAACCCAAGAAGA-3′, and reverse, 5′-ATTCAAAAATCCCTCTCCAACA-3′. After the addition of *Eco*RI and *Sac*I sites by PCR, the product was cloned into pCAMBIA3300 digested with the same enzymes, resulting in the pCambia3300-$P_{ER}$ intermediate construct. *MKK1*, *MKK2*, *MKK4*, *MKK5*, *MKK7*, and *MKK9* containing constitutively active Ser-to-Asp mutations were PCR amplified from pTA7002-*MKK1*$^{DD}$, -*MKK2*$^{DD}$, -*MKK4*$^{DD}$, -*MKK5*$^{DD}$, -*MKK7*$^{DD}$, and -*MKK9*$^{DD}$ constructs using primers that flank the omega enhancer-Flag-MKK$^{DD}$-terminator cassette (forward, 5′-CTCGAGGTATTTTTACAACAATTACCAACAAC-3′, and reverse, 5′-CAGTCACGACGTTGTAAAACGAC-3′). The pTA7002-*MKK*$^{DD}$ constructs were prepared as previously described for pTA7002-*NtMEK2*$^{DD}$ (Yang et al., 2001). The PCR products were then inserted into pCAMBIA3300-$P_{ER}$ digested with *Sma*I enzyme. Constructs with correct orientation were selected by PCR and then sequenced to ensure that no error was introduced by PCR.

The construct expressing constitutively active Δ*N-YDA* under its native promoter was kindly provided by Wolfgang Lukowitz (Lukowitz et al., 2004). Both the *MAPKK*$^{DD}$ and Δ*N-YDA* expression constructs were electroporated into *Agrobacterium tumefaciens* strain GV3101, which were used to transform wild-type and *er-105* plants by the floral dipping method (Clough and Bent, 1998). Basta-resistant T1 transgenic plants in the *er-105* background were screened for complementation of *er* mutant phenotypes. T1 plants in the wild-type background were examined for gain-of-function phenotypes (i.e., increased pedicel length). The typical lines in *er-105* and wild-type backgrounds were characterized in more detail in T2 generation.

### GUS Staining

Inflorescences were prefixed in prechilled 90% acetone for 20 min and washed with distilled water. After brief vacuum infiltration, the inflorescences were incubated in GUS staining buffer (10 mM EDTA, 0.1% Triton X-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 100 $\mu g\ mL^{-1}$ chloramphenicol, and 1 mg $mL^{-1}$ X-Gluc in 50 mM sodium phosphate buffer, pH 7.0) overnight at 37°C. After being cleared in 20% lactic acid/20% glycerol solution, the inflorescences were pictured using a digital camera.

### Histological Analysis, Cell Length, and Number Measurement

Pedicel tissue samples were fixed overnight in formalin-acetic acid-alcohol solution at 4°C, dehydrated with a graded series of ethanol, and infiltrated with Technovit 7100 resin (Heraeus Kulzer) followed by embedding and polymerization. Two-micrometer sections were prepared using a Leica Reichart Ultracut S microtome (Wetzlar). The tissue sections were stained with 0.1% toluidine blue and observed using light microscopy. Three regions of each sectioned pedicel were taken. The number of cells in a middle longitudinal cortex row was determined. This number was used to calculate the total number and average length of cells in the cortex row of each pedicel. The number of cortex cells was counted in 15 to 20 sectioned pedicels for each genotype, and the average cell number and length was determined.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: *ER* (At2g26330), *MPK3* (At3g45640), *MPK6* (At2g43790), *MKK1* (At4g26070), *MKK2* (At4g29810), *MKK4* (At1g51660), *MKK5* (At3g21220), *MKK7* (At1g18350), *MKK9* (At1g73500), and *YDA* (At1g62700).

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure 1.** Growth and Developmental Defects of $P_{ER}$:*MKK9*$^{DD}$ Transgenic Plants.

**Supplemental Figure 2.** Cell Death Phenotype Associated with the Basal-Level Expression of *MKK7*$^{DD}$ Transgene in the Steroid-Inducible pTA7002-*MKK7*$^{DD}$ Transgenic Plants.

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## AUTHOR CONTRIBUTIONS

X.M., H.W., and S.Z. designed research. X.M., H.W., Y.H., and Y.L. performed research. K.U.T. and J.C.W. contributed new tools. X.M., H.W., K.U.T., J.C.W., and S.Z. analyzed data. X.M. and S.Z. wrote the article.

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