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Mitogen-Activated Protein Kinase Regulated by the CLAVATA Receptors Contributes to Shoot Apical Meristem Homeostasis

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In Arabidopsis, the CLAVATA (CLV) pathway operates in the regulation of the size of the stem cell population in the shoot apical meristem (SAM). CLV3 functions as a small peptide ligand to negatively regulate the expression of the WUSCHEL (WUS) transcription factor through three major receptor kinase complexes of CLV1, CLV2-SUPPRESSOR OF LLP1-2 (SOL2)/CORYNE (CRN) and recently identified RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2)/TOADSTOOL 2 (TOAD2). Aiming to understand the precise molecular details of CLV3 signaling, we investigated the contribution of phospho-signaling, potentially regulated by these kinase complexes, to the CLV pathway. We detected CLV3-triggered CLV1 phosphorylation, which is also conditioned by the rest of the CLV receptors, presumably by their direct association. Our comprehensive analysis of the activities of the respective CLV receptors on mitogen-activated protein kinases (MAPKs) suggested that the precise balanced regulation of MAPK activity by the CLV receptors is likely to be key for SAM homeostasis.

Keywords: Arabidopsis • CLAVATA • CLE • MAPK • *Nicotiana benthamiana* • Shoot apical meristem.

Abbreviations: BAK1, brassinosteroid insensitive 1 associating kinase 1; BRI1, brassinosteroid insensitive 1; CaMV, cauliflower mosaic virus; CIP, calf intestinal alkaline phosphatase; CLE, CLAVATA3/embryo-surrounding region; CLV, CLAVATA; CZ, central zone; DEX, dexamethasone; ER, endoplasmic reticulum; GFP, green fluorescent protein; 3HS, triple HAs-single StrepII; IP-kinase, immunoprecipitation kinase; KAPP, kinase-associated protein phosphatase; LRR, leucine-rich repeat; MAPK/MPK, mitogen-activated protein kinase; MBP, myelin basic protein; MAPKK/MKK, mitogen-activated protein kinase kinase; OC, organizing center; PLL1, POLTERGEIST-LIKE 1; POL, POLTERGEIST; PZ, peripheral zone; RLK, receptor-like kinase; ROP, Rho GTPase-related protein; RPK2/TOAD2, RECEPTOR-LIKE PROTEIN 2/TOADSTOOL 2; RT–PCR, reverse transcription–PCR; RZ, rib zone; SAM, shoot apical meristem; SOL2/CRN, SUPPRESSOR OF LLP1-2/CORYNE; TDIF, tracheary element differentiation inhibitory factor; TFA, trifluoroacetic acid; YFP, yellow fluorescent protein; WUS, WUSCHEL.

Introduction

The shoot apical meristem (SAM) is a collection of undifferentiated cells, providing all aerial parts of the plant body, such as leaves, flowers, vasculature and stems. In Arabidopsis, the SAM is defined by three areas: the central zone (CZ) which contains undifferentiated stem cells just above the organizing center (OC), the peripheral zone (PZ) containing rapidly dividing initial cells derived from the CZ, and the rib zone (RZ). As seen in this zone structure of the SAM, two opposite activities of maintaining the undifferentiated stem cell population and promoting differentiation are precisely and carefully regulated in the SAM. Deregulation of this balance would affect the morphological structure of the plant body (Clark 2001, Miwa et al. 2008, Tucker and Laux 2007).

In Arabidopsis, the size of the stem cell population in the SAM is regulated by the CLAVATA (CLV) pathway, in which CLV3, a small signaling peptide secreted from the stem cells in the CZ, functions to restrict the expression of the WUSCHEL (WUS) homeobox transcription factor only within the OC (Clark et al. 1995, Brand et al. 2000, Schoof et al. 2000). WUS is, in turn, required for *CLV3* expression in the stem cell (Schoof et al. 2000). Perturbation in this CLV3–WUS balance results in

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altered morphology of the SAM (Schoof et al. 2000). *wus* mutants and *CLV3*-overexpressing plants exhibit the same phenotype of termination of the SAM, whereas the *clv3* mutant exhibits an enlarged SAM, a hallmark of the *clv* phenotype (Clark et al. 1995, Laux et al. 1996). Thus, this negative feedback maintains the fine balance of differentiation from the stem cells in the SAM (Schoof et al. 2000). CLV3, a founder member of the CLV3/embryo-surrounding region (CLE) family, has been demonstrated to act as a 12 or 13 amino acid arabinosylated glycopeptide within its C-terminal 14 amino acid CLE domain conserved among the CLE family members (Kondo et al. 2006, Ohyama et al. 2009). Indeed, exogenous application of synthetic CLV3 peptides, which contain at least 12 amino acids corresponding to the CLE domain, is able to mimic the *CLV3* overexpression phenotype (Fiers et al. 2005, Fiers et al. 2006, Kondo et al. 2006, Sawa et al. 2006, Sawa et al. 2008).

