Phosphorylation of SPOROCYTELESS/NOZZLE by the MPK3/6 Kinase Is Required for Anther Development[1]

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Germ cells are indispensable carriers of genetic information from one generation to the next. In contrast to the well-understood process in animals, information on the mechanism of germ cell initiation in plants is very limited. *SPOROCYTELESS/NOZZLE* was previously identified as an essential regulator of diploid germ cell (archesporial cell) differentiation in the stamens and ovules of Arabidopsis (*Arabidopsis thaliana*). Although *SPOROCYTELESS* (*SPL*) transcription is activated by the floral organ identity regulator AGAMOUS and epigenetically regulated by SET DOMAIN GROUP2, little is known about the regulation of the SPL protein. Here, we report that the protein kinases MPK3 and MPK6 can both interact with SPL in vitro and in vivo and can phosphorylate the SPL protein in vitro. In addition, phosphorylation of the SPL protein by MPK3/6 is required for SPL function in the Arabidopsis anther, as measured by its effect on archesporial cell differentiation. We further demonstrate that phosphorylation enhances SPL protein stability. This work not only uncovers the importance of SPL phosphorylation for its regulatory role in Arabidopsis anther development, but also supports the hypothesis that the regulation of precise spatiotemporal patterning of germ cell initiation and that differentiation is achieved progressively through multiple levels of regulation, including transcriptional and posttranslational modification.

Germ cells are specialized cells that undergo meiosis to give rise to gametes, carrying genetic information from one generation to the next (Twyman, 2001; Alberts et al., 2002; Bai, 2015a). Germ cell initiation is therefore a key topic in developmental biology. In many animals, germ cells differentiate once from germline progenitors during early embryogenesis. They undergo meiosis after migrating to the gonads, and the resulting haploid cells differentiate into either egg or sperm cells through gametogenesis (Cinalli et al., 2008; Juliano and Wessel, 2010). In plants, however, although both egg and sperm cells also form during sexual reproduction, there is no physically separate germline in early embryonic development (Walbot, 1985; Walbot and Evans, 2003; Bai, 2015b). Instead, meiosis and gametogenesis are separately induced from cells in the diploid sporophyte and haploid gametophyte, respectively (Bhatt et al., 2001; Rotman et al., 2005; Borg et al., 2009; Bai and Xu, 2013; Bai, 2015b; Walbot and Egger, 2016). Unlike animal germ cells, there are two types of germ cell in plants: diploid germ cells (DGCs) called archesporial cells that are committed to meiosis and haploid germ cells, which are the generative cells in pollen grains and the progenitor cells to the egg in the embryo sac of angiosperms and are specified for gametogenesis. In angiosperms, male and female archesporial cells differentiate into microsporocytes and megasporocytes, respectively, and produce microspores and megaspores through meiosis.

In contrast to the spatially independent origins of animal germ cells and the gonads into which these cells migrate for further differentiation, both DGCs and haploid germ cells in plants are produced in situ from somatic cells in the organs in which they will mature. Germ cell initiation and differentiation in plants have therefore traditionally been viewed as parts of the developmental process in reproductive organ formation.

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F.Z. conceived, designed, and performed most of the experiments; Y.-F.Z., T.Z., R.S., and J.-Y.Y. provided technical assistance and performed some of the experiments; Y.L. and D.-T.R. contributed reagents/materials/analysis tools; F.Z. and S.-N.B. analyzed data and drafted the manuscript; H.M. and Z.-H.X. contributed to the experimental design and edited the manuscript; all authors read and approved the final manuscript.

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The first key regulatory gene for archesporial cells, *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*), was identified in Arabidopsis (*Arabidopsis thaliana*; Schiefthaler et al., 1999; Yang et al., 1999). In the loss-of-function *spl/nzz* mutants, initiation and differentiation of both the microsporocytes and megasporocytes fail to occur in stamens and ovules, respectively; thus, *SPL* is an invaluable target for investigating the regulatory mechanism of germ cell initiation in plant sporophytes. Recently, SPL was shown to act as an adaptor-like transcriptional repressor regulating ovule development (Wei et al., 2015). Besides its role in the initiation of archesporial cells, SPL has been reported to alter ovule polarity by interacting with members of the YABBY family (Sieber et al., 2004) and is involved in establishing stamen identity (Liu et al., 2009). SPL is also required for auxin and cytokinin signaling in ovule development (Bencivenga et al., 2012), and its overexpression affects the auxin response in lateral organs (Li et al., 2008).

Since plant archesporial cells are initiated in situ from the somatic cells of related organs, e.g. stamens and ovules, *SPL* expression is thought to be regulated by components involved in the determination of organ identity. Accordingly, Ito et al. (2004) reported that *SPL* transcription is directly regulated by AGAMOUS (AG), a protein encoded by a C-class MADS box gene required for determining the stamen and carpel identities according to the ABC model (Coen and Meyerowitz, 1991). Interestingly, they found that microsporocytes can be induced in petals if *SPL* expression is ectopically induced in the *ag* mutant, which lacks stamens and carpels (Ito et al., 2004). The *SPL* genomic region contains a bivalent chromatin domain simultaneously enriched with the transcriptionally active marker H3K4me3 and the transcriptionally repressive marker H3K27me3 (Berr et al., 2010); the SET DOMAIN GROUP2 protein is specifically required for H3K4me3 deposition, suggesting that it could be involved in *SPL* transcriptional activation. These findings demonstrate not only the key role of SPL in archesporial cell initiation, but also its regulatory characteristics.

In addition to the floral organ identity determination genes *AG* and *APETELA3/PISTILLATA* summarized in the ABC model, other genes are also involved in early stamen development in Arabidopsis, as well as in archesporial cell initiation and differentiation. These non-ABC genes include *BARELY ANY MERISTEM1* (*BAM1*)/*BAM2* (Hord et al., 2006), *SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1/2* (Colcombet et al., 2005), *EXCESS MICROSPOROCYTES1/EXTRA SPOROGENOUS CELLS* (Canales et al., 2002; Zhao et al., 2002), *TAPETUM DETERMINANT1* (Yang et al., 2005), *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE8* (Xing et al., 2010), *ROXY1/2* (Xing and Zachgo, 2008), *ERECTA* (*ER*)/*ER-LIKE1* (*ERL1*)/*ERL2*,and *MAPK3* (*MPK3*)/*6* (Hord et al., 2008). Mutant analyses revealed that plants lacking functional copies of these genes initiated more (in *bam1 bam2* and *excess microsporocytes/extra sporogenous cells* mutants) or fewer (in *roxy1 roxy2*, *er erl1 erl2*, and *mpk3/+ mpk6/−* mutants) archesporial cells in their anthers. However, it is not known whether these genes affect archesporial cell initiation through the direct regulation of SPL.

The Arabidopsis MPK cascade is involved in responses to a variety of environmental signals, including abiotic stress such as water, salt, cold, osmotic pressure, and redox status, as well as biotic stress such as pathogens (Rodriguez et al., 2010). In addition, MPKs are involved in the developmental regulation of stomata (Wang et al., 2007; Lampard et al., 2008), ovules (Wang et al., 2008), anthers (Hord et al., 2008), and pollen tube growth (Guan et al., 2014). While the *mpk3 mpk6* double mutation is embryo lethal, Hord et al. (2008) found that *mpk3/+ mpk6/−* fails to form one or more of the four anther locules and sometimes exhibits defects in anther dehiscence. Although no molecular mechanism was elucidated, Hord et al. (2008) showed that *MPK3/6* affect *SPL* expression and are therefore good candidates for the further investigation of *SPL* regulation.

In this study, we report that *MPK3/6* do indeed affect the differentiation of archesporial cells during early anther development. We show that MPK3/6 phosphorylate SPL, which enhances SPL protein stability and is required for its function in archesporial cell differentiation. These findings uncover a new regulatory layer for SPL, and provide a novel way to decipher the SPL-centered regulatory mechanisms in Arabidopsis anther development, especially for archesporial cell initiation and differentiation.

