Mitogen-activated protein kinases MPK3 and MPK6 are required for stem cell maintenance in the Arabidopsis *shoot apical meristem*

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ORIGINAL ARTICLE

Mitogen-activated protein kinases MPK3 and MPK6 are required for stem cell maintenance in the *Arabidopsis* shoot apical meristem

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Abstract

Key message **CLV3p-mediated phosphorylation of MPK3 and MPK6 occurs via CLV1 and BAM1 receptors to regulate the maintenance of SAM development.**

Abstract The CLAVATA peptide-receptor (CLV3p-CLV1) pathway modulates a homeodomain master regulator WUSCHEL (WUS) transcription factor in the shoot apical meristem (SAM) with poorly defined signaling mechanisms. Here, we report that mitogen-activated protein kinases (MAPKs, also known as MPKs in plants) act in an intracellular signaling cascade to play an important role in the maintenance of SAM development. Interestingly, the application of exogenous CLV3p triggers rapid signaling in the SAM via dynamic activation of MPK3 and MPK6, which are positively regulated by both CLV1 and BARELY ANY MERISTEM 1 (BAM1) receptors. Surprisingly, the timing of MAPK activation is tightly correlated with the transcriptional repression of *WUS* expression in the SAM, indicating a fast CLV3p-CLV1/BAM1 signaling event. Furthermore, conditional *mpk3,6* double mutants exhibited CLV3p insensitivity in stem cell maintenance manifested by the persistent SAM growth in the presence of exogenous CLV3p signals, as well as elevated *WUS* expression and repressed WUS-specific target genes. Taken together, these results suggest that MPK3 and MPK6 activated by CLV3p signals through mainly CLV1 and BAM1 receptors are key regulators controlling stem cell homeostasis in the SAM.

Keywords *Arabidopsis* · CLAVATA receptor · MAPK cascade · Shoot apical meristem · Stem cell homeostasis

Abbreviations

MAPK (MPK)	Mitogen-activated protein kinase
SAM	Shoot apical meristem
CLV3p	CLAVATA3 peptide
WUS	WUSCHEL
BAM	Barely any meristem
LRR-RLK	Leucine-rich repeat receptor-like kinase
ER	Erecta

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Horim Lee and Ye Sol Jun contributed equally to this work.

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Introduction

Stem cells possess dual features of self-renewal and organogenesis through precisely controlled proliferation and differentiation in plants and animals (Aichinger et al. 2012; Heidstra and Sabatini 2014). Plant shoot stem cells, which are under dynamic control of the microenvironment in the primary shoot apical meristem (SAM), are the self-renewable reservoir for leaf, stem, flower and fruit organogenesis. Extensive researches have revealed a complex integration of multiple peptide receptor and hormone signaling in modulating stem cell homeostasis in the SAM (Aichinger et al. 2012; Somssich et al. 2016). The CLAVATA-WUSCHEL (CLV-WUS) regulatory pathway, involving peptide-receptor kinase (CLV3p-CLV1) and the homeodomain WUS transcription factor, plays a central role in coordinating stem cell proliferation and differentiation in the SAM of diverse

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plant species (Somssich et al. 2016). The transcript of the dual repressor-activator WUS is expressed specifically in the organizing center (OC) of the SAM, but the WUS protein represses regulatory genes promoting differentiation and migrates toward overlying stem cells via plasmodesmata to directly activate *CLV3* expression in the absence of WUS-interacting partner HAIRY MERISTEMs (HAMs) (Yadav et al. 2011, 2013; Zhou et al. 2015, 2018).

After processing, maturation and secretion, the mobile CLV3p from stem cells (Rojo et al. 2002; Kondo et al. 2006; Ni and Clark 2006; Ohyama et al. 2009) moves toward underlying cells to activate cell-surface receptor complexes and suppress *WUS* expression. Genetic and biochemical evidence supports the perception of CLV3p by leucine-rich repeat receptor-like kinase (LRR-RLK) complexes, including CLV1, BARELY ANY MERISTEM 1 and 2 (BAM1 and BAM2), RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), and a heterodimer with LRR receptor-like protein CLV2 and the transmembrane kinase CORYNE (CRN)/SUPPRESSOR OF LLP 1–2 (SOL2) (Müller et al. 2008; Guo et al. 2010; Kinoshita et al. 2010; Shinohara and Matsubayashi 2015). However, the intracellular signaling pathway restricting *WUS* expression and stem cell population via the negative feedback loop remains elusive.