Two major receptor complexes are known to perceive the CLV3 signal to limit *WUS* expression in the SAM of Arabidopsis (De Smet et al. 2009). CLV1, a leucine-rich repeat (LRR) type of receptor-like kinase (RLK) capable of binding to synthetic CLV3 peptide, forms a homomer at the plasma membrane (Clark et al. 1993, Clark et al. 1997, Ogawa et al. 2008, Bleckmann et al. 2010). The second receptor complex consists of CLV2, an LRR type of receptor protein, and SUPPRESSOR OF LLP1-2 (SOL2)/CORYNE (CRN), an RLK without an LRR (Kayes and Clark 1998, Jeong et al. 1999, Miwa et al. 2008, Müller et al. 2008). CLV2 and SOL2/CRN are primarily endoplasmic reticulum (ER)-resident proteins and depend on each other for their plasma membrane localization, where the CLV2–SOL2/CRN complex receives the extracellular CLV3 signal (Bleckmann et al. 2010). Despite the genetic evidence that *CLV1* and the *CLV2–SOL2/CRN* pathway act independently, CLV1 and CLV2–SOL2/CRN complexes can associate with each other via SOL2/CRN at the plasma membrane, which might be important for fine-tuning of CLV signaling (Miwa et al. 2008, Zhu et al. 2009, Bleckmann et al. 2010). In addition to these two genetically independent pathways, our recent genetic study using MCLV3, a 12 amino acid functional CLV3 peptide, has identified an LRR-RLK, RECEPTOR-LIKE PROTEIN 2 (RPK2)/TOADSTOOL 2 (TOAD2), that comprises the third receptor complex of CLV3 signaling (Kinoshita et al. 2010). Loss-of-function mutations in any of these CLV receptors causes weak *clv3*-like SAM enlargement phenotypes and the *clv1 clv2 rpk2/toad2* triple mutant produces a much enlarged SAM, which is almost equivalent to the SAM size of the *clv3* mutants, indicating that these three receptors transmit the CLV3 signal independently (Clark et al. 1993, Clark et al. 1995, Kayes and Clark 1998, Jeong et al. 1999, Müller et al. 2008, Kinoshita et al. 2010). However, careful genetic studies of single, double or triple mutants of the CLV receptors also suggested that there seem to be weak interactions among the three pathways (Kinoshita et al. 2010).

Downstream of the CLV receptors, several type 2C protein phosphatases are involved in transmitting the CLV3 signal. Kinase-associated protein phosphatase (KAPP) was shown to interact directly with the phosphorylated kinase domain of CLV1 and to act as a negative regulator of CLV1-triggered signaling (Williams et al. 1997, Stone et al. 1998). POLTERGEIST (POL) and POL-LIKE 1 (PLL1), both of which are negatively regulated by CLV1, CLV2, SOL2/CRN and CLV3, promote *WUS* expression (Yu et al. 2000, Yu et al. 2003, Song et al. 2006, Müller et al. 2008). In addition to these phosphatases, a Rho GTPase-related protein, ROP, was found to associate with CLV1, which suggests an involvement of the mitogen-activated protein kinase (MAPK) cascade downstream of CLV1 (Trotochaud et al. 1999).

MAPK cascades are one of the key phosphorylation signaling components for responding to various extracellular stimuli in eukaryotic cells (Lewis et al. 1998, Madhani and Fink 1998). The activation of a MAPK cascade often occurs within minutes upon stimulation, representing one of the earliest cellular responses to environmental cues. A typical MAPK cascade consists of three protein kinases, MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK) and MAPK, that sequentially phosphorylate the corresponding downstream substrates. The activated MAPKs phosphorylate various proteins such as transcription factors, protein kinases, metabolic enzymes and cytoskeletal proteins. In plants, MAPK cascades mediate various types of cell signaling including abiotic stress, innate immunity and development (Rodriguez et al. 2010). It has also been expected that CLV3 perception would trigger phosphorelay signaling to restrict the *WUS* expression domain (Clark 2001, Jun et al. 2008, Butenko et al. 2009). However, the molecular basis of the CLV signaling triggered by CLV3 stimulation leading to the restriction of the *WUS* expression domain in the SAM is still largely unknown.

In this study, we set out to examine whether phosphosignaling would play a role in the CLV pathway using complementary approaches of genetics in Arabidopsis and biochemistry in *Nicotiana benthamiana*. We detected that CLV1 associated with CLV2 and RPK2/TOAD2 in a SOL2/CRN-dependent manner in *N. benthamiana*. Transient expression of the CLV receptors with the specific ligand, CLV3, in *N. benthamiana* enabled us to detect CLV3-dependent phosphorylation of CLV1 protein, which is affected by the presence of CLV2, SOL2/CRN and RPK2/TOAD2. We revealed that MPK6, a potential downstream target of CLV signaling, is activated by the CLV3 stimuli in Arabidopsis and in *N. benthamiana*. We then demonstrated that the respective CLV receptors exert unique activities to regulate MPK6 activity in *N. benthamiana*, and some of them were also confirmed in Arabidopsis. Surprisingly, the strong effects of the respective receptor on MPK6 activity were diminished when all the CLV receptors were co-expressed in *N. benthamiana*. These findings prompted us to hypothesize that the precise control of MPK6 activity by the CLV receptors might be critical to maintain SAM homeostasis. This idea was further supported by successful transient complementation of the *clv1-11* carpel defect by transiently depressing the abnormally up-regulated activity of MAPKs, including MPK6, in the mutant. These findings shed

light on the complex SAM homeostasis maintained by CLV–MAPK signaling.

Results

The transient expression system of the CLV components in *N. benthamiana*

The transient gene expression system in *N. benthamiana* has been utilized successfully to perform biochemical interaction assays among the CLV receptors of Arabidopsis (Zhu et al. 2009, Bleckmann et al. 2010, Kinoshita et al. 2010). The CLV receptors expressed via *Agrobacterium tumefaciens* in leaf tissues of *N. benthamiana* apparently retain their biochemical nature, such as their cytological localization and the interaction specificities with their specific interaction partners (Zhu et al. 2009, Bleckmann et al. 2010, Kinoshita et al. 2010). Our previous study on RPK2/TOAD2, the fourth component of the CLV receptors, also successfully utilized this system to support biochemically our genetic-based observation that there exist three stable CLV receptor complexes, CLV1, CLV2–SOL2 and RPK2 (Kinoshita et al. 2010). To advance our understanding of the molecular events controlling the CLV signaling pathway further, we added CLV3 C-terminally tagged with yellow fluorescent protein (YFP) to the *N. benthamiana* transient expression system, in addition to CLV1, SOL2/CRN, CLV2 and RPK2/TOAD2 C-terminally fused to 3× HAs-single StrepII, 10× Myc, 3× FLAG and 10× Myc (CLV1-3HS, SOL2/CRN-10Myc, CLV2-3FLAG and RPK2/TOAD2-10Myc), respectively (**Fig. 1**).