RESULTS

MPK3/6 Affect the Differentiation of Archesporial Cells in the Arabidopsis Anther

While *MPK3/6* play crucial roles in lobe formation and anther cell differentiation (Hord et al., 2008), it is not yet clear whether or how they affect the differentiation of the archesporial cells in the anther. To clarify this, we compared cross sections of early stamen development (stages 1–6; Sanders et al., 1999; Ma, 2005) in the *mpk3/+ mpk6/−* mutant and wild-type Arabidopsis. The cellular characteristics of the mutant and wild-type plants were barely distinguishable until stage 2 (Fig. 1A), as previously reported (Hord et al., 2008). In stage 3, the adaxial differentiation of some mutant stamens was delayed; the primary parietal cell (PPC) did form, but further division and differentiation were affected, especially in adaxial locules. Strikingly, this phenotype resembled that of *spl* (Schiefthaler et al., 1999; Yang et al., 1999). After stage 4, it was clear that cell differentiation on the adaxial side of the mutant anthers was inhibited, while the abaxial side continued to differentiate similarly to the wild-type plants (Fig. 1A; Supplemental Fig. S1).

To test whether the relatively normal development on the abaxial side of anthers in the *mpk3/+ mpk6/−* mutant resulted from the heterozygosity of *MPK3*, we examined MPK3 localization using a commercial anti-MPK3 antibody, whose specificity was previously

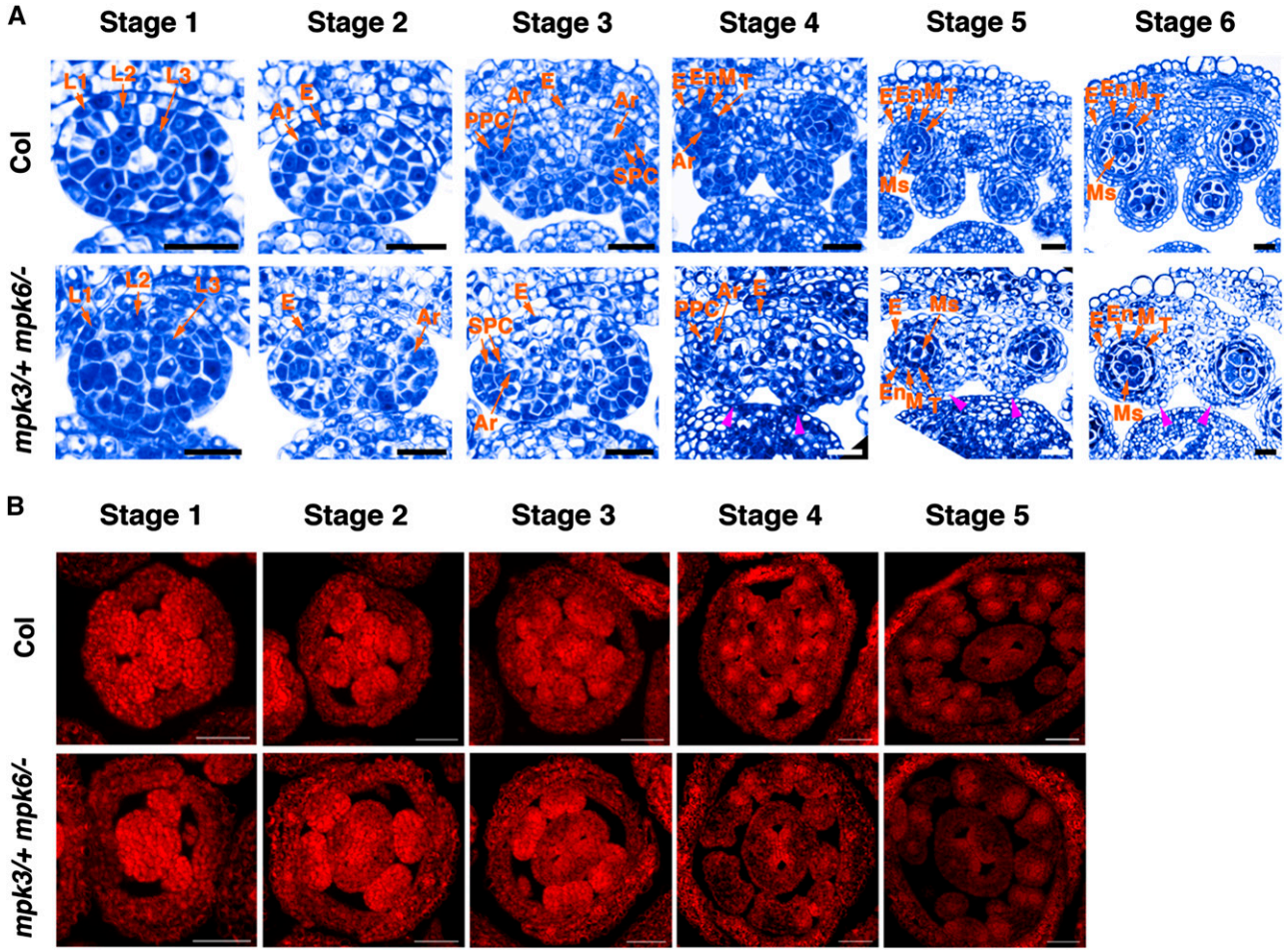

Figure 1. Phenotypic analysis of *mpk3/+ mpk6/−* anthers. A, Semithin cross sections of Col and *mpk3/+ mpk6/−* anthers. At stage 1, both Col and *mpk3/+ mpk6/−* anthers show three distinguishable types of cell layers. Archesporial cells (Ar, also called prearchesporial cells; Walbot and Egger, 2016) form in both genotypes at stage 2. Further cell division of the archesporial cells occurs in Col and *mpk3/+ mpk6/−* at stage 3; however, the PPC and Ar do not undergo further cell division and/or differentiation in *mpk3/+ mpk6/−* adaxial locules at stage 4 (magenta arrow head). Some cell differentiation defects also appear in *mpk3/+ mpk6/−* abaxial locules, which sometimes contained vacuolated cells instead of Ar. In most cases, microsporocytes (Ms) could be distinguished in *mpk3/+ mpk6/−* at stages 5 and 6; however, the surrounding cell layers, such as the middle layer, are often irregularly shaped and sometimes missing. Orange arrows indicate different cell types in serial anther developmental stages. Magenta arrowheads indicate abnormal locules without Ar (at stage 4) and Ms (at stages 5 and 6). *L1*, *L2*, and *L3*, The three cell layers in stamen primordia; Ar, archesporial cell; E, epidermis; PPC, primary parietal cell; SPC, secondary parietal cell; En, endothecium; M, middle layer; T, tapetum; Ms, microsporocytes. Bars = 20 μm. For the quantitative analysis of *mpk3/+ mpk6/−*, see Supplemental Figure S1. B, Immunolocalization of MPK3 in Col (wild type) and *mpk3/+ mpk6/−* inflorescences, using an anti-MPK3 antibody. The different fluorescence patterns shown in Figure 3E could be used as a negative control with anti-MPK6 and a second antibody. For an additional negative control, see also Supplemental Figure S2. Bars = 50 μm.

demonstrated (Wang et al., 2013). While no difference in MPK3 protein distribution was observed during stamen developmental stages 2 and 3 between the wild type and the *mpk3/+ mpk6/−* mutant, MPK3 intensity at adaxial locules was obviously reduced in stage 4 stamens in the *mpk3/+ mpk6/−* mutant (Fig. 1B; Supplemental Fig. S2). Although it is yet unknown how the localization of MPK3 changes during anther development, this pattern of distribution was consistent with the cellular differentiation phenotype of *mpk3/+ mpk6/−*. These data further demonstrated the requirement for MPK3/6 in the differentiation of archesporial cells, perhaps in a dosage-dependent manner, and implied the presence of regulatory mechanisms underlying MPK protein distribution, which was not explored further here.