Mitogen-activated protein kinase (MAPK, also called MPK in plants) cascades are evolutionarily conserved signaling modules that act as central hubs in diverse physiological and developmental processes in plants, animals and humans. Although MAPK cascades have long been proposed in SAM regulation, their molecular identities and signaling mechanisms remain unclear (Somssich et al. 2016). Over 100 *Arabidopsis* genes have been annotated to support putative MAPK cascades, including 20 *MAPK* (*MPKs*), 10 *MAPK kinases* (*MKKs*) and 80 *MAPK kinase kinases* (*MAPKKKs*) (Ichimura et al. 2002; Hamel et al. 2006). The best-characterized plant MAPKs are MPK3 and MPK6, which are redundantly involved in various signaling cascades related to innate immunity (Asai et al. 2002), abiotic stresses (Kovtun et al. 2000; Miles et al. 2005), ethylene response (Yoo et al. 2008), and stomatal and ovule development (Bergman et al. 2004; Wang et al. 2007, 2008), suggesting complexity and potentially functional redundancy of MAPK cascades in plant signal transduction networks.

In a *Nicotiana benthamiana* leaf system, it was reported that CLV3-triggered MAPK activation was negatively regulated by CLV1, but positively regulated by RPK2 based on the transient co-expression of CLV receptors and CLV3 ligands (Betsuyaku et al. 2011). In addition, the activated MPK6 phosphorylation was found in the *clv1* mutant in the absence of the application of exogenous CLV3p (Betsuyaku et al. 2011), showing that the CLV1 receptor plays a negative role in activating MAPK signaling. However, since CLV3-induced MAPK phosphorylation disappeared when all four receptors (CLV1, CLV2, CRN/SOL2 and RPK2) were co-expressed in *N. benthamiana* (Betsuyaku et al. 2011), these findings suggest not only the unique regulatory mechanism of each receptor signaling pathway, but also the antagonistic, positive roles of CLV receptors to activate MAPKs in the CLV signaling pathways. Furthermore, loss-of-function mutants of *clv1, clv2* and *rpk2* showed the additive phenotypes of enlarged SAMs toward higher-ordered mutants, indicating the overlapping positive functions of these receptors to promote CLV3p sensitivity restricting the stem cell population (Kinoshita et al. 2010). Because of technical limitations, there was no direct biochemical, molecular or genetic evidence to support the biological significance of the puzzling and complex MAPK function in the SAM of intact plants.

In this study, we report the rapid and dynamic MAPK activation and *WUS* repression in response to exogenous CLV3p treatment of the enlarged *clv3* SAM exhibiting expanded CLV1-expressing cells in "quiescence" ready to perceive exogenous CLV3p signals. We also demonstrate that the CLV3p-activated MAPKs are MPK3 and MPK6 through in-gel kinase assays in WT as well as in *mpk3* and *mpk6* mutants. Interestingly, the phosphorylation of MPK3 and MPK6 activated by CLV3p is significantly reduced in the loss-of-function *clv1 bam1* double mutant, indicating the positive role of CLV1 and BAM1 receptors in activating MAPKs. Consistently, the expression of *WUS* is elevated, but the WUS-repressing target genes are decreased in the shoot apex tissues of inducible *mpk3,6* double mutants. Moreover, the *mpk3,6* mutant plants display much less sensitive phenotype of the SAM termination mediated by the application of CLV3p and exhibit enlarged SAMs in the absence of CLV3p. Therefore, our findings suggest that both MPK3 and MPK6 activated by CLV3 signals play an important role in the intracellular CLV–WUS signaling pathway for the maintenance of SAM development.

Materials and methods

Plant materials and growth conditions

Columbia-0 (Col-0) and Landsberg *erecta* (L*er*) ecotypes were used as wild-type (WT) *Arabidopsis* plants in this study. The *mpk3* (Salk_151594), *mpk6* (Salk_062471), *fls2* (Salk_141277), *clv1* (WiscDsLox489-492B1), and *bam1*(Salk_015302) mutants and the estradiol-inducible *mpk3,6* mutants (Cheng et al. 2015) had a Col-0 background, while the *clv3-2* mutant had a L*er* background.

For liquid culture of *Arabidopsis* seedlings, seeds were germinated and grown in 6-well plates containing 1 ml of liquid medium [0.5 × Murashige and Skoog (MS) and 0.5% sucrose, pH 5.8 adjusted with KOH] at 22–23 °C and

75 µmol m^{-2} s^{-1} light intensity under a 12 h light/12 h dark photoperiod for 7 days without shaking.

RNA in situ hybridization

To amplify RNA probes, the full-length *WUS* cDNA was cloned into the pBluscript SK. Following PCR amplification with M13 forward/reverse primers, digoxigenin (DIG)-UTP labeled sense or antisense RNA probes were synthesized by T3 or T7 RNA polymerase, respectively (Roche). Section preparation, in situ hybridization and detection using NBT/BCIP were then performed according to the manufacturer's protocols (Roche).

