Transcriptional integration of paternal and maternal factors in the *Arabidopsis* zygote

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In many plants, the asymmetric division of the zygote sets up the apical–basal axis of the embryo. Unlike animals, plant zygotes are transcriptionally active, implying that plants have evolved specific mechanisms to control transcriptional activation of patterning genes in the zygote. In *Arabidopsis*, two pathways have been found to regulate zygote asymmetry: YODA (YDA) mitogen-activated protein kinase (MAPK) signaling, which is potentiated by sperm-delivered mRNA of the SHORT SUSPENSOR (SSP) membrane protein, and up-regulation of the patterning gene *WOX8* by the WRKY2 transcription factor. How SSP/YDA signaling is transduced into the nucleus and how these pathways are integrated have remained elusive. Here we show that paternal SSP/YDA signaling directly phosphorylates WRKY2, which in turn leads to the up-regulation of *WOX8* transcription in the zygote. We further discovered the transcription factors HOMEODOMAIN GLABROUS11/12 (HDG11/12) as maternal regulators of zygote asymmetry that also directly regulate *WOX8* transcription. Our results reveal a framework of how maternal and paternal factors are integrated in the zygote to regulate embryo patterning.

[*Keywords*: zygote asymmetry; MAPK signaling; WOX8; maternal factors]

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Unlike animal zygotes, where transcription is blocked and maternally derived mRNAs and proteins stored in the unfertilized egg drive early embryo development (Riechmann and Ephrussi 2001; Tadros and Lipshitz 2009), plant zygotes are transcriptionally active, including the transcription of embryo patterning genes (Ueda et al. 2011; Nodine and Bartel 2012). Therefore, fundamental developmental strategies that have evolved in the plant kingdom are different from those in the animal kingdom, implying the evolution of mechanisms that ensure the timely transcriptional initiation of patterning programs in the zygote.

Initiation of the main body axis is one of the first patterning steps in developing an organism from a unicellular zygote. In many plants, including *Arabidopsis thaliana*, *Fucus* (brown algae), *Physcomitrella patens* (moss), or rice (Brawley et al. 1977; He et al. 2007; Sato et al. 2010; Sakakibara et al. 2014), the zygote divides asymmetrically. In *Arabidopsis*, this division yields a small apical daughter cell that forms the embryo and a large basal daughter cell that will give rise mostly to the extra-embryonic suspensor (Mansfield and Briarty 1991). The zygote expresses paralogs of the stem cell transcription factor WUSCHEL (WUS); namely, WUS HOMEOBOX2 (WOX2) and the redundant WOX8 and WOX9 proteins (also named STIMPY-LIKE and STIMPY) (Haecker et al. 2004; Wu et al. 2007; Ueda et al. 2011). After the zygotic division, *WOX2* expression becomes confined to the apical cell, where it regulates embryo development, whereas expression of *WOX8* becomes restricted to the basal cell. WOX8 is a master regulator of early embryogenesis, controlling suspensor development and root initiation in the basal lineage. In addition, WOX8 non-cell-

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autonomously regulates development of the embryo, including the expression of *WOX2* and patterning of the phytohormone auxin (Breuninger et al. 2008).

Maternal parent-of-origin-effect genes have been identified that regulate endosperm development (Waters et al. 2011) or cotyledon development in mid-stage embryos (Ray et al. 1996) but, unlike in animals, none that specifically affect the zygote or early embryo patterning. Recent studies discovered several factors that are required for zygote asymmetry. The zinc finger transcription factor WRKY2 regulates symmetry breaking and unequal division of the zygote in part by directly activating transcription of the *WOX8* gene (Ueda et al. 2011). In *wrky2* mutants, zygotes fail to polarize after a transient symmetric stage and eventually divide in a more symmetric manner compared to wild type. The YODA (YDA) mitogen-activated protein kinase kinase kinase (MAPKKK) with the downstream MAPKs MPK3/6 is required for zygote elongation and suspensor specification (Lukowitz et al. 2004; Wang et al. 2007; Bayer et al. 2009). Activation of YDA/MPK signaling in the zygote requires the membrane-associated receptor-like kinase SHORT SUSPENSOR (SSP), whose mRNA is delivered from the sperm cell and translated in the zygote (Bayer et al. 2009). In addition, the EMBRYO-SURROUNDING FACTOR1 (ESF1) peptide and the plasma membrane receptor kinase ZYGOTE ARREST1 (ZAR1) are required for *WOX8* expression and zygote development (Costa et al. 2014; Yu et al. 2016). These examples imply that zygote development depends on external cues. However, the molecular mechanisms linking external cues and transcriptional regulation in the zygote have remained elusive.

In this study, we dissected the molecular relationship between SSP/YDA–MAPK signaling and the WRKY2/WOX8 transcription module in zygote asymmetry and embryo patterning by combining molecular and biochemical approaches with genetic studies in *Arabidopsis*. Our results indicate that WRKY2 bridges MAPK signaling to the nucleus of the zygote and that *WOX8* transcriptional control integrates paternal and novel maternal inputs during the initiation of zygote asymmetry.

Results

Both WRKY2 *and* SSP *up-regulate* WOX8 *expression to polarize the zygote*

To address the relationship between the WRKY2 and SSP pathways in regulating *Arabidopsis* zygote asymmetry, we first compared the respective mutant phenotypes. We measured the ratio of apical to basal daughter cell lengths as an inverse proxy for the asymmetry of the zygotic division. Because no further elongation occurs at this stage (Mansfield and Briarty 1991), we measured the total length of one-cell stage embryos as a proxy for zygote elongation. Both *wrky2-1* and *ssp-2* display shorter zygotes and divide less asymmetrically than the wild type (Fig. 1A,B; Supplemental Table S1; Lukowitz et al. 2004; Bayer et al. 2009; Ueda et al. 2011). Notably, despite the reduction of the total length of the zygote, the length of the apical cell in these mutants is increased compared with wild type, and the basal cell is strongly reduced (Fig. 1A,B; Supplemental Table S1). This indicates that the loss of zygote asymmetry is not simply the consequence of its reduced elongation and suggests that the two processes might be independently regulated, although both are under the control of *WRKY2* and *SSP*.