Hord et al. (2008) reported the decreased expression of *SPL* in the *mpk3/+ mpk6/−* mutant; however, they tested *SPL* expression in inflorescences, which included older flowers expressing *SPL* in the microsporocytes. To further explore the relationship between *MPK3/6* and *SPL*, we carried out detailed analyses of *SPL* expression very

early in stamen development. We first generated a new *SPL* promoter cassette, which included the whole genomic sequence except for the first exon (Supplemental Fig. S3A) and then cloned GUS and *SPL-Myc* fusion DNA sequences into it. In P_{SPL}:*GUS* transgenic plants, the observed signal was identical to the previously reported *SPL* expression pattern (Fig. 2A; Schiefthaler et al., 1999; Yang et al., 1999; Ito et al., 2004). Moreover, the P_{SPL}:*SPL-Myc* transgene could rescue the *spl* mutant phenotype (Supplemental Fig. S3B), confirming the biological activity of this promoter. We crossed the P_{SPL}:*GUS* marker line with the *mpk3/+ mpk6/−* mutant and found that *SPL* promoter activity was not significantly altered in the young stamen of *mpk3/+ mpk6/−* (Fig. 2A). In situ hybridization revealed that the *SPL* expression level was comparable in the mutant and the wild-type anthers from stage 1 to stage 3 (Fig. 2B), although cell differentiation was retarded as early as stage 3 in *mpk3/+ mpk6/−* (Fig. 1A). These results suggested that the phenotypes of the *mpk3/+ mpk6/−* mutant stamen were not likely to be caused by a decrease in *SPL* transcription during early stamen development.

MPK3/6 Interact with SPL

To explore whether *MPK3/6* and *SPL* genetically interact to affect the differentiation of archesporial cells, we crossed heterozygous *spl/+* with *mpk3/+ mpk6/−* and found that the F2 plants with the *spl/− mpk3/+ mpk6/−* genotype had a similar anther phenotype to *spl* (Supplemental Fig. S4). This demonstrates that *MPK3/6* function through *SPL* in the same genetic pathway during the early development of the Arabidopsis stamen.

SPL was previously identified as key regulator in the initiation and differentiation of archesporial cells (Schiefthaler et al., 1999; Yang et al., 1999), and our above findings indicated that it genetically interacts with *MPK3/6*. Following the finding that *MPK3/6* affect the differentiation of archesporial cells without affecting *SPL* transcription, we tested whether MPK3/6 and SPL proteins interact by carrying out a yeast two-hybrid assay. Both MPK3 and MPK6 interacted with SPL (Fig. 3A), which was further demonstrated in vitro and in vivo by pull-down and bimolecular fluorescence complementation assays, respectively (Fig. 3B; Supplemental Fig. S5; Fig. 3C).

Interestingly, MPK3 and MPK6 exhibited differing overlaps in expression with SPL during early stamen development: MPK3 was distributed throughout the stamen primordium before stage 4, significantly more widely than SPL and then became concentrated in the area in which archesporial cells and related cells differentiated (Fig. 3D). By contrast, MPK6 did not colocalize with SPL in the stamen primordium before stage 4, but did during stage 5 (Fig. 3E). Intriguingly, MPK3, but not MPK6 or SPL, was detectable in the clearly differentiated archesporial cells (Fig. 3, D and E). This result echoed the correlation of MPK3 distribution with the differentiation of archesporial cells in the anther.

MPK3/6 Phosphorylate SPL

Since MPK3 and MPK6 interacted with SPL and are protein kinases, we reasoned that MPK3/6 were likely to affect SPL function through phosphorylation. To test this hypothesis, we carried out in vitro phosphorylation assays (Mishra et al., 2006) using recombinant SPL and MPK3 or MPK6. Both MPK3 and MPK6 phosphorylated the recombinant SPL protein (Fig. 4, A and B). By searching the SPL protein sequence, we found two phosphorylation sites commonly targeted by MPKs (Gonzalez et al., 1991; Supplemental Fig. S6A). To test whether the phosphorylation by MPK3/6 occurs at these sites, we generated site-specific mutant forms of the recombinant SPL protein and performed in vitro kinase assays with MPK3 and MPK6. The phosphorylation of SPL was significantly reduced when the candidate MPK-target serines 141 (S141) and 304 (S304) of SPL were substituted with alanines (S141A and S304A), and almost disappeared when both S141 and S304 were mutated to alanines (Fig. 4, C and D). These results suggest that the SPL protein could be phosphorylated by MPK3/6 at S141 and S304.

MPK3/6-Mediated SPL Phosphorylation Is Necessary and Sufficient for the Differentiation of Archesporial Cells on the Adaxial Side of the *mpk3/+ mpk6/−* Mutant Anther

If the phosphorylation of SPL were necessary and sufficient for its function in the differentiation of archesporial cells, the dephosphorylated SPL would not be able to rescue the *spl* mutant phenotype, and the mimic of phosphorylated SPL should rescue the *mpk3/+ mpk6/−* mutant phenotype. Accordingly, we generated transgene constructs driven by the native *SPL* promoter with either a single Ser-to-Ala point mutation (SPL^{S141A} or SPL^{S304A}) or mutations at both S141 and S304 (SPL^{AA}) to mimic the dephosphorylated state (Dayhoff, 1978). In addition, we generated the analogous Ser-to-Asp single mutations (SPL^{S141D} and SPL^{S304D}) or mutations at both Ser sites (SPL^{DD}) to mimic the phosphorylated state (Thorsness and Koshland, 1987). The three phosphorylation mimics and the three dephosphorylated forms of SPL were fused with a Myc tag and integrated into the *mpk3/+ mpk6/−* and *spl* mutants. Wild-type *SPL* fused to a *Myc* tag was used as a control for the functional assessment of SPL phosphorylation.

We analyzed the anther phenotypes of the transgenic plants using locule number and pollen density as criteria. We found that, unlike the wild-type SPL and the SPL^{DD}, SPL^{S141A}, SPL^{S304A}, and SPL^{AA} were unable to rescue the *spl* mutant phenotype (Fig. 5A), indicating that SPL phosphorylation is necessary for its function in the differentiation of archesporial cells in the anther. A similar investigation revealed that mimics of phosphorylated SPL (SPL^{S141D}, SPL^{S304D}, and SPL^{DD}) successfully rescued the *mpk3/+ mpk6/−* mutant phenotype while the dephosphorylated SPL did not (Fig. 5B), indicating that SPL phosphorylation is sufficient for its function in the differentiation of archesporial cells in the anther. The