CLV1 is associated with CLV2, SOL2/CRN and RPK2 in *N. benthamiana*

Association specificities amongst the CLV receptors have been established by a number of groups including ours using *N. benthamiana*. CLV2 has been shown to interact with SOL2/CRN by fluorescent-assisted microscopic techniques and immunoprecipitation in *N. benthamiana* (Zhu et al. 2009, Bleckmann et al. 2010). Co-immunoprecipitation experiments have demonstrated that RPK2/TOAD2 interacts with itself, but not with CLV1 and CLV2 (Kinoshita et al. 2010). CLV1 was shown to associate weakly with CLV2 only in the presence of SOL2/CRN (Zhu et al. 2009, Bleckmann et al. 2010). However, all the experiments were targeted analyses to examine specific associations between two or three selected proteins in certain conditions. There might be a possibility that the CLV receptors behave differently when all the other CLV receptors co-exist in the same cells. Thus, before exploring CLV3-triggerred responses through the CLV receptors, we decided to assess this possibility by co-expressing all the CLV receptors together in *N. benthamiana*. First, we performed an immunoprecipitation assay using CLV1-3HS to clarify which receptors are co-purified with CLV1-3HS (**Fig. 2**). As reported previously for the fluorescent-tagged versions, CLV1-3HS was also co-purified with CLV2-3FLAG only in the presence of SOL2/CRN-10Myc (Bleckmann et al. 2010) (**Fig. 2**, top and second from the top). Surprisingly, in the same immunoprecipitates, we detected SOL2/CRN-10Myc as well as RPK2/TOAD2-10Myc (**Fig. 2**, second from the bottom and bottom). Therefore, we concluded

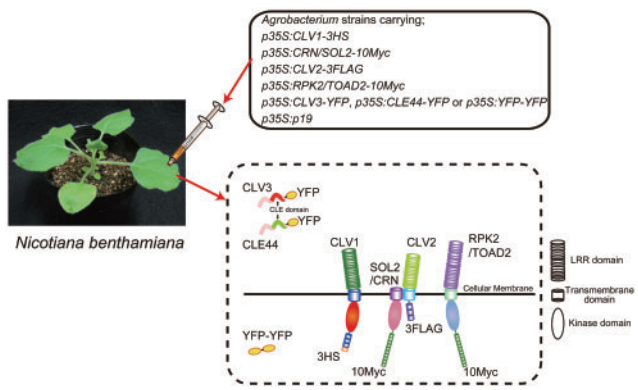

Fig. 1 A schematic diagram of our experimental system using *Nicotiana benthamiana*. We transiently expressed the *CLAVATA* components via *Agrobacterium tumefaciens* in *N. benthamiana* leaves. We used CLE44–YFP and YFP–YFP as negative controls of CLV3–YFP. All the constructs were C-terminally epitope tagged and were driven under the control of the *CaMV 35S* promoter. A mixture of *Agrobacterium* strains carrying the respective *CLV* constructs as well as the *p19* silencing suppressor were infiltrated into leaves of *N. benthamiana*. The leaf samples were harvested 3 d after infiltration. All the epitope-tagged CLV components used in this study are schematically drawn in a dashed box. The picture shows three stable CLV receptor complexes proposed by a number of studies.

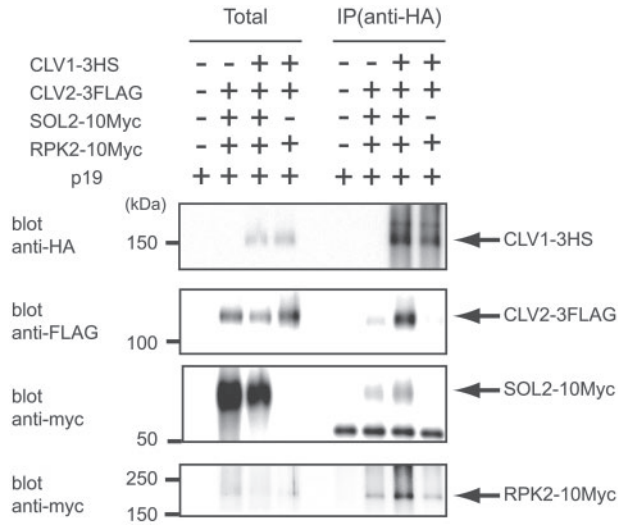

Fig. 2 CLV1 associates with CLV2, SOL2/CRN and RPK2/TOAD2 in the presence of SOL2/CRN. CLV1-3HS, CLV2-3FLAG, SOL2/CRN-10Myc and RPK2/TOAD2 were co-expressed in *N. benthamiana*, and total protein extracts were subjected to immunoprecipitation using anti-HA antibody. The resulting immunocomplexes were analyzed by Western blot with anti-HA, anti-Myc or anti-FLAG antibody. CLV1-3HS was immunoprecipitated with CLV2, SOL2/CRN-Myc and RPK2/TOAD2 only when SOL2/CRN-10Myc was also expressed. This experiment was repeated at least twice with a similar result.

that CLV1 is able to associate with CLV2, SOL2/CRN and RPK2/TOAD2 when all the CLV receptors were co-expressed in *N. benthamiana*. In other words, SOL2/CRN is able to promote association not only of CLV2 but also of RPK2/TOAD2 with CLV1. The SOL2/CRN-mediated association of CLV1 with CLV2, SOL2/CRN and RPK2/TOAD2 might have a biological impact on the CLV signaling pathway in the SAM of Arabidopsis.