To test whether *WRKY2* and *SSP* genetically interact, we first analyzed double mutants. We found that defective zygote asymmetry in the *wrky2-1* mutant is not

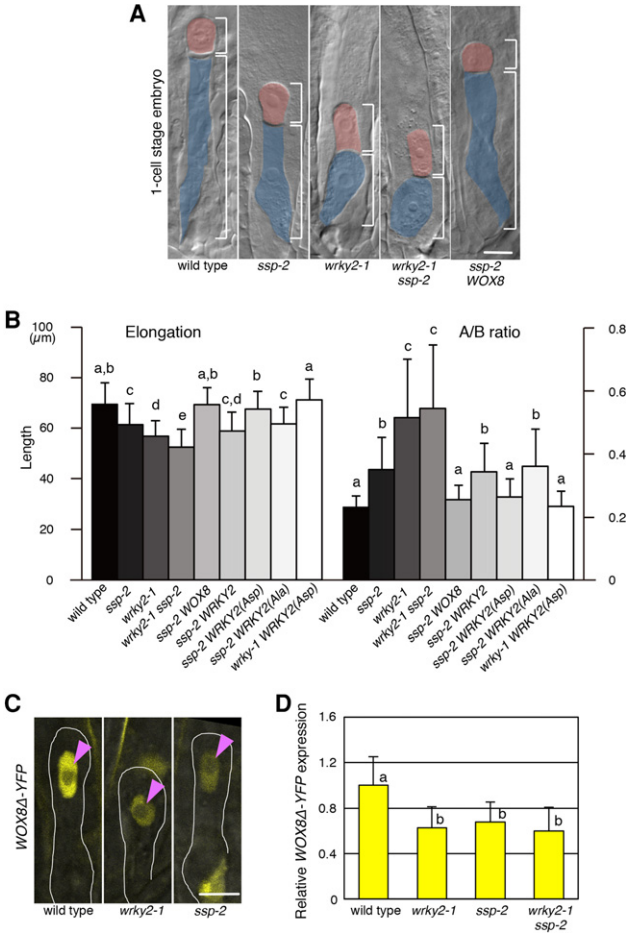

Figure 1. WRKY2 and SSP/MAPK signaling regulate zygote elongation and asymmetric division by square brackets. *WOX8* expression. (*A*) One-cell stage embryos of the indicated genotypes. The apical (red) and basal (blue) cells are color-coded, and their lengths are marked by square brackets. (*WOX8*) *pEASE:WOX8-YFP*. (*B*) The total length of one-cell stage embryos (sum of apical and basal cell lengths), denoted as elongation, and the ratio of apical divided by basal cell lengths, denoted as A/B ratio, are shown. (*WRKY2*) *pWRKY2:WRKY2-YFP*; [*WRKY2*(*Ala*)] *pWRKY2:WRKY2(Ala)-YFP*; [*WRKY2*(*Asp*)] *pWRKY2:WRKY2(Asp)-YFP*. (*C*) *WOX8Δ-YFP* expression in zygotes of the indicated genotypes. The zygotes are outlined, and the nuclei are indicated by arrowheads. (*D*) Relative *WOX8Δ-YFP* signal intensities in the zygote. Wild type is set as 1. Bars, 10 μm. Error bars represent SD. $n \geq 80$ (*B*); $n \geq 22$ (*D*). Letters on columns indicate significantly associated categories. $P < 0.05$ by the Tukey-Kramer test.

significantly enhanced by *ssp-2*, suggesting a single pathway (Fig. 1A,B; Supplemental Table S1). In contrast, the effects on zygote elongation are stronger in the double mutant than in each single mutant (Fig. 1A,B; Supplemental Table S1). Because WRKY2 regulates the zygote and the extraembryonic suspensor through direct transcriptional activation of the patterning gene *WOX8* (Ueda et al. 2011), we tested whether *SSP* might also act through *WOX8* in this process by using the *WOX8Δ-YFP* reporter gene, which faithfully mimics the endogenous *WOX8* mRNA pattern (Breuninger et al. 2008; Ueda et al. 2011). We found that both *ssp-2* and *wrky2-1* zygotes display a similarly strong reduction in *WOX8Δ-YFP* expression compared with wild type (Fig. 1C,D). Notably, *WOX8Δ-YFP* expression in the *wrky2-1 ssp-2* double mutant is not significantly altered compared with either single mutant (Fig. 1D), supporting the hypothesis that *SSP* and *WRKY2* act in a single pathway regulating *WOX8*. Moreover, restoring expression of *WOX8* in the zygote through the *pEASE:WOX8-YFP* transgene suppresses all *ssp-2* zygote defects (*ssp-2 WOX8* in Fig. 1A,B; Supplemental Table S1), similar to the previously reported suppression of *wrky2* zygote defects by the same transgene (Ueda et al. 2011).

Together, these results suggest that *WRKY2* and *SSP* activities act in one genetic pathway required for the asymmetric division of the zygote through positively regulating *WOX8* expression, whereas they appear to act at least in part independently in zygote elongation.

WRKY2 binds to MPK3/6 and is activated by phosphorylation

Previous studies showed that, in the zygote, SSP acts through the YDA MAPKKK and the MAPKs MPK3 and MPK6 (Wang et al. 2007; Musielak and Bayer 2014). Therefore, one possible way in which WRKY2 and SSP could interact is that SSP/YDA–MAPK signaling targets the WRKY2 protein. In yeast two-hybrid assays, we found that WRKY2 binds to MPK3 and MPK6 but not to MAPK MPK9 as a negative control (Fig. 2A) and confirmed this interaction by bimolecular fluorescence complementation (BiFC) in *Arabidopsis* protoplasts (Supplemental Fig. S1A).

The WRKY2 protein contains a cluster of five serine–proline motifs (SP cluster) as putative MPK phosphorylation sites at the N terminus that is in close proximity to predicted MPK-docking sites (Supplemental Fig. S1B; Eulgem et al. 2000; Sharrocks et al. 2000; Ishihama and Yoshioka 2012). To investigate whether MPK3 can phosphorylate the WRKY2 protein, we used an in vitro assay with proteins synthesized in *Escherichia coli*. We obtained two main WRKY2 products of slightly different sizes that are phosphorylated only in the presence of both MPK3 and the constitutively active MKK4/5DD that activates MPK3 (Fig. 2B; Supplemental Fig. S1C,D). To analyze whether the SP cluster is important for in vitro WRKY2 phosphorylation, we exchanged the serine residues of the five SP motifs within the cluster with alanines to block