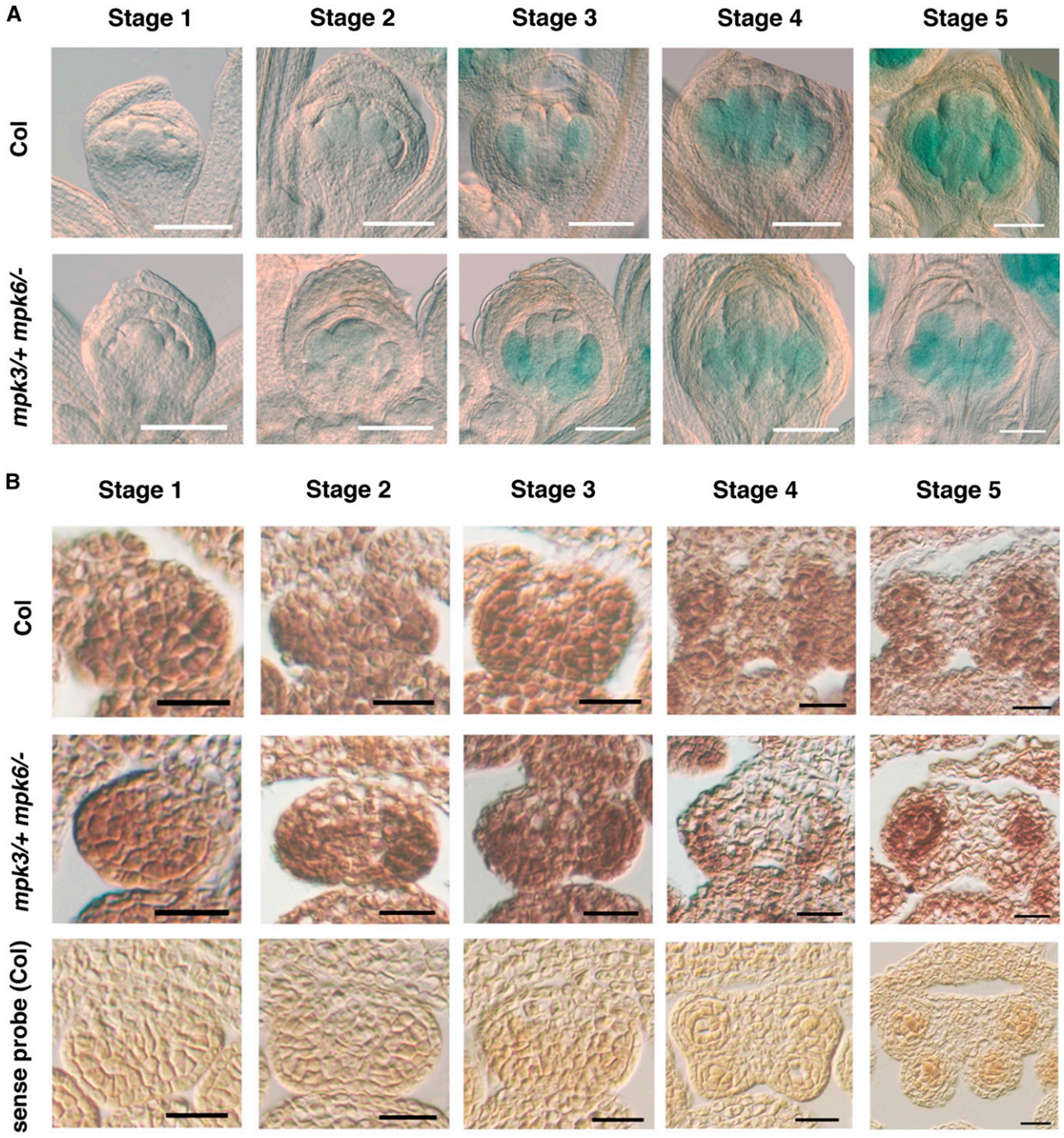

Figure 2. Transcript levels of *SPL* in *mpk3/+ mpk6/−*. A, P_{SPL}:*GUS* in different backgrounds. A P_{SPL}:*GUS* line was crossed with *mpk3/+ mpk6/+*. In F3, inflorescences of P_{SPL}:*GUS* plants in the *mpk3/+ mpk6/−* background were selected for further GUS staining. The GUS signals are comparable in *mpk3/+ mpk6/−* stamens and Col, indicating the presence of the *SPL* promoter construction and its activity (see also Supplemental Fig. S3). Bars = 100 μm. B, *SPL* in situ hybridization in different backgrounds. The *SPL* transcript levels in Col and the *mpk3/+ mpk6/−* mutant anther are indistinguishable before stage 3. The sense probe is the negative control. Bars = 20 μm.

latter experiment further demonstrated that SPL is a target of MPK3/6 phosphorylation and functions downstream of MPK3/6. Interestingly, compared to S304 site, S141 showed stronger rescue effects in both complementation assays (Fig. 5, A and B), indicating that the extent of in vivo phosphorylation of SPL may be different at these two sites.

While little is known about the molecular mechanism of SPL involved in the differentiation of archesporial cells, the expression of several genes required for anther

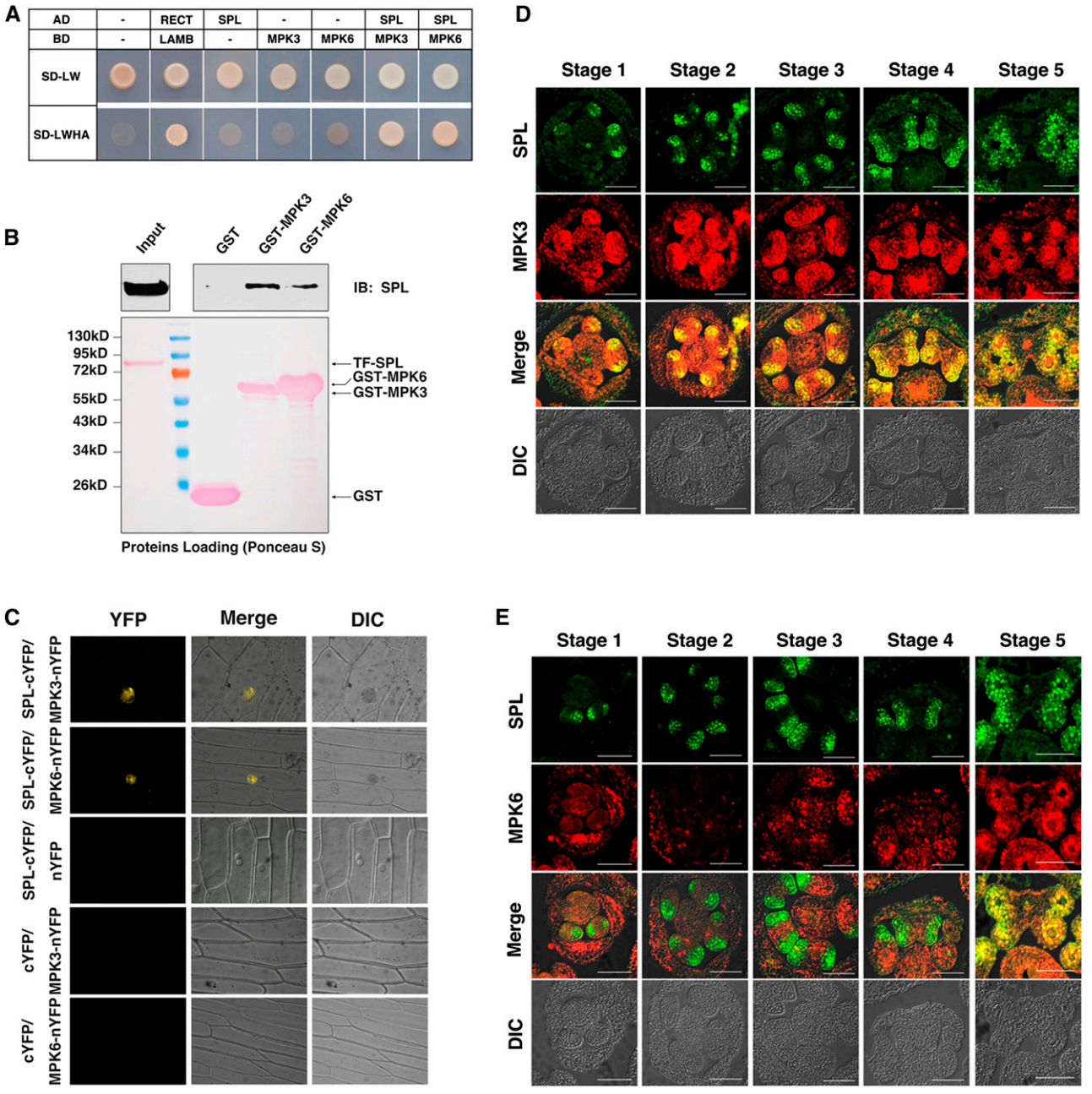

Figure 3. MPK3/6 interact with SPL. A, Yeast two-hybrid assay of MPK3/6 and SPL interaction in yeast. *RECT/LAMB* pairs serve as a positive control, with pGBKT7 and pGADT7 empty plasmids pairs as negative controls. SD-LW, Synthetic dropout medium without Leu and Trp; SD-LWHA, synthetic dropout medium without Leu, Trp, His, and adenine. B, Pull-down assay, which shows that MPK3/6 interact directly with SPL in vitro. Ponceau S indicates the loading control. The negative control is shown in Supplemental Figure S5. IB, Immunoblot of recombinant SPL with a SPL antibody; TF-SPL, SPL protein fused with trigger factor (TF) at N-terminal part; GST, glutathione *S*-transferase. GST-MPK3/MPK6, GST fusion at the N terminus of MPK3 or MPK6. C, Bimolecular fluorescence complementation assay in onion epidermal cells showing that MPK3/6 interact with SPL in plant cells. cYFP, C-terminal version of YFP; nYFP, N-terminal version of YFP; DIC, differential interference contrast. D and E, Colocalization of MPK3 (D) and MPK6 (E) with SPL in transverse section anthers of P_{SPL}:*SPL-Myc/spl* transgenic plants. The specificity of anti-MPK3 and anti-MPK6 antibodies was previously shown (Wang et al., 2013). The different signals of MPK3 and MPK6 can be used as negative controls for each other. For further negative controls, see also Supplemental Figure S2. Bars = 50 μm.

development is known to be regulated by *SPL* function (Ge et al., 2010; Chang et al., 2011). To further verify that SPL phosphorylation is required for its function, we examined expression levels of genes downstream of SPL activity in the transgenic SPL-phosphorylation mimics in the *spl* background. The expression levels of