SOL2/CRN functions not only as an adaptor protein but also as a signaling molecule

Our data imply that SOL2/CRN-mediated association of SOL2/CRN, CLV2 and RPK2/TOAD2 with CLV1 might play an important role in CLV signaling (**Fig. 2**). A previous study indicated that SOL2/CRN is required not only for recruitment of CLV1 into the CLV2–SOL2/CRN complex but also for localization of the CLV2–SOL2/CRN complex at the plasma membrane, where the complex can meet CLV1 and is exposed to the apoplastic CLE ligands (Bleckmann et al. 2010). However, it is still unclear whether SOL2/CRN functions simply as an adaptor protein to guide the CLV2–SOL2/CRN receptors to the plasma membrane for proper assembly of the CLV receptor complex(es) there or possesses its own signaling function to mediate CLV signaling. In the former scenario, the mutant phenotype of the *sol2/crn* plants might simply reflect loss of potential CLV2-dependent (and also RPK2/TOAD2-dependent) modulation of CLV1 activity in the absence of the SOL2/CRN adaptor protein. To answer this question, we performed a complementation test of the *sol2-1* mutants with the C-terminally 10× Myc-tagged *SOL2/CRN* gene containing a mutation which causes an amino acid substitution of the conserved catalytic lysine to glutamic acid at position 146 under the control of the cauliflower mosaic virus (CaMV) *35S* promoter ($SOL2_{K146E}$-10Myc) (**Fig. 3**). As a positive control, we prepared a non-mutated version of *SOL2/CRN-10Myc* in the same vector backbone. The selected T_1 *sol2-1* plants transformed with either the non-mutated version of *SOL2-10Myc* or the mutant version of *$SOL2_{K146E}$-10Myc* were analysed for the *clv*-like carpel phenotype of the *sol2* mutants (**Fig. 3C**). Protein and mRNA expression levels of the transgenes in the individual transformants were analyzed in the following generation using the young seedlings of the segregating T_2 populations since none of the flowering T_1 *sol2-1* plants carrying *$SOL2_{K146E}$-10Myc* accumulated detectable levels of $SOL2_{K146E}$-10Myc protein (**Fig. 3A, B**). The wild-type *SOL2* construct with a 10× Myc tag complemented the *sol2-1* carpel phenotype, when expressed at a higher level, as observed for the 10-1 plant (**Fig. 3**). This is consistent with the previous report that *CaMV35S*-driven *SOL2/CRN* is able to complement *sol2/crn* without causing additional effects (Müller et al. 2008). However, T_1 transformants expressing the mutated version did not fully complement the *sol2-1* phenotype, at least under our conditions (**Fig. 3C**). We also confirmed that one of the *$SOL2_{K146E}$-10Myc* plants, the KD-2 plants, accumulated the mutant $SOL2_{K146E}$-10Myc protein even slightly more than the complementing 10-1 plant using anti-Myc (**Fig. 3A**). Thus, our data suggested that SOL2/CRN is not merely an adaptor

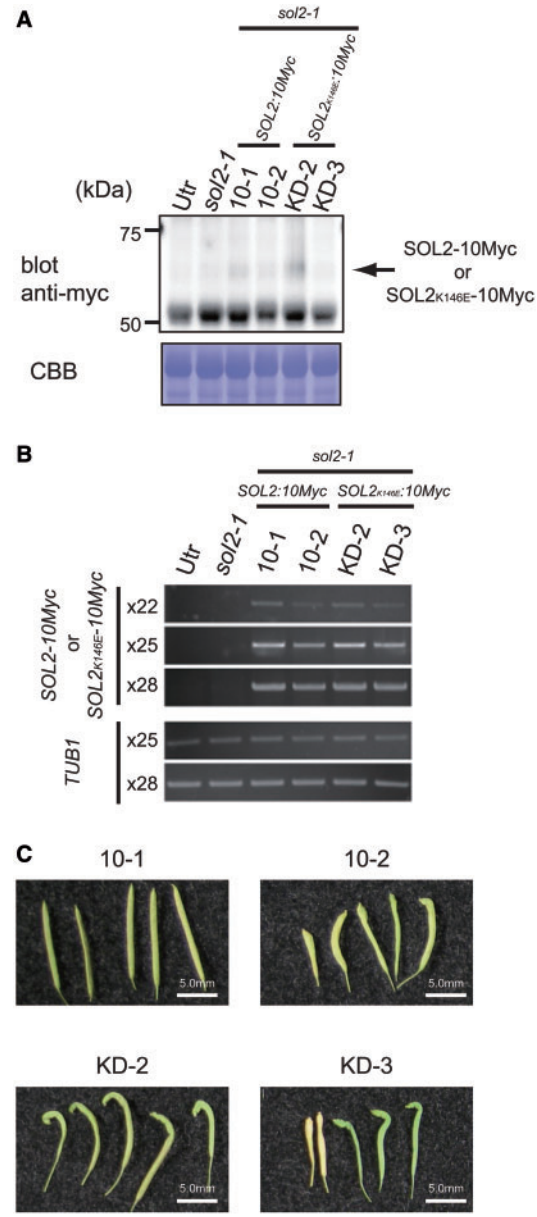

Fig. 3 SOL2/CRN kinase activity is required for SOL2/CRN function in CLV signaling. The stable transgenic *sol2* plants expressing either *SOL2/CRN-10Myc* or *$SOL2/CRN_{K146E}$-10Myc* under the control of the *CaMV 35S* promoter were generated and the resulting plants were analyzed. (A) Immunoblot analysis of 7-day-old seedlings of the respective genotypes. The T_2 segregating transgenic populations were analyzed with the wild-type and the *sol2* mutant plants. Total protein extracts were detected with anti-Myc. Equal loadings were confirmed by Coomassie Brilliant Blue (CBB) staining of the blot. (B) Semi-quantitative reverse transcription–PCR (RT–PCR) analysis using specific primer sets detecting only *SOL2-10Myc* fusion transcripts. Total mRNAs were extracted from the same set of plants as in A. The numbers of PCR cycles are shown on the left side of every gel picture. (C) The silique phenotypes of T_1 transgenic plants. Five representative siliques from the transgenic plants are shown. Lines 10-2, KD-2 and KD-3 showed the *clv*-like carpel phenotype of the *sol2* mutants, while line 10-1 restored the *sol2* defect. The *sol2* carpel phenotype was clearly detected by the shape of the siliques.

protein among the CLV receptors, but requires its own kinase activity to mediate CLV signaling in the SAM.