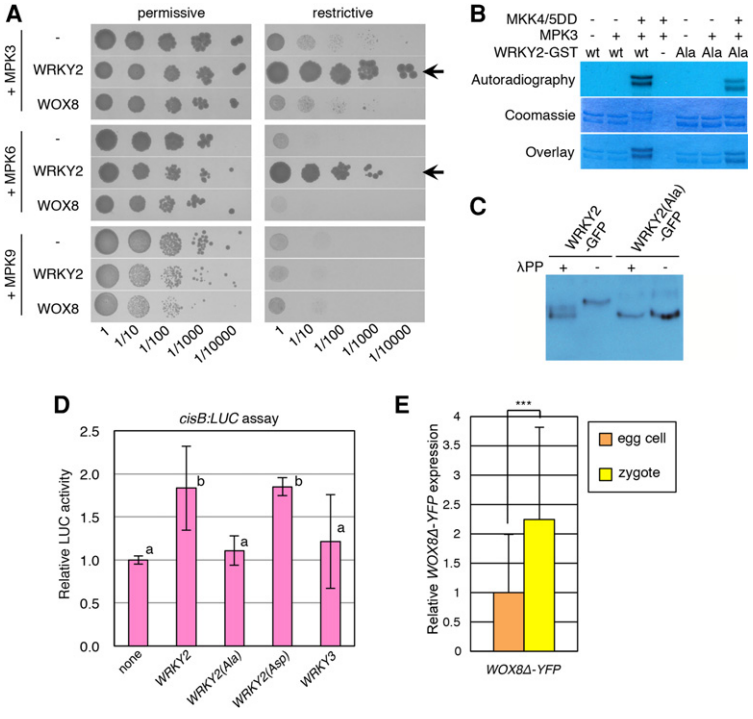

Figure 2. MPK3/6 phosphorylate WRKY2 to promote zygote asymmetry via transcriptional activation of *WOX8*. (*A*) Yeast two-hybrid assay of interactions between WRKY2 and MPK3 or MPK6. Panels show serial decimal dilutions of yeast on media with (permissive) and without (restrictive) histidine. Arrows mark growth on the restrictive medium. WOX8 and MPK9 were used as negative controls for WRKY2 and MPK3/6, respectively. (*B*) In vitro phosphorylation of WRKY2. (+) The presence of constitutively active MKK4 and MKK5 (MKK4/5DD) or MPK3; (–) the absence of constitutively active MKK4 and MKK5 (MKK4/5DD) or MPK3. WRKY2-GST (wt) and WRKY2(Ala)-GST (Ala) expressed in *Escherichia coli* show two main products, possibly due to partial degradation. An image of the entire gel is shown in Supplemental Figure S1C. (*C*) In vivo phosphorylation of WRKY2-GFP. *Arabidopsis* protoplasts were cotransformed with MPK3 and MKK4DD. The five serine residues of the serine–proline motifs (SP cluster) were replaced with alanine in WRKY2(Ala)-GFP. WRKY2-GFP, but not WRKY2(Ala)-GFP, shows a mobility shift due to phosphorylation that disappears after incubation (+) with λ phosphatase (λPP). (*D*) Luciferase (LUC) assay of the indicated effector proteins on the *WOX8 cisB* element in protoplasts. Expression of control without effector (none) is set as 1. Unmodified WRKY2, phospho-blocked WRKY2(Ala), and phosphomimic WRKY2(Asp) variants were used. (WRKY3) Negative control. The characters on the graph indicate the significantly associated categories. $P < 0.05$ by the Tukey-Kramer test. (*E*) Relative intensity of *WOX8Δ-YFP* signals in the zygote and the egg cell. Egg cell is set as 1. (***) $P < 0.001$, Student's *t*-test. Error bars represent SD. $n \geq 3$ (*D*); $n \geq 30$ (*E*).

phosphorylation [WRKY2(Ala)] (Fig. 2B; Supplemental Fig. S1C). This resulted in a reduced phosphorylation of WRKY2(Ala) protein by MPK3 (Ala in Fig. 2B; Supplemental Fig. S1C), indicating that, in vitro, MPK3 targets at least some of the exchanged SP sites within the SP cluster of WRKY2. Nevertheless, the remaining detectable phosphorylation of WRKY2(Ala) in this assay suggests additional phosphorylation sites outside the SP cluster. Mass spectrometric analysis of *Arabidopsis* WRKY2 transiently expressed in tobacco (*Nicotiana benthamiana*) leaves suggests that phosphorylation occurs on several SP sites—including Ser116 and Ser120 within the SP cluster—and several other Ser and Thr residues (Supplemental Figs. S1B, S2). We confirmed that WRKY2 protein is phosphorylated also in *Arabidopsis* protoplasts using a phospho-retardation assay (Fig. 2C). Importantly, WRKY2(Ala) does not show any phosphorylation in protoplasts (Fig. 2C), suggesting that, in this experimental system, the SP cluster is the main phosphorylation target site of WRKY2. Therefore, we focused our functional analysis on the WRKY2 (Ala) variant.

To assess whether phosphorylation of the SP cluster is crucial for the transcriptional activity of WRKY2, we investigated the WRKY2 sequence variants using a luciferase (LUC) transcription assay in *Arabidopsis* protoplasts (Fig. 2D). We found that unmodified WRKY2 or the constitutively active phospho-mimic version WRKY2(Asp), where aspartates replace all serine residues of the SP sites in the SP cluster, significantly activates transcription from a *WOX8* intron fragment containing the WRKY2-binding site (*cisB*) (Fig. 2D; Supplemental Fig. S3A). In contrast, the phospho-blocked WRKY2(Ala) or WRKY3 as negative control does not (Fig. 2D). Thus, phosphorylation of the WRKY2 SP cluster is essential for transcriptional activation of the *WOX8* regulatory region in protoplasts.

To investigate whether WRKY2 phosphorylation by the SSP/MAPK cascade is crucial for the regulation of zygote asymmetry, we analyzed whether the WRKY2 sequence variants can rescue the *ssp-2* zygote defects. We used a functional WRKY2-YFP protein as a backbone, enabling us to select transgenic plants with similar expression levels (data not shown). We found that expression of the phospho-mimic WRKY2(Asp)-YFP from the endogenous *WRKY2* promoter not only fully rescues the *wrky2* zygote but also suppresses the elongation and asymmetry defects of *ssp-2* zygotes (Fig. 1B; Supplemental Table S1). In contrast, expression of unmodified WRKY2-YFP or the phospho-blocked WRKY2(Ala)-YFP does not rescue the *ssp-2* zygote defects (Fig. 1B; Supplemental Table S1).

Because SSP activity is derived from the sperm-delivered mRNA (Bayer et al. 2009), we tested whether *WOX8* transcription increases after fertilization. We found that *WOX8* expression levels in the wild-type zygote is increased about twofold compared with the egg cell (Fig. 2E) but is low in *wrky2-1* and *ssp-2* zygotes (Fig. 1D).

Together, these findings suggest that the phosphorylation of the SP cluster of WRKY2 triggered by sperm-delivered SSP causes up-regulation of *WOX8* expression after fertilization and that this step is crucial for zygote elongation and asymmetry.