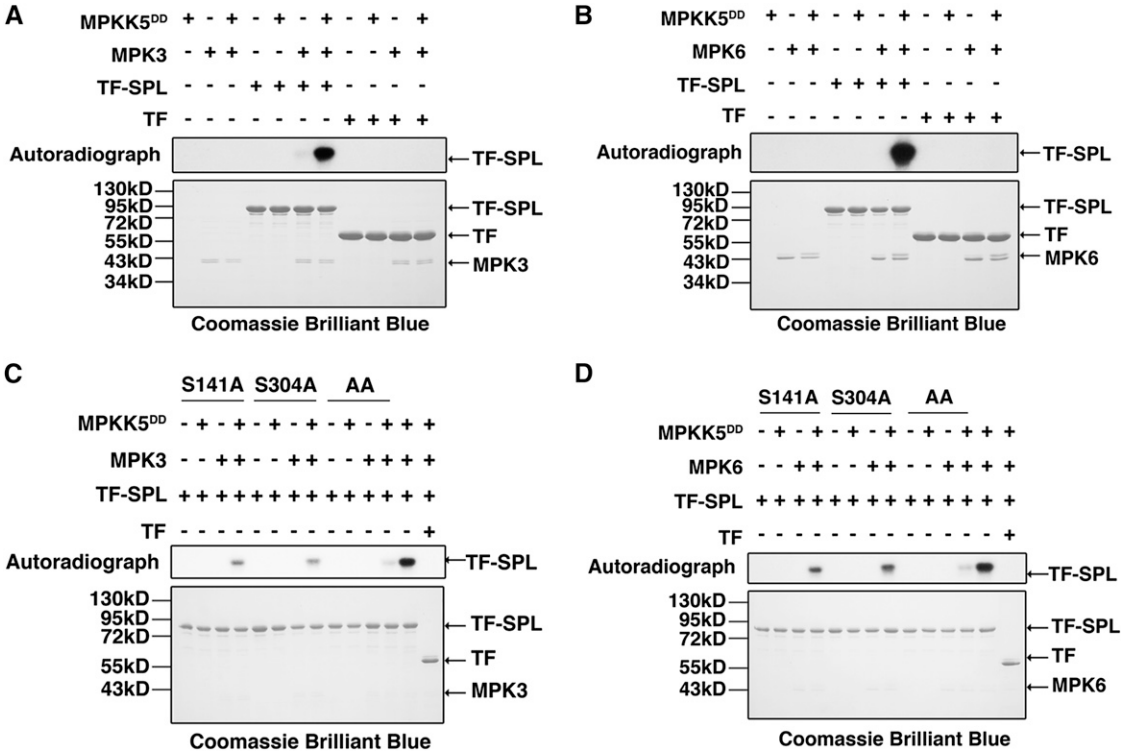

Figure 4. In vitro kinase assay. A and B, SPL is phosphorylated by MPK3 (A) and MPK6 (B) in vitro. Recombinant proteins with FLAG (for MPKK5^{DD}), His (for MPK3 and MPK6), and trigger factor (TF; for SPL) tags were purified for in vitro kinase assay as described (Xu et al., 2008). Constitutively active MPKK5 (MPKK5^{DD}) was used to activate MPK3 and MPK6. After the reaction, phosphorylated proteins were visualized using autoradiography. Reactions with various components omitted (−) were controls. The same type and amount of proteins were incubated with unlabeled ATP. After electrophoresis, the gels were stained by Coomassie Brilliant Blue as a loading control. C and D, MPK3 (C) and MPK6 (D) kinase assays were carried out as in A and B, except with mutant SPL proteins in which S141 and S304 were converted into alanines (S141A and S304A, respectively). AA, S141A and S304A double mutant protein.

all five genes, *ROXY2*, *DYSFUNCTIONAL TAPETUM1*, *MYB35*, *AMS*, and *MALE STERILITY1*, were restored in the lines containing mimics of phosphorylated SPL (SPL^{DD}) to almost the same levels as those in wide-type Arabidopsis, but not in the lines containing dephosphorylated SPL (SPL^{S141A}, SPL^{S304A}, and SPL^{AA}; Fig. 5C).

To precisely monitor the effects of mutated SPL on anther development, we examined paraffin sections of *SPL^{AA}* transgenic lines in the *spl* background and *SPL^{DD}* in the *mpk3*/+ *mpk6*/− background. We found that, consistent with the above quantitative analyses, *SPL^{AA}* did not rescue the *spl* mutant phenotype (Fig. 5D). This finding further strengthened the conclusion that SPL phosphorylation is necessary for its function in the differentiation of archesporial cells. Observations of anther development in transgenic *SPL^{DD}* lines in the *mpk3*/+ *mpk6*/− background revealed that the mutant defect in adaxial anther development was successfully rescued by *SPL^{DD}* (Fig. 5E). This supported the conclusion that SPL is a target of MPK3/6 and that SPL phosphorylation is sufficient to rescue the defective differentiation of archesporial cells in the *mpk3*/+ *mpk6*/− mutant. Furthermore, detailed observations revealed that, although archesporial cells differentiated on the abaxial side of anthers in the *mpk3*/+ *mpk6*/− mutant, the cellular differentiation was still affected to some extent (Figs. 1A and 5E). This minor defect was also partially rescued by the introduction of *SPL^{DD}* (Fig. 5E).

Phosphorylation Affects SPL Protein Stability

The above results demonstrate that SPL phosphorylation is necessary and sufficient for its function in the differentiation of archesporial cells in the context of MPK3/6. However, the question of how phosphorylation affects the function of SPL remained. We have found a dosage-dependent defect in *mpk3*/+ *mpk6*/− mutant (Fig. 1; Supplemental Fig. S1). Together with the observation that the SPL protein was quite unstable and readily degraded, we speculated that phosphorylation might affect its stability. To test this hypothesis, we first conducted an in vitro assay with a cell-free system (Wang et al., 2009) in which the recombinant proteins were tested for their degradation rate using an antibody against SPL. The recombinant SPL was degraded gradually, and this degradation was inhibited by the