CLV3 co-expression results in mobility shift of CLV1 and CLV2 in *N. benthamiana*

We next examined the effect of CLV3 co-expression on the CLV receptors in *N. benthamiana*. Although CLV3 was shown to exist as an active 12 or 13 amino acid peptide corresponding to the conserved CLE domain in planta through a possible maturation process after translation, a *CLV3–green fluorescent protein* (*GFP*) construct has been demonstrated to complement the *clv3* mutant, indicating that the CLV3 maturation machinery is likely to generate the functional CLV3 peptide from the CLV3–YFP fusion protein in the plant tissue (Rojo et al. 2002, Lenhard and Laux 2003). In our transient expression system, CLV3–YFP was detectable in an immunoblot using GFP antibody; however, it accumulated to very low levels compared with YFP–YFP expressed from the same vector. CLV3–YFP might not accumulate well or it might be degraded and/or cleaved by an unknown CLV3 maturation machinery (**Fig. 4E, F**). Together with the successful complementation of the *clv3* defects by CLV3–GFP, CLV3–YFP possibly produces the functional CLV3 peptide in *N. benthamiana*, which is supported by our subsequent experiments (Rojo et al. 2002, Lenhard and Laux 2003) (**Figs. 4–6**). Co-expression of the functional ligand and the functional receptor complexes of

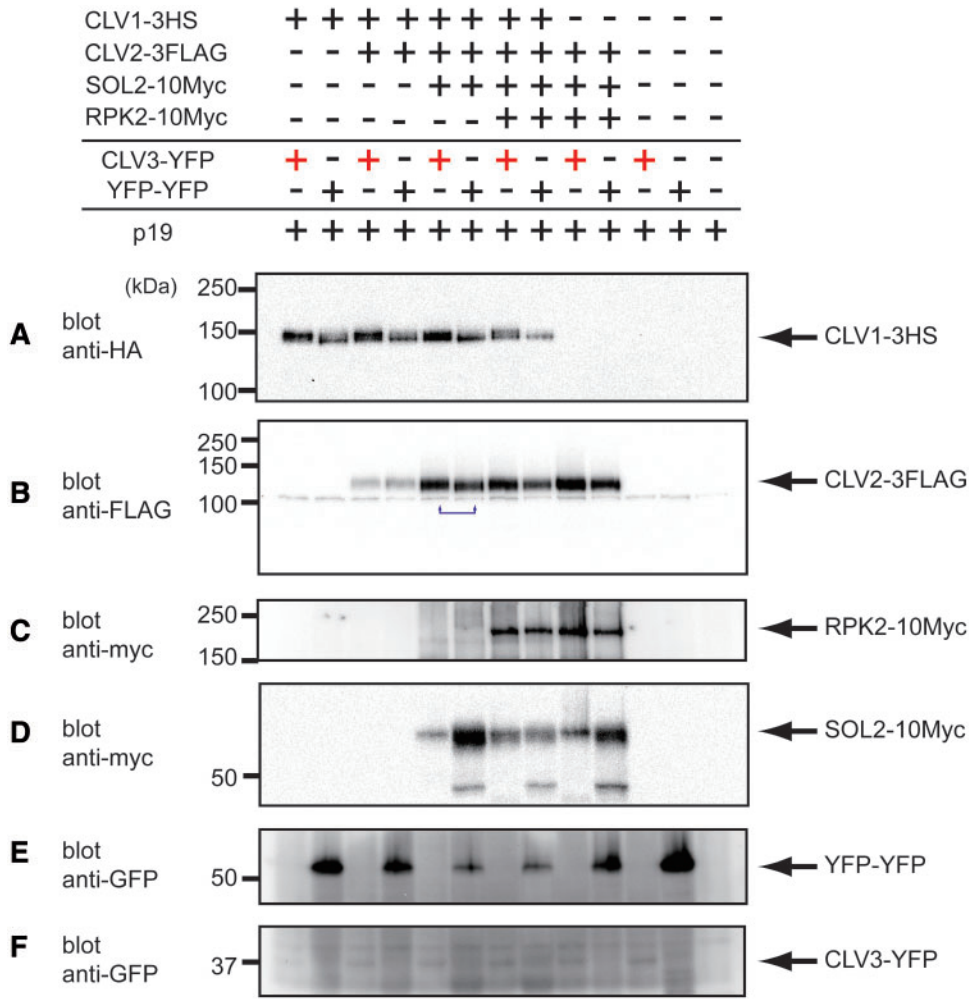

Fig. 4 Co-expression of the CLV components detected CLV3-induced mobility shifts of CLV1-3HS and CLV2-3FLAG. CLV1-3HS was expressed with different combinations of CLV receptors in *N. benthamiana*. Expression levels of the respective receptors and effects of CLV3–YFP co-expression on the CLV receptors were analyzed using immunoblotting. YFP–YFP was used as a negative control of CLV3–YFP co-expression. (A) Immunoblot (8% gel) detected with anti-HA for CLV1-3HS. The CLV3-triggered electrophoretic mobility shift of CLV1 was detected with all combinations of the CLV receptors tested here. (B) Immunoblot (8% gel) with anti-FLAG. CLV2 mobility on SDS–PAGE was also affected by CLV3 co-expression. A clear band shift by CLV3 co-expression is indicated by the blue double-sided arrow. (C) Immunoblot (8% gel) with anti-Myc to detect RPK2/TOAD2-10Myc. (D) Immunoblot (8% gel) detected with anti-Myc for SOL2/CRN-10Myc. (E) Blot (15% gel) with anti-GFP for YFP–YFP expression. (F) Immunoblot (15% gel) with anti-GFP for CLV3–YFP expression.