HOMEODOMAIN GLABROUS11 (HDG11) and HDG12 are direct regulators of WOX8 *transcription*

In addition to the WRKY2-binding site in *cisB*, the second *WOX8* intron contains the 85-base-pair (bp)-long *cisC* element (Supplemental Fig. S3A) that is also sufficient for the correct spatial and temporal *WOX8* expression pattern during embryogenesis (Ueda et al. 2011). Using a yeast one-hybrid approach (see the Supplemental Material), we identified the class IV homeodomain–leucine zipper transcription factor family (HD-ZIP IV) protein HDG11 (At1g73360) as a *cisC*-binding protein (Fig. 3A). In contrast, HDG11 does not bind to a mutated *cisC* sequence where the core sequence of the predicted HD-ZIP IV-binding motif TAAA was changed ($cisC_m$ in Fig. 3A; Supplemental Fig. S3A; Nakamura et al. 2006) or to the unrelated *cisB* motif (Fig. 3A). We confirmed that HDG11 activates transcription from *cisC* in *Arabidopsis* protoplasts (Supplemental Fig. S3B). A similar activation was found with the closely related HDG12 (At1g17920) but not with HDG6/FLOWERING WAGENINGEN (FWA) as a negative control (Supplemental Fig. S3B; Kinoshita et al. 2004).

These results show that HDG11/12 bind to the *cisC* regulatory site of *WOX8* in yeast and activate transcription from *cisC* in *Arabidopsis* protoplasts.

HDG11/12 regulate zygote development through WOX8

To investigate the role of *HDG11/12* genes in embryogenesis, we first analyzed their expression patterns in the

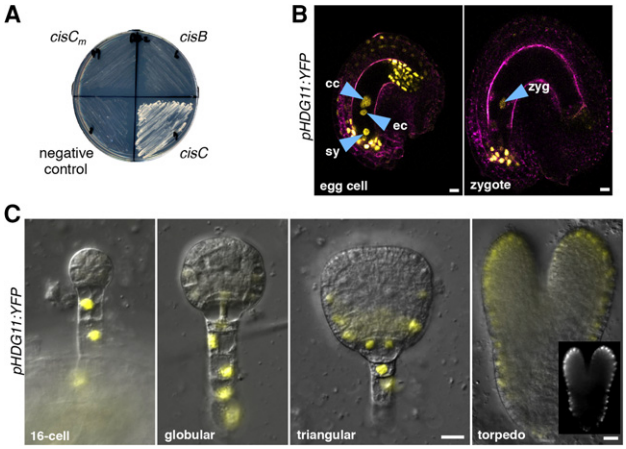

Figure 3. HDG11 binds to the *WOX8 cisC* fragment and is expressed in the female gametophyte, the zygote, and the embryo. (*A*) Yeast one-hybrid assay of interactions between HDG11 and the indicated *WOX8 cis*-regulatory fragments. (*B*) Two-photon microscope images showing *pHDG11:YFP* expression in ovules containing a mature embryo sac with the egg cell (female gametophyte) or the zygote. Autofluorescence is shown in magenta. Arrowheads point to the nucleus of the egg cell (ec), synergid cell (sy), central cell (cc), and zygote (zyg). (*C*) *pHDG11:YFP* expression during embryogenesis at the indicated stages. (*Inset*) Enhanced YFP signal without differential interference contrast image. Bars, 10 µm.

female gametophyte and during embryogenesis. We detected expression of a *pHDG11:YFP* reporter in the egg cell, synergid cells, and central cell of the embryo sac and in cells at the micropylar and antipodal side of the integuments (Fig. 3B). After fertilization, *pHDG11:YFP* expression remains high in the zygote and the integument at the micropyle but ceases in the endosperm (Fig. 3B). In preglobular and globular embryos, expression is restricted to the suspensor (Fig. 3C), similar to *WOX8* and *WRKY2* expression (Ueda et al. 2011). At the triangular embryo stage, *pHDG11:YFP* expression becomes gradually visible in the epidermis (Fig. 3C). Expression of a functional HDG11-YFP fusion protein from the endogenous promoter fully rescues the *hdg11-1 hdg12-2* double mutant (*hdg11/12 HDG11* in Fig. 4B; Supplemental Table S1) and is expressed similarly to the transcriptional reporter, albeit weaker, in the embryo (Supplemental Fig. S4A), indicating that the observed expression pattern is sufficient for gene function. The expression pattern of a *pHDG12:YFP* reporter gene is strikingly similar to that of *pHDG11:YFP* (Supplemental Fig. S4B).

We then analyzed the phenotypes of the strong loss-of-function T-DNA insertion mutants *hdg11-1* and *hdg12-2* (Nakamura et al. 2006; Khosla et al. 2014). In both single mutants, the zygotes are indistinguishable from wild type, whereas zygotes of the *hdg11-1 hdg12-2* double mutant do not fully elongate and divide more symmetrically than wild type (Figs. 4A, 5A; Supplemental Table S1). Furthermore, double-mutant zygotes show a strong reduction of *WOX8Δ-YFP* expression (Fig. 4C,D) similar to *wrky2-1* and *ssp-2* zygotes (Fig. 4D). Importantly, the asymmetry defects of *hdg11-1 hdg12-2* zygotes are completely suppressed and elongation defects are partially suppressed by restoring *WOX8* expression through the *pEASE:WOX8-YFP* transgene (Fig. 4A,B; Supplemental Table S1), again similar to *wrky2-1* and *ssp-2* (Fig. 1A,B; Ueda et al. 2011). Thus, HDG11/12 directly activate *WOX8* transcription, which in turn affects zygote elongation and asymmetric division.

Because all zygote phenotypes of the *hdg11-1 hdg12-2* double mutant are strikingly similar to the mutants of the SSP/WRKY2 pathway and because all factors appear