Protein in situ immunohistochemistry

The shoot apex tissues were harvested, fixed and embedded using the same procedures as for the SAM arrest assay (Kim et al. 2017). Antigen unmasking using sodium citrate was conducted according to the manufacturer's instructions (Zymed). Sections were incubated with blocking buffer (5% [w/v] BSA in 1 × PBS) for 3 h at room temperature, then incubated with the primary rabbit α-phospho-MAPK antibody (Life technologies, #36-8800) diluted 1:100 in blocking buffer overnight at 4 °C in a humidified chamber. Slides were subsequently washed three times in 1 × PBS with 0.3% [v/v] Triton X-100 with gentle shaking for 10 min. Next, slides were incubated with secondary goat α-rabbit IgG antibody diluted 1:2000 in blocking buffer overnight at 4 °C (CALTAG Laboratories). Finally, slides were washed three times and detected using NBT/BCIP according to the manufacturer's instructions (Roche).

RNA extraction and RT-qPCR assay

Total RNAs from tissues were extracted with TRIzol reagent (Invitrogen) and first-strand cDNAs were synthesized using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays were performed with the gene-specific primers listed in Table 1 using the Step One Plus™ real time PCR detection system with Power SYBR® green master mix (Applied Biosystem).

Protein extraction and immunoblot assay

Total proteins were extracted using E buffer [135 mM Tris–HCl pH 8.8, 1% (w/v) SDS, 10% (v/v) glycerol, 50 mM $Na_2S_2O_5$]. After extracts were centrifuged at maximum speed in a microfuge (13,000*g*, 10 min, 4 °C), the supernatant was transferred into a new tube, after which we added 1/10 volume of Z buffer [125 mM Tris–HCl pH 6.8, 12% (w/v) SDS, 10% (v/v) glycerol, 22% (v/v) β-mercaptoethanol, 0.001% (w/v) bromophenol blue]. Protein quantities were measured using a DC protein assay kit (Bio-rad). Immunoblot assays were then performed using an α-phospho-MAPK antibody (Cell signaling, #9101) or an α-TUB antibody (Sigma, T9026).

SAM termination and SAM size assays

Wild-type Col-0 and *mpk3,6-es1/2* mutant seedlings were grown on MS plates (0.5 × MS, 0.5% Sucrose and 0.8% phytoagar) without estradiol for 1 week to avoid embryonic lethality, then transferred into new MS plates with or without 5 µM CLV3p in the presence of 10 µM estradiol under a 12 h light/12 h dark photoperiod for 4 weeks.

For measurement of the SAM size, seedlings were grown on MS plate for 1 week and transferred into new MS plates with 10 µM estradiol under a 12 h light/12 h dark photoperiod for 10 days. Shoot apices of seedlings were sampled and fixed in 4% paraformaldehyde/4% DMSO solution at 4 °C for 16 h. Paraffin embedding and sections were performed as described previously (Kim et al. 2017). Sectioned samples were stained with 0.1% Giemsa (Sigma) for 2 min and rinsed briefly with water. The width and height of SAMs were observed using a microscope (Olympus X22LED) and measured the distance between flanking primordia using Image J program.

Table 1 Primers used for RT-qPCR analysis in this study

Gene name	Forward primer	Reverse primer
WUS (At2g17950)	TGCAAGCTCAGGTACTGAATG	ATGATCCATGTTTGCCCATC
ARR7 (At1g19050)	CCTCGTATACAAGAATGTCTCAAAG	CTGCTAGCTTCACCGGTTTC
ARR15 (At1g74890)	CAGCACTCAGAGAAATCCCAGT	TCATACATTGTTCTATACGAGGTTG
GRF6 (At2g06200)	GCCTCCTCCTACTCAATTCACTCCAAATCT	ATGATCTCTTTGTTTCAGCTTCTCCTCTAA
YAB3 (At4g00180)	ACTGGGCTCATTTCCCTCACATACAC	CCAAGAGAAAATTCTAGTAGTTCCGAAA
ACT2 (At3g18780)	TCCCTCAGCACATTCCAGCAGAT	AACGATTCCTGGACCTGCCTCATC

Statistical analysis

Statistical analyses were performed with one-way ANOVA or unpaired *t* test using the Prism software (version 7.0b) and significant differences were determined by Turkey's multiple comparison test or two-tailed test, respectively.