CLV signaling in *N. benthamiana* enabled us to molecularly dissect CLV3 recognition by the receptors and, potentially, the resulting downstream signaling.

Activation of the RLKs often involves autophosphorylation, which can be detected as an electrophoretic mobility shift in SDS–PAGE (Wang et al. 2001). Phosphorylated protein often migrates more slowly than the non-phosphorylated protein in SDS–PAGE (Wang et al. 2001). In order to test whether the CLV receptors are also activated through autophosphorylation upon stimulation of CLV3, we focused on CLV1, amongst the CLV receptors, and expressed CLV1-3HS with or without CLV3–YFP in *N. benthamiana*, since CLV1 has been shown to bind directly to the synthetic CLV3 peptide (**Fig. 4A, F**). Additionally, our data indicated that CLV2, SOL2/CRN and RPK2/TOAD2 are able to associate with CLV1, suggesting a potential modulation of CLV1 activity by the other CLV receptors (**Fig. 2**).

Considering the potential effects of the other CLV receptors on CLV1 activity, we also co-expressed CLV2-3FLAG, SOL2/CRN-10Myc and RPK2/TOAD2-10Myc in various combinations (**Fig. 4B–D**). Immunoblot analyses showed that, indeed, CLV1-3HS migrated more slowly on SDS–PAGE only when co-expressed together with CLV3–YFP, independently of the other CLV receptors (**Fig. 4A, F**). Similar band shifts correlating with CLV3–YFP co-expression were also observed for CLV2-3FLAG, but not for SOL2/CRN-10Myc and RPK2/TOAD2-10Myc (**Fig. 4B–D**). CLV2 might also be post-translationally modified upon CLV3 stimulation (**Fig. 4B**; a clear band shift is shown by the blue double-sided arrow). However, the shift of the CLV2-3FLAG band by CLV3–YFP co-expression was weaker than that of CLV1-3HS (**Fig. 4A, B**). We also observed that CLV2-3FLAG consistently accumulated more when co-expressed with SOL2/CRN-10Myc, suggesting that the CLV2–SOL2/CRN interaction might stabilize CLV2 protein (**Fig. 4B**). Additionally, it should be noted that CLV1 co-expressed with all the other CLV receptors reproducibly narrowed the CLV3-dependent electrophoretic mobility shift of CLV1 compared with CLV1 expressed without the rest of the CLV receptors, prompting us to carry out further detailed analysis (**Figs. 4A, 5C**).

Thus, the CLV3-dependent mobility shift of CLV1 might represent CLV1 phosphorylation, possibly autophosphorylation, upon CLV3 recognition. Moreover, the CLV1 potential phosphorylation is likely to be regulated by the presence of the other CLV receptors. In addition, our finding of CLV1 association with CLV2, SOL2/CRN and RPK2/TOAD2 implied that CLV2, SOL2/CRN and RPK2/TOAD2 might directly modulate CLV1 phosphorylation (**Fig. 2**).

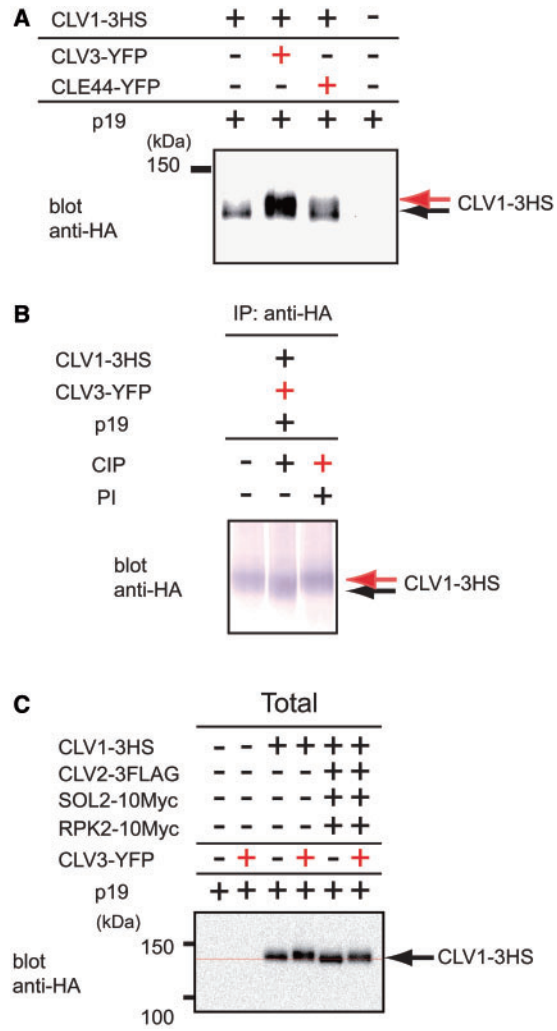

Fig. 5 CLV3 specifically triggers CLV1 phosphorylation. (A) Immunoblot analysis of CLV1-3HS protein in the presence/absence of different CLE proteins. The protein extracts from *N. benthamiana* expressing the constructs (+) were separated by SDS–PAGE (6%) and were detected using anti-HA. (B) Phosphatase treatment of immunoprecipitated CLV1-3HS protein co-expressed with CLV3–YFP. Immunoprecipitated CLV1-3HS protein was mock treated, or treated with recombinant calf intestinal alkaline phosphatase (CIP) or CIP together with phosphatase inhibitors (PIs). The resulting protein samples were separated by SDS–PAGE (6%) and analyzed by immunoblotting with anti-HA. (C) Immunoblot (6% gel) of CLV1-3HS detected with anti-HA. The protein samples from *N. benthamian* expressing the various constructs shown above were analysed by immunoblotting for CLV1-3HS mobility. A red line is shown to visualize clearly the differences in the CLV1-3HS electrophoretic mobility.