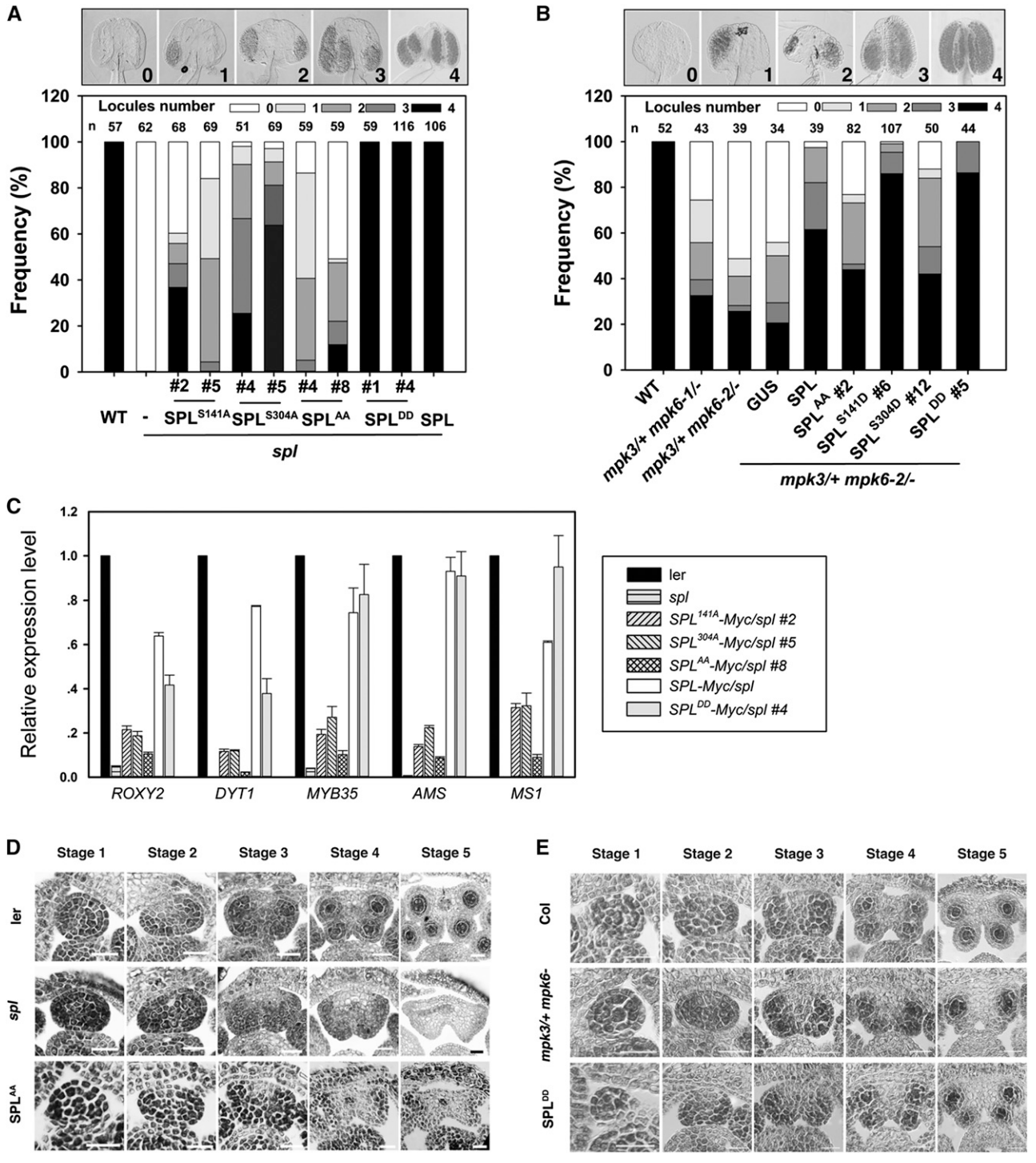

Figure 5. The phosphorylation of SPL is necessary and sufficient for archesporial cell differentiation. A and B, Quantitative analysis of the number of locules bearing pollen in transgenic plants in the *spl* background (A) and the *mpk3/+ mpk6/−* background (B). Top, Alexander staining assay to indicate the pollen density, numbers in the pictures indicate the number of locules with stained pollen; the n values in diagrams indicate the number of stamens counting in different plants. For the *SPL* transcript levels in the indicated lines, see also Supplemental Figure S7, A and B. All variants of SPL shown in A and B were fused with the Myc tag and were driven by the *SPL* promoter. "−" indicates empty *SPL* promoter cassette. "GUS" indicates P_{SPL}:*GUS* construction. C, Quantitative real-time PCR analysis of the expression of genes downstream of *SPL* in the inflorescences of plants with normal and nonphosphorylatable SPL. The materials in diagram correspond to those designated in A. The Arabidopsis *GAPDH* gene was used as an internal control. Error bars represent SD from three independent experiments. D, Paraffin sections of anthers of the transgenic line SPL^{AA} in a *spl* background, as in A. L*er* is the wild-type control. *spl* is the background control. Bars = 20 μm. E, Paraffin sections of anthers of the transgenic line SPL^{DD} in *mpk3/+ mpk6/−* background, as in B. Col is the wild-type control. *mpk3/+ mpk6/−* is the background control. Bars = 20 μm.

protease inhibitor MG132 (Fig. 6A), implying that the ubiquitin-mediated 26S proteasome system (Palombella et al., 1994) is involved in SPL degradation. Similar degradation was detected in transgenic plants containing Myc-tagged SPL (SPL-Myc) using an antibody against the Myc-tag (Fig. 6B). These results indicated that SPL degradation is mediated by the 26S proteasome in vitro and likely in vivo.

Using this assay for SPL degradation, we measured the degradation rate in the SPL-rescued *spl* mutant and the SPL-transformed *mpk3/+ mpk6/−* mutant. The former was assumed to have functional in vivo phosphorylation machinery (wild-type *MPK3/6*) and the latter to have defective machinery (mutated *MPK3/6*). SPL protein degradation was significantly faster in the SPL-transformed *mpk3/+ mpk6/−* mutant line than in the SPL-rescued *spl* mutant line (Fig. 6, C and D), indicating that SPL protein stability was correlated with the presence of MPK3/6. These results suggest that MPK3/6-mediated phosphorylation promotes SPL stability.

To further explore the effect of phosphorylation on SPL stability, we measured the degradation rate of wild-type SPL, the three phosphorylation mimics, and the three dephosphorylated forms in the *spl* mutant background. While mimics of phosphorylated SPL (SPL^{DD}) were more stable than wild-type SPL, the mimic of dephosphorylation at the two S141 and S304 sites (SPL^{AA}) resulted in a significantly faster degradation than wild-type SPL (Fig. 6, D and E), as rapid as that of SPL in the *mpk3/+ mpk6/−* mutant background. Dephosphorylation at either of the S141 or S304 single sites led to an intermediate rate of degradation. This quantitative analysis further demonstrated that phosphorylation affects SPL protein stability in a site-specific and dose-dependent manner.

DISCUSSION

In this work, to elucidate the regulatory mechanism of germ cell differentiation in plants, we confirmed that MPK3/6 are required for the differentiation of archesporial cells (Hord et al., 2008) and demonstrated that MPK3/6 interact with and phosphorylate SPL. We revealed that phosphorylated SPL is necessary and sufficient for the differentiation of archesporial cells in the anther and that phosphorylation maintains the stability of the SPL protein. These findings suggest that a comprehensive SPL-centered regulatory mechanism is present in Arabidopsis to ensure the spatiotemporally and quantitatively precise differentiation of archesporial cells. Several characteristics of such regulation could be identified based on our findings.

SPL Phosphorylation Might Strengthen the Spatial and Quantitative Stability of Archesporial Cell Differentiation in the Anther

After Yang et al. (1999) and Schiefthaler et al. (1999) revealed the key role of *SPL* in germ cell initiation, Ito et al. (2004) demonstrated that the transcriptional regulation of *SPL* expression by the AG protein is required for stamen and carpel identity. Here, we found that posttranslational phosphorylation is required for SPL function. Although multiple mechanisms regulating the function of a gene are not rare in nature, why is the SPL function regulated at multiple layers?

During anther development, archesporial cells (DGCs) are initiated at the four corners of the anther in most angiosperms (Esau, 1977; Goldberg et al., 1993), but it is not clear how the pattern is formed and/or regulated. *AG* expression is restricted to the region where germ cells will initiate (Bowman et al., 1991), consistent with *SPL* expression upon activation by AG; our current results suggest that posttranslational phosphorylation provides an additional parameter to precisely restrict SPL localization and facilitate the proper spatiotemporal pattern of archesporial cell differentiation. This conclusion is supported by the findings that the MPK3 protein is localized to the region of germ cell induction (Fig. 1B; Col), the SPL protein is colocalized with MPK3 (Fig. 3D), and MPK3 localization is affected by MPK6 (Fig. 1B; *mpk3/+ mpk6/−*). These results suggest that at least two steps are required for the spatiotemporally regulated differentiation of the archesporial cells in the Arabidopsis anther. First, AG activates *SPL* transcription in the proper microenvironment for germ cell initiation. Second, after the production of the RNA and protein, the phosphorylation of SPL strengthens the spatial and quantitative stability of the protein for the differentiation of archesporial cells in the Arabidopsis anther. Similar multicellular regulation has been observed for DNase activity in female flower development of cucumber (*Cucumis sativus*), where the expression of DNase is induced by ethylene in both the carpel and stamen primordia, but the enzymatic activity is restricted to anthers specifically after posttranslational modification (Gu et al., 2011).