Results

The CLV3p signal mediates rapid MAPK activation and *WUS* suppression in the SAM

It has been challenging to investigate the primary CLV3p-CLV1 signaling events, as the key receptor CLV1 is dynamically internalized and degraded in response to endogenous CLV3 ligands in the wild-type (WT) SAM (Nimchuk et al. 2011), and CLV3p has also been shown to induce SAM immunity via the FLS2 receptor (Lee et al. 2011, 2012a, b). To analyze the synchronized, dynamic and physiological stem cell signaling between the receptor and its target gene *WUS* in the SAM, we exploited the enlarged SAMs of the *clv3* mutant, in which CLV1 receptors are stably accumulated on the plasma membrane and the expression domains of *CLV1* and *WUS* are overlapped (Fig. 1a). Because the prolonged treatment of synthetic 12-amino acid (aa) CLV3p and 13-aa arabinosylated CLV3p was found to effectively complement the enlarged *clv3* SAM growth in previous reports (Fig. 1a) (Kondo et al. 2006; Ohyama et al. 2009; Kim et al. 2017), we adopted 12-aa CLV3p as an input signal in this study. Although we previously showed that FLS2 signaling does not affect the regulation of *WUS* expression involved in stem cell homeostasis (Lee et al. 2011), to uncouple the CLV3p-triggered FLS2 immune response from SAM development, we used the *clv3 fls2* double mutant. Importantly, the defect of the enlarged SAM in *clv3-2* was fully rescued by the WT genomic *CLV3* transgene, validating the key role of CLV3p signaling in the *clv3-2* mutant (Song et al. 2012).

Remarkably, we detected rapid and dynamic MAPK phosphorylation activated by the application of exogenous CLV3p within 30 min in the enlarged *clv3 fls2* SAM along CLV1-expressing cells (Nimchuk et al. 2015) by in situ immunolocalization using an α-phospho-MAPK antibody, and these phosphorylation signals were eventually disappeared at 60 min (Fig. 1b). Furthermore, the expanded *WUS* expression in the background of *clv3* SAM (Schoof et al. 2000) was rapidly reduced by treatment with CLV3p within 30–60 min when MAPKs were highly activated (Fig. 1b, c). Taken together, these results suggest a correlation between CLV3p-induced MAPK activation and transcriptional repression of the downstream target *WUS*.

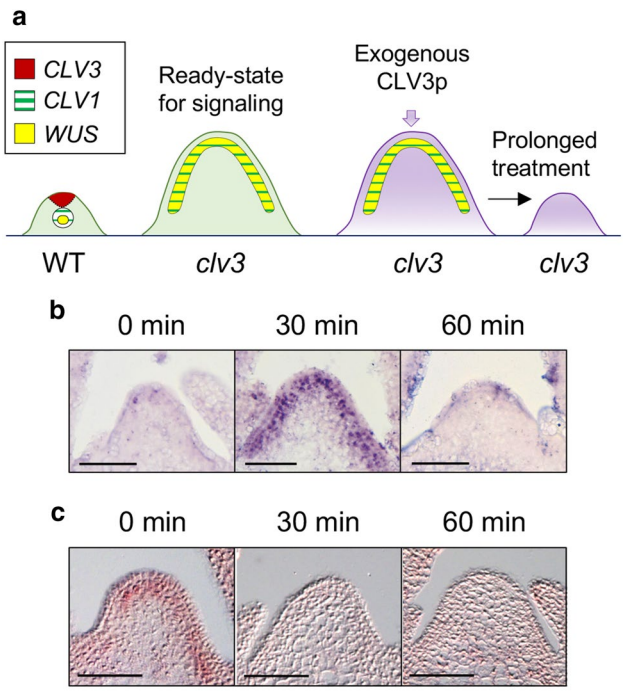

Fig. 1 The CLV3p signal mediates rapid MAPK activation and *WUS* suppression in the SAM. **a** A schematic strategy for studying stem cell signaling using the *clv3* SAM. *CLV3, CLV1* and *WUS* genes related to stem cell signaling exhibit different expression domains in the SAM. Overlapped expression of *CLV1* and *WUS* domains in the *clv3* SAM show the ready state for direct response triggered by CLV3p signals. **b** MAPK phosphorylation by CLV3p in *clv3-2 fls2-24* SAMs treated with 1 μM CLV3p for 0 min, 30 min or 60 min. Protein in situ immunohistochemistry was performed using an α-phospho-MAPK antibody. **c** *WUS* repression by CLV3p in the same context with (**b**) through RNA in situ hybridization. Scale bar, 50 μm (**b, c**)

MPK3 and MPK6 are activated by treatment with CLV3p signals

Using immunoblot assays, we further confirmed the CLV3p-triggered MAPK activation in the enriched *Arabidopsis* shoot apex tissues. Interestingly, we could find that MAPKs are also phosphorylated by the treatment of CLV3p in the WT Col-0 shoot apices (Fig. 2a). Importantly, because the specifically phosphorylated MAPK bands shown in WT were eliminated in *mpk3* or *mpk6* mutants (Fig. 2a), CLV3p-activated MAPKs were identified as MPK3 and MPK6. These findings exhibit that CLV3p signals can activate MPK3 and MPK6 to transmit intracellular signaling in the SAM.