CLV1 is phosphorylated by CLV3 co-expression in *N. benthamiana*

The CLV3-dependent CLV1 mobility shift in SDS–PAGE indicated CLV1 autophosphorylation in response to CLV3 in planta. In order to investigate this possibility further, we next tested the specificity of this effect by co-expressing a CLV3-unrelated CLE gene with CLV1-3HS in *N. benthamiana* (**Fig. 5A**). The tracheary element differentiation inhibitory factor (TDIF) encoded by CLE41/44 is a member of the CLE family; however, TDIF inhibits differentiation from the stem cells, while CLV3 promotes differentiation (Ito et al. 2006, Fukuda et al. 2007, Hirakawa et al. 2008). Additionally, TDIF exhibits no CLV3 activity in the SAM when exogenously applied to the SAM, suggesting that TDIF should be an appropriate negative

control of CLV3-triggered responses (Ito et al. 2006, Sawa et al. 2006, Kinoshita et al. 2007). Using SDS–PAGE with a better resolution for the CLV1 mobility shift, we immunodetected CLV1-3HS in the lysates of *N. benthamiana* infiltrated with either CLV1-3HS, CLV1-3HS + CLV3–YFP or CLV1-3HS + CLE44–YFP (**Fig. 5A**). Co-expression of CLV3–YFP strongly induced mobility shift(s) of CLV1-3HS in SDS–PAGE, resulting in the appearance of slower migrating band(s) (**Fig. 5A**). There still remained a weak faster migrating band in the CLV1-3HS + CLV3–YFP sample; however, the majority of the CLV1-3HS signal shifted to the higher molecular weight band(s) (**Fig. 5A**). In contrast, co-expression of CLE44–YFP did not cause such notable mobility shifts of the CLV1-3HS signal (**Fig. 5A**).

Next, we investigated the possibility that the CLV1 mobility shift might denote CLV3-dependent phosphorylation of CLV1. CLV1-3HS co-expressed with CLV3–YFP was immunoprecipitated using the anti-HA antibody and treated with or without alkaline phosphatase (**Fig. 5B**). Phosphatase inhibitors were used to show the specific activity of phosphatases. Immunoblot analysis of the resulting samples revealed that phosphatase activity specifically abolished the mobility shift of the CLV1-3HS co-expressed with CLV3–YFP, indicating that phosphorylation at least contributes to the CLV3–YFP-dependent CLV1-3HS mobility shift in SDS–PAGE (**Fig. 5B**).

To distinguish whether CLV3–YFP-dependent phosphorylation of CLV1-3HS is autophosphorylation upon direct CLV3 binding to CLV1-3HS or indirect phosphorylation by another *N. benthamiana* protein kinase activated by CLV3–YFP, we generated a kinase-dead version of CLV1-3HS by substituting the conserved catalytic lysine at position 720 with glutamic acid ($CLV1_{K720E}$-3HS); however, we were never able to detect the $CLV1_{K720E}$-3HS protein in immunoblots with anti-HA in multiple experiments using independently generated multiple clones (data not shown) (Williams et al. 1997, Stone et al. 1998). Thus, we cannot rule out the possibility that CLV1-3HS is transphosphorylated by another protein kinase of *N. benthamiana* upon stimulation of CLV3. Nevertheless, our data demonstrated that CLV1 is modified specifically by CLV3-dependent phosphorylation in *N. benthamiana*.

CLV1 phosphorylation status is modified not only by CLV3 but also by the other CLV receptors in *N. benthamiana*

Having found that the CLV1 mobility shift was caused by CLV1 phosphorylation, we then investigated in detail the differential CLV1-3HS phosphorylation status observed in **Fig. 4**. The plant lysates from *N. benthamiana* transiently expressing CLV1-3HS with or without CLV3–YFP in the presence or absence of CLV2, SOL2/CRN and RPK2/TOAD2 were analyzed by anti-HA immunoblotting (**Fig. 5C**). The immunoblot showed clearly that co-expression of all the CLV receptors, in the absence of CLV3–YFP, accelerated the electrophoretic mobility of CLV1-3HS compared with expression of CLV1-3HS alone (**Fig. 5C**). A similar effect due to co-expression of all the CLV receptors on CLV1-3HS mobility in SDS–PAGE was also observed when co-expressed together with CLV3–YFP (**Fig. 5C**). As we have already shown in **Fig. 5B**, CLV3-dependent phosphorylation is able to cause the electrophoretic mobility shift of CLV1-3HS. The different electrophoretic mobility of CLV1-3HS might reflect its various states of phosphorylation. Thus, the presence of the other CLV receptors may contribute to modify the CLV1-3HS phosphorylation status.