MPK3/6 May Mediate Environmental Stress Signals for DGC Induction

DGCs are cells destined to undergo meiosis. Although it is not yet clear how meiosis originated in early evolution (Dacks and Roger, 1999; Cavalier-Smith, 2002; Marston and Amon, 2004; Ma, 2005; Ramesh et al., 2005; Cooper et al., 2007), the increased genetic variation among the progeny derived from meiosis was ultimately selected as a mechanism for adaptation to environmental stresses (Bai and Xu, 2013; Bai, 2015b). Unlike unicellular organisms such as yeast that are directly challenged at the individual cell level by environmental stresses, animal germ cells are well protected during embryogenesis (Ermolaeva et al., 2013), and plant germ cells are protected by various reproductive organs in plants ranging from moss to angiosperms (Gifford and Foster, 1989; Goldberg et al., 1993; Bai, 2015a, 2015b). It is therefore interesting to consider whether germ cell induction is affected by environmental stresses in multicellular animals and plants. While little information is available in animals, Kelliher and Walbot (2012) reported that a reduced redox status is required for the induction of archesporial cells in the

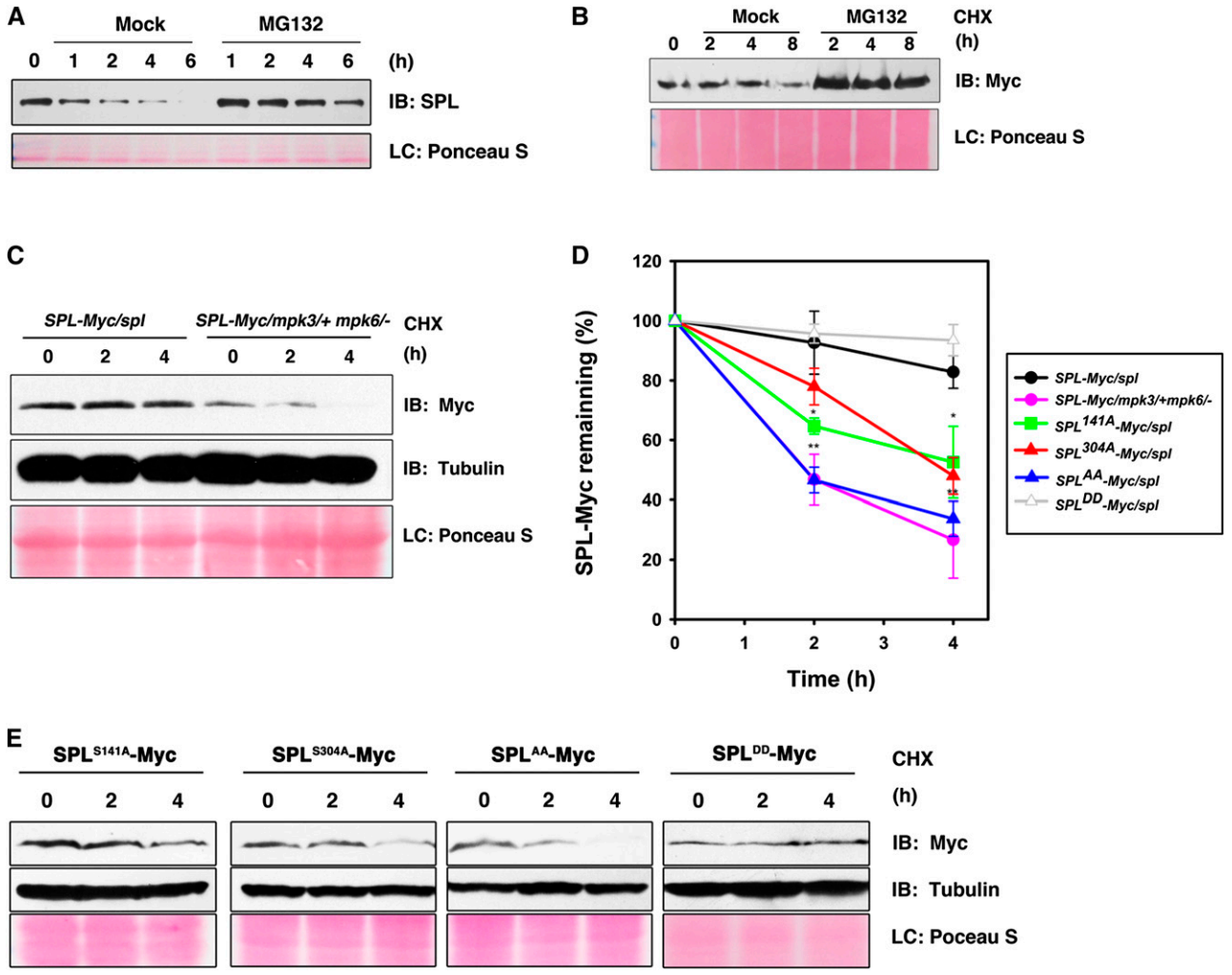

Figure 6. Phosphorylation affects SPL stability. A, Cell-free degradation assay of a recombinant SPL protein. Protein extracts were prepared from Col inflorescences and then incubated with or without MG132 over the indicated time course. Ponceau S indicates the extracted protein loading control. IB, Immunoblot with SPL antibody; LC, loading control. B, In vivo SPL degradation assay. The inflorescences of P_{SPL}:*SPL-Myc/spl* transgenic plants were incubated in liquid Murashige and Skoog medium with or without MG132 over the indicated time course. DMSO was used as a negative control. CHX (cycloheximide) was used to block protein biosynthesis. Ponceau S indicates the extracted protein loading control. IB, Immunoblot with anti-Myc antibody; LC, loading control. C, In vivo SPL degradation in *mpk3/+ mpk6/−* occurred faster than in the P_{SPL}:*SPL-Myc/spl* complementation line. The inflorescences were incubated in liquid Murashige and Skoog medium using CHX to block protein biosynthesis and then harvested at the indicated time course for immunoblotting. IB, Immunoblot with c-Myc antibody to detect endogenous SPL protein, with an anti-α-tubulin antibody as the internal control. Ponceau S indicates the extracted protein loading control. D, A statistical analysis of the SPL degradation rate in C and E. Error bars represent SD from three experiments. $*P < 0.05$, $**P < 0.01$, Student's *t* test. E, Nonphosphorylated SPL degraded faster and phosphorylated SPL degraded slower than wild-type SPL protein in vivo.

maize anther, consistent with the notion that meiosis originated as a stress-responsive process.

Among the highly divergent roles of MPK in plant signaling, their involvement in responses to ozone and reactive oxygen species are well documented (Wang et al., 2013). Plants are photoautotrophic organisms and their default pathway for energy acquisition is photosynthesis. If the induction of archesporial cells requires a hypoxia condition as indicated by Kelliher and Walbot (2012), there must be a mechanism to create a proper microenvironment for germ cell initiation. The Arabidopsis anther has well-differentiated chloroplasts (Chen et al., 2015) and is thought to be photosynthetically active; thus, the anthers would constantly release oxygen. If this is the case, it is reasonable to hypothesize that MPK3/6 may play a role in early stamen development in Arabidopsis, responding to the oxidative status and stabilizing the SPL protein via phosphorylation to ensure germ cell formation. Moreover, it has been reported that not only could oxidative signals change protein phosphorylation under certain conditions (Hardin et al., 2009), but protein phosphorylation could in turn regulate redox homeostasis

(Grieco et al., 2012). From this perspective, it will be important to investigate whether the MPK3/6-mediated phosphorylation of SPL is essential for the establishment of a hypoxic niche for germ cell initiation, as found in maize.

The SPL-Centered Regulatory Mechanism for the Archesporial Cell Differentiation Is Likely Conserved in the Brassicaceae Family

In angiosperms, stamen development is highly conserved; not only in the morphogenetic processes, but also in genes determining organ identity (Goldberg et al., 1993; Causier et al., 2010; Litt and Kramer, 2010; Rijpkema et al., 2010). It is therefore reasonable to anticipate similarity in mechanism for the initiation of archesporial cells (DGCs). However, sequence comparison of SPL reveals that while distantly related sequences can be traced back to the mosses (*Physcomitrella patens*; Supplemental Fig. S6B), there is dramatic sequence divergence in the examined species. Nevertheless, SPL homologs from members of the Brassicaceae family have a conserved domain containing the predicted MPK phosphorylation sites (Supplemental Fig. S6C). Although MPKs have highly conserved functional motifs among angiosperms (MAPK Group, 2002), we did not find a predicted *SPL* homolog with any known functional domain in the grass genomes (Supplemental Fig. S6D). This result suggests that while the stamen developmental process is conserved, as is meiosis, some of the regulatory mechanisms of the induction and differentiation of archesporial cells might vary from species to species. Such a scenario points to exciting opportunities for future comparative studies into the mechanisms of induction and differentiation of archesporial cells, not only between animals and plants, but also among plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The Arabidopsis (*Arabidopsis thaliana*) mutant lines *spl* (Yang et al., 1999), *mpk3/+ mpk6-1/−*, and *mpk3/+ mpk6-2/−* (Hord et al., 2008) were described previously. Except where mentioned in Figure 5B, "*mpk3/+ mpk6/−*" refers to the *mpk3/+ mpk6-2/−* line. Col plants were used as the wild-type control for the *mpk* mutants. L*er* plants were used as wild-type control for the *spl* mutant. The seeds were transplanted to soil after germination on Murashige and Skoog medium and were grown under long-day conditions (16 h light/8 h dark; light bulb, Philips 28 W 840 neon, 4,000K, 103 lm/W) at 22°C.