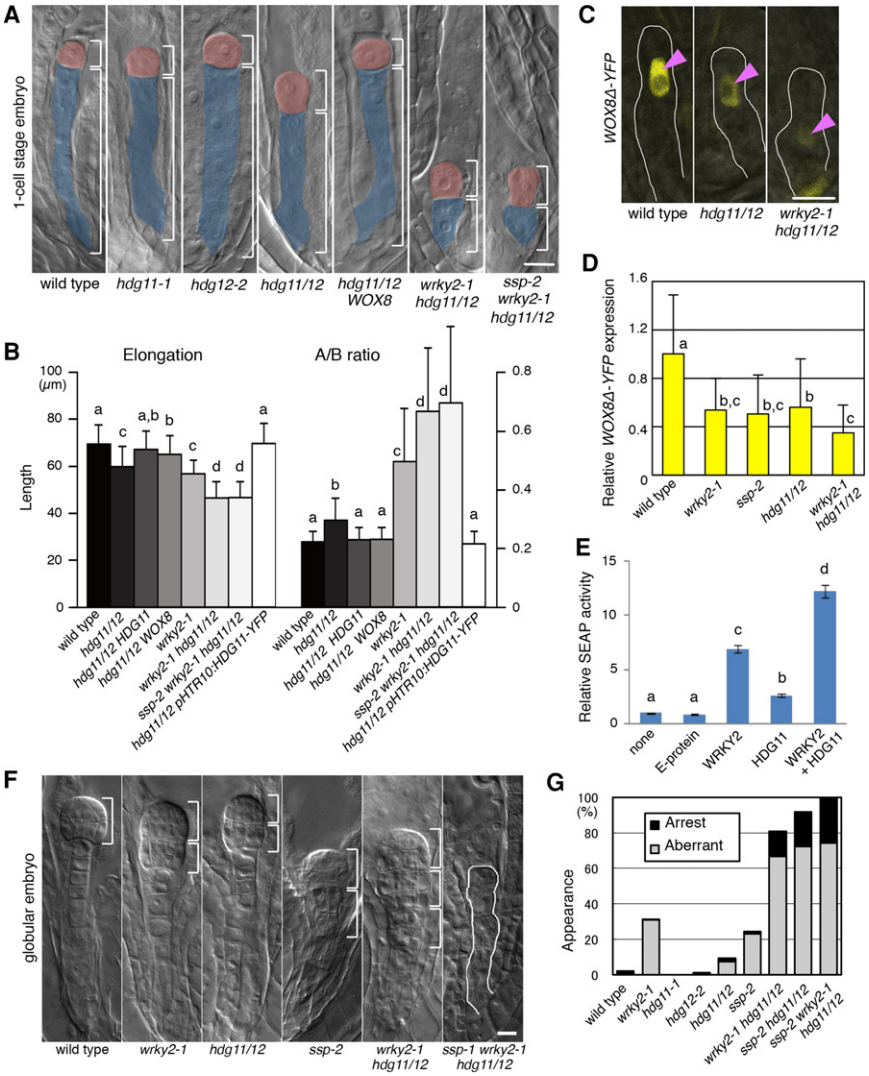

Figure 4. *HDG11/12* regulate zygote asymmetry and embryo patterning. (*A*) One-cell stage embryos of the indicated genotypes. The apical (red) and basal (blue) cells are color-coded, and their lengths are marked by square brackets. (*hdg11/12*) *hdg11-1 hdg12-2* double mutant; (*WOX8*) *pEASE:WOX8-YFP*. (*B*) Elongation and the A/B ratio of one-cell stage embryos of the indicated genotypes. (*HDG11*) *pHDG11:HDG11-YFP*. (*C*) *WOX8Δ-YFP* expression in zygotes. The zygote is outlined, and arrowheads indicate the nuclei. (*D*) Relative *WOX8Δ-YFP* signal intensities in the zygote. Wild type is set as 1. (*E*) Secreted alkaline phosphatase (SEAP) assay of the indicated effector proteins on the reporter with the *WOX8* intron fragment containing *cisB* and *cisC*. Mammalian E protein was used as a negative control. (*F*) Early globular stage embryos of the indicated genotypes. The sphere-like structures are marked by square brackets, and the arrested embryo is outlined. (*G*) Frequency of mutant embryo phenotypes. (Aberrant) Embryo whose cell division pattern is different from wild type; (arrest) embryo arrested with <10 cells. Bars, 10 µm. Error bars represent SD. $n \geq 80$ (*B*); $n \geq 28$ (*D*); $n \geq 4$ (*E*); $n \geq 238$ (*G*). Wild type and *wrky2-1* in *B* correspond to Figure 1B because these measurements were performed in one experiment. The characters on graphs indicate the significantly associated categories. $P < 0.05$ by the Tukey-Kramer test in *B*, *D*, and *E*.

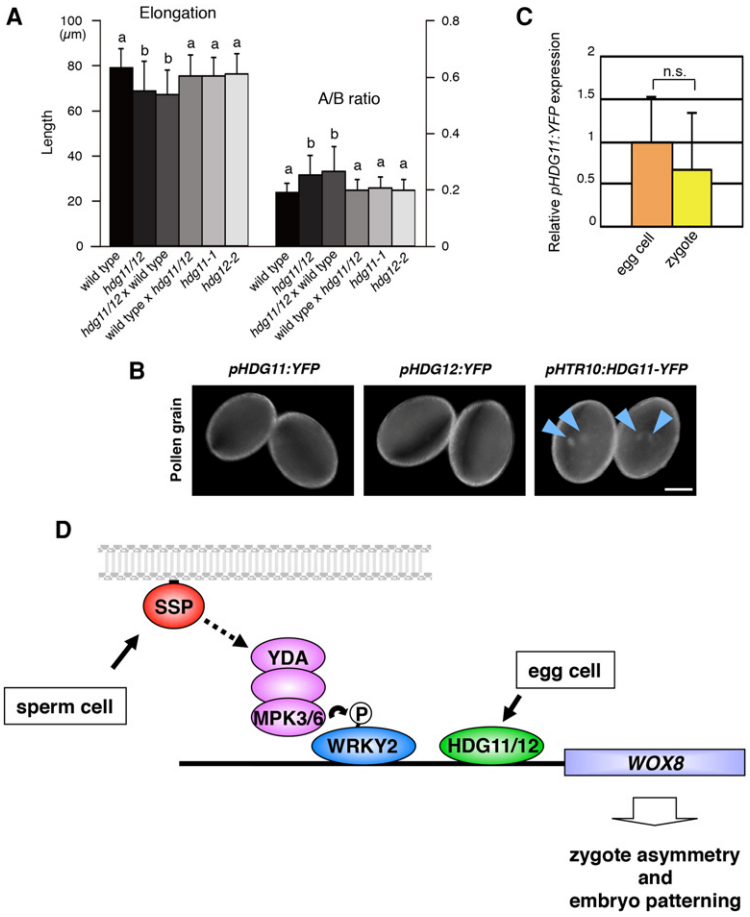

Figure 5. HDG11/12 act as maternal parent-of-origin factors in zygote asymmetry. (*A*) Elongation and the A/B ratio of one-cell stage embryos of the indicated genotypes. Crosses are denoted as female × male. (*hdg11/12*) *hdg11-1 hdg12-2*. (*B*) Expression of the indicated reporter genes in pollen grains. Arrowheads point to the sperm cell nuclei. (*C*) Relative intensity of *pHDG11:YFP* signals in the zygote and the egg cell. Egg cell is set as 1. (*D*) Model of the regulation of zygote asymmetry. Sperm-derived SSP triggers the YDA–MPK3/6 cascade in the zygote to phosphorylate WRKY2. WRKY2 and maternally derived HDG11/12 directly bind to the *WOX8* intron and activate its transcription to regulate elongation and asymmetry of the zygote and embryo patterning. Bar, 10 µm. Error bars represent SD. $n \geq 80$ (*A*); $n \geq 19$ (*C*). The characters on columns indicate the significantly associated categories. $P < 0.05$ by the Tukey-Kramer test. (n.s) Not significant (Student's *t*-test).

to act through the regulation of *WOX8* transcription, we asked how these pathways genetically interact. At the zygote stage, we found that the *wrky2-1 hdg11-1 hdg12-2* triple mutant and the *ssp-2 wrky2-1 hdg11-1 hdg12-2* quadruple mutant display stronger elongation and asymmetry defects than either *wrky2-1* or *ssp-2* single mutants or the *hdg11-1 hdg12-2* double mutant (Fig. 4A,B; Supplemental Table S1). In a complementary experiment, we confirmed additive transcriptional activation by WRKY2 and HDG11/12 from a *WOX8* intron fragment encompassing *cisB* and *cisC* (Supplemental Fig. S3A) using a transcriptional assay in human embryonic kidney 293 (HEK-293T) cells (Fig. 4E).