Recent findings have revealed that CLV1 and BAM1 seem to act as major receptors for CLV3p signaling, because the loss-of-function *clv1 bam1* double mutant showed an

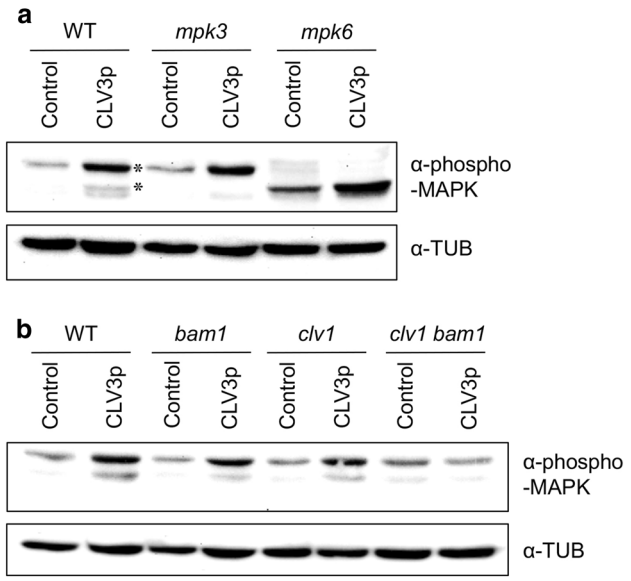

Fig. 2 MPK3 and MPK6 are activated by treatment with CLV3p signals. **a** Immunoblot assays using the shoot apex tissues harvested from WT Col-0, *mpk3* and *mpk6* mutant seedlings treated without (Control) or with 10 μM CLV3p for 10 min. Asterisks, endogenous MPK3 and MPK6. **b** Immunoblot assays using the shoot apex tissues harvested from WT Col-0, *bam1, clv1* and *clv1 bam1* mutant seedlings treated without (control) or with 10 μM CLV3p for 10 min. Activated MAPKs were detected by an α-phospho-MAPK antibody and loading amounts of proteins were detected by an α-TUB antibody. These experiments were repeated at least three times with similar results

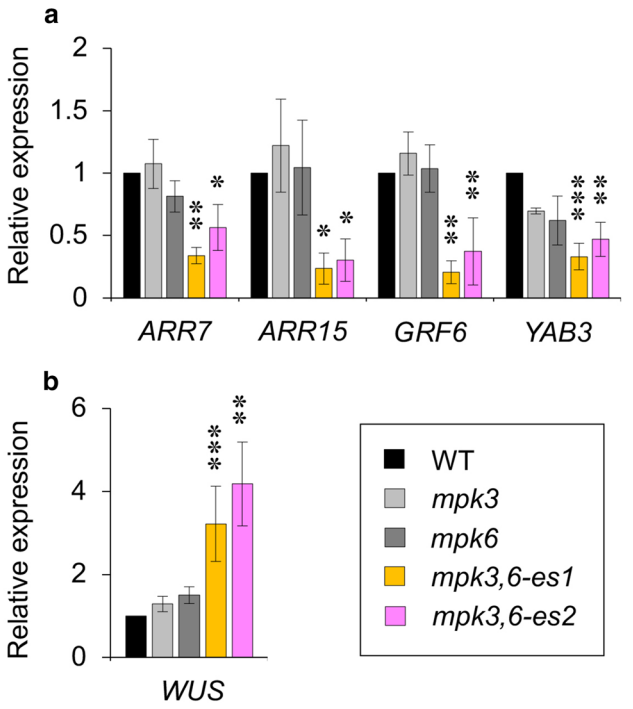

Fig. 3 Expression of *WUS* and WUS-target genes is impaired in the loss-of-function *mpk3,6* mutants. The expression of WUS-specific target genes (**a**) and *WUS* (**b**) in WT, *mpk3, mpk6* and *mpk3,6-es1/2* seedlings treated with 10 μM estradiol for 5 days. RNAs were isolated from the shoot apex tissues. Relative expression was normalized by *ACT2*. Error bars are ±S.D. ($n=3$). Significant differences compared to WT were determined via one-way ANOVA followed by Tukey's test: $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$

almost insensitive response to the repression of *WUS* expression induced by CLV3p (Shinohara and Matsubayashi 2015). Interestingly, CLV3p-triggerred MAPK phosphorylation was dramatically reduced in the shoot apex tissues of the *clv1 bam1* mutant compared to those of WT and each individual single mutant (Fig. 2b). Therefore, these results suggest that CLV1 and BAM1 mainly serve as corresponding receptors for CLV3p signals to induce intracellular MAPK phosphorylation.