Characterization of the Anther Phenotype

Pollen viability was determined by staining flowers with Alexander's staining solution (Alexander, 1969) for at least 10 h. Stamens were then dissected from the flowers onto slides and sealed with chloral hydrate solution (4 g chloral hydrate, 1 mL glycerol, and 2 mL deionized water). Images were taken with differential interference contrast optics using an Olympus BX51 microscope. The number of locules (with pollen) was counted, and quantitative analysis was carried out using Microsoft Excel. Inflorescences and flower buds were prepared for paraffin and semithin sectioning as described previously (Xu et al., 2005; Wang et al., 2010). Images were captured using a Zeiss microscope (Axio Imager D2), processed by ZEN Lite 2011 (blue edition; Carl Zeiss), and edited using Photoshop CS6 (Adobe Systems).

Quantitative Real-Time PCR

Total RNA was extracted from inflorescences using the RNeasy Plant Mini Kit (Qiagen). After digestion by DNase I (Promega), 4 μg of RNA was used for reverse transcription with SuperScript III Reverse Transcriptase (Invitrogen). Reverse transcription products were diluted 10-fold for quantitative real-time PCR. Quantitative real-time PCR analyses were performed with the SYBR *Premix Ex Taq* (TaKaRa) using the Applied Biosystems 7500 real-time PCR system. The sequences of primers are listed in Supplemental Table S1.

Immunofluorescence Localization Assay

Inflorescences were soaked in a fixation solution (4% paraformaldehyde in 1× PBS, pH 7.4, and 0.1% Triton X-100), vacuum infiltrated for 5 min, and incubated overnight at 4°C. Paraffin sectioning was carried out as described previously (Xu et al., 2005; Wang et al., 2010), except that the 8-μm sections were spread on poly-Lys-coated slides. After blocking in 1× PBS containing 3% (w/v) BSA for 1 h (at room temperature) or overnight (at 4°C), the sections were incubated with primary antibodies in blocking solution (MPK3 antibody dilution, 1:500; MPK6 antibody dilution, 1:2,000; Myc antibody dilution, 1:500) for 3 to 4 h (at room temperature) or overnight (at 4°C). Secondary anti-rabbit Alexa Fluor 594 (Jackson) and anti-mouse Fluor 488 (Jackson) antibodies were diluted at 1:500 in PBS and incubated for 4 h at room temperature. Images were photographed using a Zeiss microscope with ZEN 2011 software and edited using Photoshop CS6. Similar results were obtained from at least three biological replicates.

In Vitro Pull-Down Assays

The cDNA fragments of *MPK3* and *MPK6* were cloned into pGEX-4T-2 (Amersham Biosciences), generating expression vectors for *GST-MPK3* and *GST-MPK6*. The expression vector for *Trigger Factor* (*TF*)*-SPL* was generated by cloning the *SPL* cDNA fragment into pColdTF (TaKaRa). The pGEX-4T-2 and pColdTF vectors were used to express *GST* and *TF*, which served as negative controls. Recombinant proteins were expressed in *Escherichia coli* BL21 cells. The expression of *GST-MPK3*, *GST-MPK6* and *GST* were induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside under 20°C for 16 h. The expression of *TF-SPL* and *TF* were induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside under 16°C for 20 h, following the manufacturer's instructions. Recombinant GST and GST-fused proteins were purified using a centrifuge tube method provided by the GST Gene Fusion System Handbook (Amersham Biosciences). Recombinant TF and TF-SPL protein were purified according to the QIAexpressionist Handbook (Fifth Edition; Qiagen). For the pull-down assays, purified His-fusion proteins were incubated with either purified GST or GST-fusion proteins and with Glutathione-Sepharose 4B beads (Pharmacia Biotech) in binding buffer (1× PBS, pH 7.0, 100 mM NaCl, 1 mM DTT, 0.5 mM EDTA, and 0.5% NP-40) for 2 h at 4°C. After washing three times with binding buffer, proteins were eluted from beads using 2× SDS loading buffer and analyzed by immunoblotting and antibodies against SPL (SAIERBIO) and TF (Takara). For details of SPL antibody, CKPGSKTGQQKQKKPT peptide of SPL protein was synthesized as immunogen and injected into rabbits. After the antigen affinity purification and specificity selection, anti-SPL polyclonal antibody was obtained. The sequences of primers are listed in Supplemental Table S1.

In Vitro Kinase Assays

The in vitro kinase assay was performed as described previously (Xu et al., 2008). Recombinant His-tagged MPK3 and MPK6 (8 μg) was activated by incubation with recombinant FLAG-MKK5^{DD} (0.8 μg) in the reaction buffer (20 mM HEPES, pH 7.5, 10 mM $MgCl_2$, and 1 mM DTT) containing 50 μM ATP at 25°C for 30 min. Activated MPK3 and MPK6 were mixed with the SPL variants at a 1:10 ratio and diluted to a total volume of 20 μL in reaction buffer. Each reaction contained 50 μM ATP and 0.1 μM Ci [γ-^{32}P]ATP and was incubated at 25°C for 30 min. The reactions were stopped by the addition of 5× SDS loading buffer and incubating at 80°C for 10 min. Samples were separated on 12% SDS-PAGE gels, which were dried prior to being exposed to film.

Protein Extraction and Degradation Assay

Approximately 0.02 g of Arabidopsis inflorescence was ground in liquid nitrogen and redissolved in 50 μL SDS sample loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 20% Suc, 0.02% bromophenol blue, and 10% β-mercaptoethanol), as previously described (Ng et al., 2009). The sample was boiled for 10 min before

being loaded onto an SDS-PAGE gel. The cell-free degradation assay was performed using a previously reported method (Wang et al., 2009). Approximately 0.09 g of Col inflorescence was harvested into a centrifuge tube and ground into a fine powder in liquid nitrogen. Total proteins were subsequently extracted by adding 200 μL degradation buffer (25 mM Tris-HCl, pH 7.5, 10 mM NaCl, 10 mM $MgCl_2$, 4 mM phenylmethylsulfonyl fluoride, 5 mM DTT, and 10 mM ATP) to the tube. After centrifugation, the supernatant was collected. For degradation of TF-SPL purified from *E. coli*, 5 μg of the recombinant TF-SPL protein was incubated in 100 μL of the extracts mentioned above. MG132 (Sigma-Aldrich) was added to block the 26S proteasome system, and DMSO was used as the negative control. The extracts were incubated at 25°C, with 20 μL samples taken at the indicated intervals; 5 μL 5× loading buffer was added for SDS-PAGE. The abundance of SPL protein was estimated by immunoblots using a SPL antibody. To test for the in vivo degradation of the SPL-Myc variants, the inflorescences were placed in liquid Murashige and Skoog medium with 100 μM cycloheximide (Sigma-Aldrich). MG132 was added to block the 26S proteasome system and DMSO was used as a control. Samples were taken at the indicated intervals and weighed after drying with filter paper. Proteins were extracted using loading buffer as described above and equal amounts of supernatant were loaded onto 12% SDS-PAGE gels for electrophoresis. The abundances of SPL-Myc variants were estimated using a western blot and a c-Myc monoclonal antibody (Santa Cruz Biotechnology). The results were quantified using Image J software, according to the manual. The results were obtained from three biological replicates.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Quantitative analysis of the *mpk3/+ mpk6/−* phenotype.

Supplemental Figure S2. Immunohistochemistry analysis of *MPK3* and *MPK6* expression patterns.

Supplemental Figure S3. *SPL* promoter activity.

Supplemental Figure S4. Genetic interaction between *SPL* and *MPK3/6*.

Supplemental Figure S5. Pull-down assay.

Supplemental Figure S6. Conservation of SPL protein phosphorylation sites in Arabidopsis and other *Brassicas*.

Supplemental Figure S7. *SPL* expression levels in transgenic plants.

Supplemental Table S1. Primer sequences.

Supplemental Table S2. Species and gene information for Supplemental Figure S6.

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