During subsequent embryo development, we observed aberrant cell division patterns in *ssp-2* and *wkry2-1* single mutants and the *hdg11-1 hdg12-2* double mutant but not *hdg11-1* or *hdg12-2* single mutants (Fig. 4F,G). These abnormal embryos generated spherical structures reminiscent of secondary proembryos (Fig. 4F) that lack *WOX8Δ-YFP* expression (Supplemental Fig. S5A). The defects are strongly enhanced in the *wrky2-1 hdg11-1 hdg12-2* and *ssp-2 hdg11-1 hdg12-2* triple and *ssp-2 wrky2-1 hdg11-1 hdg12-2* quadruple mutants, with the strongest defects being arrest of embryo development with <10 cells formed (Fig. 4F,G).

In summary, we conclude that HDG11/HDG12 and SSP/WRKY2 activities together are required to promote *WOX8* expression and regulate zygote asymmetric division and early embryo patterning.

HDG11/12 are maternal effect factors promoting zygote polarization

Because WRKY2 activity in the zygote is under control of the sperm-supplied factor SSP, we examined whether HDG11/12 also have a parent-of-origin effect. Fertilization of *hdg11-1 hdg12-2* egg cells with wild-type pollen does not suppress zygote defects (Fig. 5A; Supplemental Table S1). In contrast, the reciprocal cross of wild-type egg cells with *hdg11-1 hdg12-2* pollen does suppress all zygote defects (Fig. 5A; Supplemental Table S1). This indicates that the maternal *HDG11/12* gene copies are sufficient for normal zygote development, whereas the paternal copies are not. In contrast to the zygote defects of the *hdg11-1 hdg12-2* double mutant, pollination with wild-type pollen did complement later embryo defects at the globular stage (Supplemental Fig. S5E).

To investigate why the paternal *HDG11/12* gene copies are unable to complement the mutant zygote defects, we analyzed their expression before and after fertilization. In self-pollinated plants, *pHDG11:YFP* is expressed at similar levels in the egg cell and the zygote (Fig. 5C). In the sperm cells, expression of *pHDG11:YFP* and *pHDG12:YFP* reporters is undetectable (Fig. 5B). After fertilization,

paternally provided *pHDG11:YFP*, *pHDG12:YFP*, and *pHDG11:HDG11-YFP* reporters remain unexpressed in the young (short) zygotes (Supplemental Fig. S5B) when zygote polarization occurs (Kimata et al. 2016). However, expression of *pHDG11:YFP*, *pHDG11:HDG11-YFP*, and *pHDG12:YFP* becomes detectable in the mature elongated zygotes, and, during subsequent embryo stages, expression patterns were identical to those in self-pollinated embryos (Supplemental Fig. S5B–D; cf. Fig. 3C and Supplemental Fig. S4A,B). Unlike wild-type pollen, pollination of *hdg11-1 hdg12-2* mother plants with pollen carrying the *pHTR10:HDG11-YFP* transgene (Fig. 5B), which expresses the functional HDG11-YFP fusion protein in sperm cells (Ingouff et al. 2007), fully complements all mutant zygote defects (Fig. 4B; Supplemental Table S1).

Discussion

Embryogenesis requires the initiation of specific patterning programs in the zygote. Because plant zygotes, unlike their animal counterparts, are transcriptionally active (Autran et al. 2011; Nodine and Bartel 2012; Del Toro-De Leon et al. 2014), control mechanisms must have evolved to ensure transcriptional regulation of patterning genes. Our results reveal that a collaboration of paternal and maternal factors in the zygote underlies the transcriptional up-regulation of the *WOX8* patterning gene in controlling zygote asymmetry.

WRKY2 bridges paternally potentiated MAPK signaling to transcriptional regulation in the zygote

MAPK signaling cascades are highly conserved intracellular modules that connect external signals to transcriptional regulation in plants and animals. The YDA–MAPK pathway has been studied intensively in stomata development, where it connects peptide signals from neighboring cells to the regulation of cell fate through phosphorylation of the SPEECHLESS transcription factor (Lampard et al. 2008). In the zygote, YDA–MAPK signaling is required for the development of the zygote and the basal descendants (Lukowitz et al. 2004). Our finding that the activator of *WOX8* transcription, WRKY2, is a direct target of SSP/YDA/MPK3/6 phosphorylation connects external signals to the transcriptional output of the zygote (Fig. 5D). Importantly, because *SSP* mRNA is delivered by the sperm and is a limiting factor of YDA signaling, the transcriptional up-regulation of *WOX8* in the zygote is under paternal control and thus requires fertilization. What are the signals activating this process and how are they perceived? The membrane-associated IRAK/Pelle kinase SSP, required for YDA/MPK3/6 activation, is thought to function in assembly of the receptor complex (based on the function of mammalian homologs) rather than as receptor itself (Bayer et al. 2009). A recent study shows that the membrane receptor kinase ZAR1 is required for normal *WRKY2* and *WOX8* expression levels in the zygote and interacts physically with SSP in pull-down assays (Yu et al. 2016), but whether ZAR1 might provide the receptor for YDA/MAPK signaling still needs to be addressed. The recently discovered ESF1 peptides that are produced mainly by the central cell and the endosperm (nutrient tissue that surrounds the embryo) promote *WOX8* expression and zygote asymmetry (Costa et al. 2014). However, ESF1 and SSP appear to act synergistically, suggesting that ESF1 might function in parallel to a putative SSP/receptor complex and not through it. Moreover, *ESF1* mRNAs are also detectable in early embryonic transcriptome data (Bayer et al. 2016). Thus, the receptor and the signal causing WRKY2 phosphorylation through the YDA–MAPK pathway in the zygote remain to be determined.