Expression of *WUS* and WUS-target genes is impaired in the loss-of-function *mpk3,6* mutant

MPK3 and MPK6 have shown the functional overlap in various signaling pathways, including those associated with growth and development, abiotic stresses, hormones and pathogen defenses (Pitzschke et al. 2009; Meng et al. 2012; Lampard et al. 2014), and the *mpk3,6* double homozygous mutant is known to show the embryonic lethal phenotype under normal growth conditions (Wang et al. 2007). Therefore, to further investigate the function of MPK3 and MPK6 in the shoot stem cell homeostasis, we performed an RT-qPCR analysis to examine the expression of *WUS* and WUS-target genes in the enriched shoot apex tissues of WT, *mpk3, mpk6* and the estradiol-inducible *mpk3,6* double mutants (referred to as *mpk3,6-es1* and *mpk3,6-es2* for two independent lines), which bypassed the embryo lethality of *mpk3,6* (Wang et al. 2007; Cheng et al. 2015). Interestingly, the RT-qPCR analysis showed that the expression of two A-type *ARABIDOPSIS RESPONSE REGULATOR* (*ARR*) genes, *ARR7* and *ARR15*, as well as that of *GROWTH-REGULATING FACTOR6* (*GRF6*) and *YABBY3* (*YAB3*) were significantly decreased in the absence of MPK3/6 activities, but not MPK4 (Fig. 3a, Fig. S1), whereas the expression of *WUS* was significantly promoted in conditional *mpk3,6-es1/2* double mutants (Fig. 3b). A-type *ARR* genes encoding negative regulators of cytokinin signaling are known to be directly repressed by WUS (Leibfried et al. 2005). Moreover, it has been reported that WUS directly represses differentiation-promoting regulatory genes, such as *GRF6* and *YAB3* (Yadav et al. 2013). Because the expression of *WUS* and WUS-target genes was not significantly influenced in the *mpk3* or *mpk6* single mutant compared to WT (Fig. 3a, b), these results indicate that MPK3 and MPK6 may redundantly affect the expression of marker genes involved in SAM development via enhanced WUS activity.

MPK3 and MPK6 are involved in CLV3p-mediated SAM maintenance

To examine the long-term effects of CLV3p treatment in the SAM development of *mpk3,6-es1/2*, we developed a CLV3p-dependent SAM termination assay based on the SAM transition to a visible inflorescence stem. To accomplish this, WT Col-0 and *mpk3,6-es1/2* mutant seedlings were first grown on MS plates for 7 days without the application of estradiol to bypass the embryonic and seedling lethality. The majority (69%) of WT seedlings grown on MS medium containing 5 µM CLV3p for additional four weeks in the presence of 10 µM estradiol showed terminated SAMs without the inflorescence stem (Fig. 4a, b). In contrast, most estradiol-induced *mpk3,6-es1/2* plants (88.9%) retained SAM growth and produced inflorescence stems in the same condition (Fig. 4a, b, Fig. S2). Moreover, the overall vegetative SAM size of *mpk3,6-es1* mutants was significantly increased compared to that of WT in the presence of 10 µM estradiol (Fig. 4c, d). Taken together, these results indicate that CLV3p-triggered signaling is notably blocked in the *mpk3,6* mutant, and MPK3/6 play critical roles in the regulation of stem cell homeostasis involved in SAM development.

Discussion

We have provided several lines of evidence to demonstrate the biological function of MPK3 and MPK6 in regulating stem cell homeostasis during SAM development. In situ immunostaining and in-gel kinase assays showed clear and dynamic MAPK activation by phosphorylation and an RNA in situ hybridization illustrated rapid *WUS* repression mediated by CLV3p in the enlarged *clv3* SAMs (Fig. 1b, c). Because CLV1 receptors perceiving CLV3p ligands are immediately diminished by endocytosis in the WT (Nimchuk et al. 2011), the loss-of-function *clv3* mutant background made it feasible to analyze direct, synchronized, rapid and dynamic signaling responses triggered by exogenous CLV3p not previously possible. Interestingly, activated MAPKs in the SAM were revealed as MPK3 and MPK6, and the phosphorylation of these MPKs was induced by CLV1 and