Because WRKY proteins have been shown to be targets of MAPK signaling in pathogen defense, our findings support the concept that the genetic repertoire involved in fertilization is similar to the one active in pathogen defense (Okuda et al. 2009; Kessler et al. 2010), which has been interpreted as an evolutionary relationship of plant responses to the invasions of pollen tubes or pathogens (Dresselhaus and Marton 2009).

Maternal control of HDG11/12

Only very few cases of maternal parent-of-origin mutants are known to affect plant embryo development. As one example, expression of the microRNA processing factor DICER-LIKE1 in the sporophytic mother plant is required for normal cotyledon formation at mid-embryo stages (Ray et al. 1996). However, unlike in animals, where maternal control of early embryo patterning is well documented (Riechmann and Ephrussi 2001; Tadros and Lipshitz 2009), maternal effects on earlier embryo stages in plants have not been reported previously.

Our results that *WOX8* transcription and zygote asymmetry are directly regulated by maternally, but not paternally, derived HDG11/12 activity show that there is maternal control of early embryogenesis also in plants. What is the basis of this maternal parent-of-origin effect? Our data suggest that the paternal *HDG11/12* gene copies are not expressed in the sperm cells and during early zygote stages and thus are unable to drive zygote polarization but are expressed and thus able to complement *hdg11/12* mutant defects thereafter. This interpretation is in line with recent findings that zygote polarization, such as cytoskeleton rearrangement and nuclear migration, is evident already a few hours after fertilization, whereas the zygotic division takes place ~20 h after fertilization (Gooh et al. 2015; Kimata et al. 2016). Silencing of parental gene copies is common in animals and plants and has been suggested for several paralogs of *HDG11/12* (Gehring et al. 2009, 2011). In this view, *HDG11/12* might be similar to a recently reported group of genes that display transient maternal parent-of-origin effects due to delayed transcription of the paternal gene copy (Del Toro-De Leon et al. 2014).

Two mechanisms, not mutually exclusive, can explain the maternal parent-of-origin effect of HDG11/12 in zygote asymmetry. First, the maternal gene copies could be transcribed in the zygote and then regulate its

development. The fact that transcription initiated in the zygote can regulate its own asymmetric division has been shown by the ability to restore *WOX8* expression and zygote polarity through a sperm-delivered *WRKY2* gene copy (Ueda et al. 2011). Second, the zygote could have inherited *HDG11/12* mRNA or protein from the egg cell, similar to animal zygotes. This mechanism has not been described yet in plants, but because HDG11 expression is detected already in the egg cell, it is conceivable that at least some of the egg cell HDG11/12 reservoir could be passed on to the zygote.

A complex network regulates zygote asymmetry and embryo patterning

Comparison of the different mutant combinations indicates that zygote asymmetry requires a complex regulatory network with redundant and synergistic activities. For example, the *wrky2 ssp* double mutant displays a more severe reduction in zygote elongation compared with the *wrky2* single mutant, suggesting that SSP affects additional factors functionally redundant with WRKY2. One potential candidate might be WRKY34, which, in pollen development, is phosphorylated by MPK3/6, acts redundantly to WRKY2 (Guan et al. 2014), and is also expressed at levels similar to that of WRKY2 in the zygote (Xiang et al. 2011). Nevertheless, despite the redundancy within the regulatory network, the complete loss of SSP/WRKY2 and HDG11/12 activities cannot be compensated for, as indicated by the strong defects and the early developmental arrest of *ssp wrky2 hdg11/12* quadruple-mutant embryos. Because *WRKY2*, *HDG11/12*, and *WOX8* are also expressed after the division of the zygote, these defects could be either the consequence of the defective zygote or due to additional functions of these genes in specifying later events in embryo development. One example for the latter possibility is the expression of *HDG11/12* in the protoderm at the triangular stage, where they redundantly regulate epidermis development with their paralogs, *ATML1* and *PDF2* (Lu et al. 1996; Abe et al. 2003; Khosla et al. 2014; Ogawa et al. 2015).

WOX8 *transcriptional regulation as an integrator for maternal and paternal cues into embryogenesis*

The absence of either WRKY2 activation or HDG11/12 in the zygote similarly reduces *WOX8* transcription and causes asymmetry defects, indicating that both inputs must be present to reach the *WOX8* transcription levels necessary for zygote development (Fig. 5D). Because *HDG11* expression levels are unchanged between the egg cell and zygote, it appears plausible that the up-regulation of *WOX8* after fertilization might be caused mainly by the sperm-triggered phosphorylation of WRKY2.

Thus, regulation of *WOX8* transcription serves as an integrator of different regulatory inputs from the egg cell and the sperm. One possible advantage of transcriptional control of *WOX8* through both parents could be to ensure that the *WOX8* expression level necessary for zygote asymmetry is faithfully reached after fertilization. Comparison of a large set of publicly available transcriptome data and our own expression studies (data not shown) indicates that in early embryos and the suspensor, *WOX8* expression is indeed higher than in all other tissues, where it is usually at the detection limit. Paradoxically, however, some of these other tissues coexpress *HDG11/12*, *WRKY2*, and *MPK3/6*, raising the question of why *WOX8* is not up-regulated there. A plausible hypothesis is that up-regulation of *WOX8* transcription is limited to the zygote by the combination of HDG11/12 transcription factors, WRKY2 phosphorylation, and a transcriptionally competent chromatin. Because plant zygotes are deeply embedded in maternal tissue and cannot be isolated in sufficient amounts, future development of sensitive chromatin analysis methods will be necessary to test this hypothesis.

The parental conflict theory proposed for plants and mammals holds that, for embryo nourishment, the two parental genomes act antagonistically (Haig and Westoby 1989; Moore and Haig 1991). Whereas paternal genes favor nutrient supply to their own offspring, maternal genes favor the distribution of resources among all offspring. Our results—that cues from both parents conjointly regulate zygote asymmetry through *WOX8* transcription—suggest that during early embryo patterning, a parental cooperation model applies.

Materials and methods

Growth conditions and strains

Plants were grown on soil under continuous light at 18°C–20°C. *WOX8Δ-YFP* and *pEASE:WOX8-YFP* were described previously (Ueda et al. 2011). The *Arabidopsis* lines generated in this study are described in Supplemental Table S2.

Microscopy

Nomarski microscopy analysis was performed after clearing seeds (1:8:3 glycerin:chloral hydrate:water) with a Zeiss Imager A1 or Axioskop2 microscope. For fluorescent analysis, the seeds were placed in 10% glycerin. A two-photon microscope (Zeiss LSM780) was used to observe female gametophytes and zygotes, and intensity of nuclear YFP signal was measured by using ImageJ (http://rsb.info.nih.gov/ij) software. For BiFC assays, an Olympus IX71 microscope was used. Cell lengths of one-cell stage embryos were measured with AxioVision (Zeiss) or ImageJ software.

Yeast assays

The yeast one-hybrid screen was performed as described (Lopato et al. 2006) using the synthetic library REGIA (Regulatory Gene Initiative in *Arabidopsis*), which contains ~1500 *Arabidopsis* transcription factors (Paz-Ares 2002). The yeast two-hybrid assay was performed according to the manufacturer's guidebook (Clontech). For all constructs, full-length cDNAs were cloned into pGADT7 for prey or Clontech pGBKT7 for bait. The detailed methods are described in the Supplemental Material.

BiFC assay

BiFC constructs were created as described previously (Taoka et al. 2011). The 35S CaMV promoter was used to drive the full-length

genes fused to the VENUS-C-terminal fragment for bait or the VENUS-N-terminal fragment for prey. For transient expression analysis, 5 µg each of bait, prey, and tagRFP (transformation marker; Evrogen) plasmids were simultaneously introduced into 100 µL of protoplast suspension, generated from the *Arabidopsis* suspension culture "deep cells" by PEG-mediated transformation (Walter et al. 2004; Saito et al. 2011).

After overnight incubation, YFP-positive (representing interaction) cells were counted among the cells expressing tagRFP (representing transformed cells).

LUC assay

The effector containing transcription factors and the reporter plasmids, including firefly LUC, were prepared as described previously (Yamaguchi et al. 2010). As the effectors, full-length cDNAs were placed after the 35S CaMV promoter. For the reporter plasmids, the quadruple repeat of the 84-bp-long *cisB* fragment or the 85-bp-long *cisC* fragment was connected to a 35S minimal promoter, LUC, and a NOS terminator (Ueda et al. 2011). A plasmid containing the *Renilla* LUC gene driven by the 35S CaMV promoter was used as an internal control. Transient expression in *Arabidopsis* suspension culture "deep cells" was performed as for the BiFC assays (see above). LUC activity was assayed using the dual-LUC reporter assay system (Promega) and a Mithras LB940 reader (Berthold Technologies). To assess the efficiency of protoplast transformation, the data were used for analysis only when the firefly and *Renilla* LUC values both exceeded 1000.

Secreted alkaline phosphatase (SEAP) assay

For the effectors, a triple de novo assembly was designed by generating three PCR-amplified linear DNA fragments: coding sequence (CDS) of WRKY2 or HDG11, VP16 with a nuclear localization signal (NLS) sequence, and the SV40 promoter-driven mammalian expression vector. Each pair of adjacent fragments shared 32 bp of overlapping sequence. For the reporter, four times the repeats of the second intron of *WOX8* and pAP1(PMA)-SEAP that encodes the SEAP reporter gene (Clontech, catalog no. 631907) were amplified and assembled following the AQUA cloning procedure as described previously (Beyer et al. 2015). A plasmid containing a mammalian E protein driven by SV40 promoter was used as a negative control. HEK-293T cells were maintained in DMEM (Cell Culture Technologies) supplemented with 10% fetal bovine serum (PAN Biotech, catalog no. P30-3602, batch no. P101003TC), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (PAN Biotech). Seventy-five-thousand cells were seeded per well of a 24-well plate 1 d prior to transfection. Cells were transfected with the plasmids (0.75 µg of DNA in total) using polyethylenimine. After 24 h of incubation, SEAP reporter activity was determined as described previously (Schlatter et al. 2002).

Plasmid construction for transgenic plants

Details of cloning procedures are in the Supplemental Material, and all constructs for transgenic plants are listed in Supplemental Table S2.

In vivo phosphorylation assay

Protoplasts were isolated as described previously (Wu et al. 2009). Protoplasts were transfected with plasmids carrying 35S:WRKY2-GFP or 35S:WRKY2(Ala)-GFP, 35S:MKK4DD, and 35S:MPK3 and incubated for 16 h at room temperature. The protoplasts were resuspended in Tris buffer (50 mM Tris-HCl at pH 7.5, 0.15 M NaCl) containing 1% Igepal CA-630 (Sigma-Aldrich) and sonicated three times for 15 sec. All buffers were supplemented with 2× Complete protease inhibitor cocktail (Roche), 1 mM PMSF, and phosphatase inhibitors (25 mM sodium fluoride, 1 mM sodium orthovanadate, 50 mM β–glycerophosphate, 10 mM sodium pyrophosphate). The protoplast solution was diluted to a final concentration of 0.2% Igepal CA-630. The debris was separated by centrifugation, and the supernatant was incubated with anti-GFP microbeads (Miltenyi Biotec) on a rotating wheel for 15 min. The lysate was applied on a column in the magnetic field (Miltenyi Biotech) and washed with Tris buffer containing 0.1% Igepal CA-630. WRKY2-GFP or WRKY2(Ala)-GFP was eluted in HEPES buffer by removing the columns from the magnetic field. The eluate was supplemented with λ phosphate buffer (New England Biolabs) and 1 mM $MnCl_2$ and treated with λ protein phosphatase (New England Biolabs) for 5 min at 30°C. Proteins were separated on 7.5% SDS-PAGE supplemented with 50 µM Phos tag and 100 µM $MnCl_2$ and visualized with anti-GFP antibody (Abcam, ab290) and anti-rat-HRP antibody (Thermo).

In vitro phosphorylation assay

Recombinant proteins for the in vitro phosphorylation assay were expressed from the pGEX4T1 vector in *E. coli* BL21 (DE3) pLysS cells and purified as described in the manufacturer's instructions (GE Healthcare). Protein expression was induced by adding IPTG to a final concentration of 0.5 mM and incubation for 3 h at 28°C. The culture was pelleted, resuspended in lysis buffer, and sonicated three times for 30 sec with 50% pulses. The GST-tagged proteins were purified using GraviTrap columns according to the manufacturer's instructions (GE Healthcare). MPK3 was activated by incubation in kinase buffer (20 mM HEPES at pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 25 µM ATP) in the presence of MKK4DD and MKK5DD for 2 h at room temperature. Phosphorylation of WRKY2 and MBP was tested by incubation of WRKY2 or MBP protein with activated MPK3 in kinase buffer supplemented with 1 µCi of ATP-γ32P per reaction for 30 min and autoradiography. Uninduced *E. coli* lysate was processed in parallel and tested for background signals in the phosphorylation assay.

Mass spectrometry and data analysis

N. benthamiana leaves were infiltrated with p35S:WRKY2-GFP, p35S:MKK4DD-p35S:MPK6, and p35S:MKK5DD-p35S:MPK3 as described previously (Leuzinger et al. 2013). The leaf samples were harvested, and the phospho-enrichment experiment was performed as described previously (Vu et al. 2016). Detailed methods are in the Supplemental Material.

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