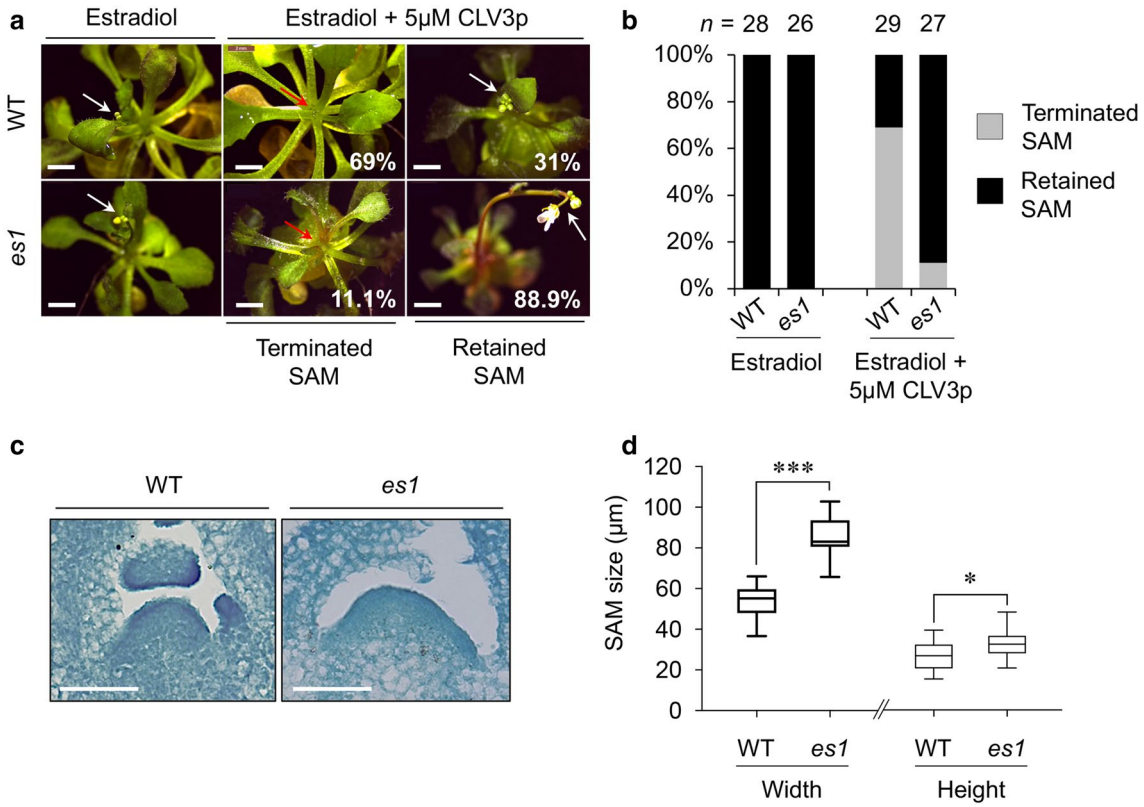

Fig. 4 MPK3 and MPK6 are involved in CLV3p-mediated SAM maintenance. **a** Significant arrest of CLV3p-mediated SAM termination in *mpk3,6-es1* (*es1*) compared to that of WT Col-0. Red arrows, terminated SAMs. White arrows, retained SAMs. **b** Quantification of terminated or retained SAM growth of WT and *es1* in the presence of exogenous CLV3p ($n = 26–29$). **c** The phenotype of enlarged SAM shown in *es1* compared to WT. Scale bar 50 µm. **d** The width and height of vegetative SAMs in WT and *es1* seedlings. Bands inside the boxes represent medians. Boxes represent the interquartile ranges and whiskers indicate the minimum and maximum values ($n = 5–8$). Significant differences were determined by two-tailed *t* test: $^{*}P < 0.05$ and $^{***}P < 0.001$

BAM1 receptors (Fig. 2b). However, since CLV3p-mediated MAPK phosphorylation was not completely eliminated in the shoot apex tissues of the *clv1 bam1* double mutant, it indicates that other CLV receptors may also be involved in MAPK phosphorylation in CLV signaling. Recent findings have shown that CLV1 and BAM1 serve as direct receptors for arabinosylated CLV3p, and the *clv1 bam1* double mutants reveal remarkably enlarged SAMs similar to those of the *clv3* single mutant (Shinohara and Matsubayashi 2015). Although these data represent the exclusive roles of CLV1 and BAM1 as the main receptors to perceive CLV3p signals, it is possible that other CLV receptors, including CLV1 homologues BAM2/3, CLV2 and RPK2, could be directly or indirectly involved in CLV3p-mediated MAPK phosphorylation. Moreover, it has been reported that another LRR-RLK ER-family receptor kinases, including ERECTA (ER), ERECTA-LIKE1 (ERL1) and ERL2, regulate stem cell maintenance in the SAM via a WUS-independent manner, because the loss-of-function *wus er erl1 erl2* quadruple mutant restored the SAM loss found in the *wus* single mutant (Kimura et al. 2018). ER-family receptors are also known to function as an upstream of the YODA-MKK4/5/7/9-MPK3/6 cascade in stomatal development (Pillitteri and Torii 2012; Zoulias et al. 2018). Therefore, future researches will be required to determine how multiple receptor complexes may participate in the regulation of MAPK activation related to the complex peptide-receptor networks modulating stem cell maintenance in the SAM.

It has been reported that CLV3-mediated MAPK phosphorylation occurs in a complex manner in *N. benthamiana* leaves and *Arabidopsis* seedlings (Betsuyaku et al. 2011). However, it was shown that CLV1 receptor acts as a "negative" regulator of CLV3-dependent MPK6 phosphorylation. For example, the transient co-expression of *CLV1* and *CLV3* genes in *N. benthamiana* leaves led to the partial suppression of CLV1-dependent MAPK phosphorylation detected in the absence of *CLV3* expression. In addition, elevated MPK6 activity was found in *clv1* mutant seedlings, even in the absence of treatment with exogenous CLV3p. Unlike the negative role of CLV1 for MPK6 activation, our results displayed that CLV3p-mediated phosphorylation of both MPK3 and MPK6 was dramatically reduced in the *clv1 bam1* mutant in the SAM, and there was no obvious MPK6 activity in the *clv1* mutant without CLV3p stimulation (Fig. 2b). Because the isolated, enriched shoot apex tissues were used in this study but not whole seedlings, the use of different tissues may lead to the different results in the *clv1* mutant. In addition, *CLV1* has been reported to be expressed outside the SAM (Chou et al. 2016). Based on our observation of MAPK activation, these results showed that CLV3p-mediated phosphorylation of MPK3 and MPK6 is positively regulated by both CLV1 and BAM1 receptors. Moreover, since the phosphorylation of MPK3 and MPK6 was not significantly reduced in the *clv1* or *bam1* single mutants, it suggests that both CLV1 and BAM1 are redundantly required for the CLV3p-mediated activation of MPK3 and MPK6.

In the plant MAPK signaling pathways, well-known MAPKs such as MPK3 and MPK6 have been shown to be involved in different MAPK cascade modules induced by flg22, H_2O_2, phytohormones (ethylene and jasmonate), abiotic stresses (ozone and osmotic shock) and developmental signals (epidermal patterning factors 1 and 2) (Colcombet and Hirt 2008; Lee et al. 2015; Zoulias et al. 2018). Indeed, the physical interactions between MPK3/6 and upstream MKKs are not sufficient to represent the unique characteristics of each signaling pathway. For example, previous results have shown that among ten MKKs, MPK3 interacts with six MKKs and MPK6 interacts with nine MKKs via in vitro protein microarrays (Popescu et al. 2009). One possible explanation of how the same CLV3p input signals are differently transduced via the positive role of CLV1/BAM1 on MPK3/6 activation in stem cell signaling is the regulation by scaffold proteins to specify and facilitate each signaling pathway in different MAPK cascades. Recent advances have shown that receptor for activated C kinase 1 (RACK1) functions as a scaffold protein involved in a novel plant immune pathway to link the upstream Gβ subunit and the downstream MAPK cascade module, which is distinct from the previously known flg22-induced MAPK cascade (Cheng et al. 2015). Moreover, in stomatal development, the polarity protein BREAKING OF ASYMMETRY IN THE STOMATAL LINEAGE (BASL) has also been reported to act as a scaffold protein physically interacting with MAPKKK YDA and MPK3/6 to spatially concentrate MAPK signaling for asymmetric cell division of stomatal precursor stem cells (Dong et al. 2009; Zhang et al. 2015). Since MPK3 and MPK6 have been shown to be activated by various upstream sets such as MKK4/5 or MKK7/9 in distinct stress responses and stomatal development (Colcombet and Hirt 2008; Yoo et al. 2008; Pitzschke et al. 2009; Rodriguez et al. 2010; Zoulias et al. 2018), it would be interesting to identify putative scaffold proteins and MKKs acting as an upstream of MPK3/6 activation via CLV1 and BAM1 receptors involved in stem cell homeostasis in the SAM.

Author contribution statement HL and JS designed the experiments and wrote the manuscript. YSJ, OKC and HL performed the experiments.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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