MAPK is activated by CLV3 stimuli in both *N. benthamiana* and Arabidopsis

Activation of the cellular surface receptor upon stimulation immediately turns on subsequent intracellular signaling to modify the cellular status accordingly (Asai et al. 2002, Tor et al. 2009). In general, it has been implicated that RLK including CLV receptors regulates phosphorylation signals as downstream factors (Shiu and Bleecker 2001, Jun et al. 2008, Butenko et al. 2009). Therefore, to unravel the downstream events of CLV3–CLV1 signaling, we analyzed the activity of MAPK, which is one of the candidates for the CLV-mediated signaling (Clark 2001, Jun et al. 2008, Butenko et al. 2009). In-gel kinase assay using myelin basic protein (MBP) as a substrate revealed that transient expression of CLV3–YFP in *N. benthiamiana* caused the activation of a 46 kDa protein kinase, which corresponds to the molecular mass of *N. benthamiana* NbSIPK and Arabidopsis MPK6 (**Fig. 6A**, bottom and second from the bottom, lanes 1 and 2) (Sharma et al. 2003). This suggested that CLV3 activates the MAPK-like activity. To confirm further CLV3-dependent activation of MPK6 in planta, 7-day-old seedlings of Arabidopsis Col-0 wild-type grown in liquid medium were mock treated or treated with 10 μM MCLV3, the functional synthetic CLV3 peptide, for 30 min and then the extracted proteins were subjected to in-gel kinase assay using MBP as a substrate (**Fig. 6B**, top, lanes 1 and 2). As in the case of *N. benthamiana*, a 46 kDa protein kinase was activated by MCLV3 treatment in Arabidopsis. An immunoprecipitation kinase (IP-kinase) assay using the MPK6-specific antibody Ab6NT1 indicated that this 46 kDa protein kinase was MPK6 (**Fig. 6B**, bottom and second from the top, lanes 1 and 2) (Ichimura et al. 2000, Teige et al. 2004, Takahashi et al. 2007). The protein levels of MPK6 were almost equal during this experiment (**Fig. 6B**, second from the bottom, lane 1 and 2). Therefore, these results suggested that the exogenous application of MCLV3 has an ability to activate MPK6 in Arabidopsis.

The activation of MAPK is differently regulated by the respective CLV receptors

We investigated whether CLV receptors regulate MAPK activation through the effect of CLV3 expression. The respective CLV receptors were expressed with or without CLV3–YFP in *N. benthamiana* (**Fig. 6A**). The resulting protein extracts were analyzed for the expression of the respective receptors (**Fig. 6A**, top four panels) and were also subjected to in-gel kinase assay (**Fig. 6A**, bottom and second from the bottom). CLV1-3HS and SOL2/CRN-10Myc weakly activated the 46 kDa protein kinase without CLV3–YFP expression, probably by a dose-dependent

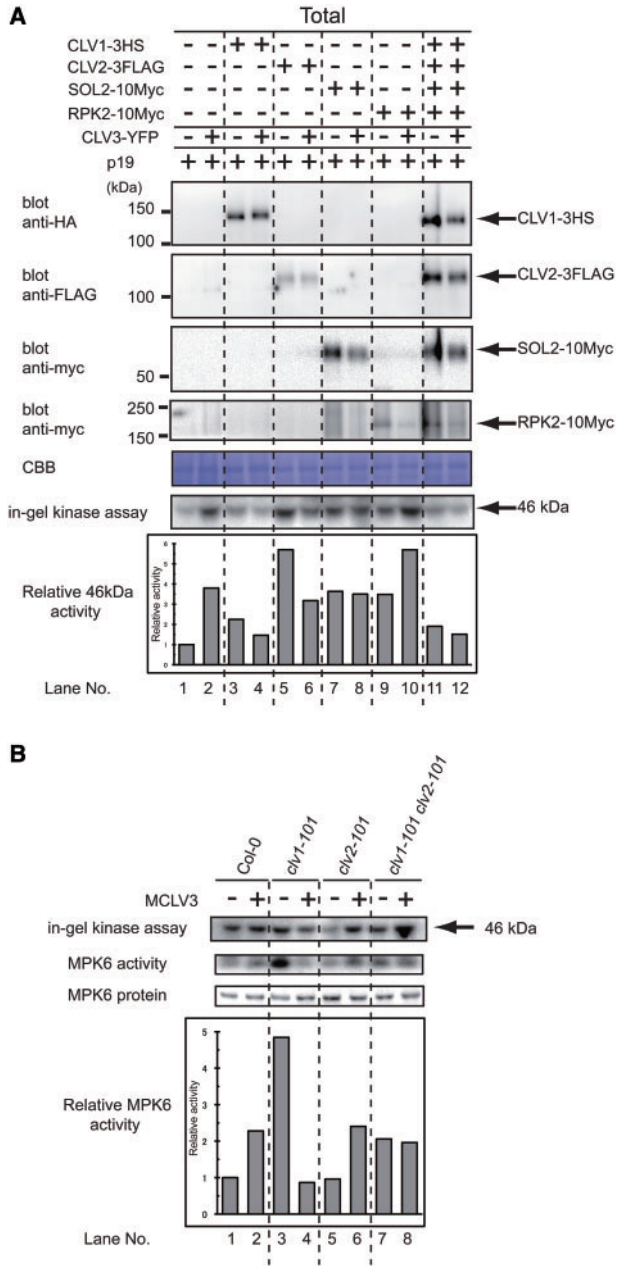

Fig. 6 CLV3 activates MPK6 and the CLV receptors modulate MPK6 differently. (A) The respective CLV receptors were expressed with or without CLV3–YFP and analyzed by immunoblot and by in-gel kinase assay. All the CLV receptors were also co-expressed together with or without CLV3–YFP and analyzed accordingly. (B) Seven-day-old seedlings of Arabidopsis were mock treated (–) or treated with the MCLV3 peptides (+) for 30 min. The resulting total proteins were analyzed by in-gel kinase assay, IP-kinase assay using anti-MPK6 antibody and immunoblot with anti-MPK6. Relative MPK6 activity is shown.

autoactivation (**Fig. 6A**, bottom and second from the bottom, lanes 3 and 7). Co-expression of CLV3–YFP partially suppressed this CLV1-triggered weak activation of 46 kDa protein, while it did not affect SOL2/CRN-dependent activation of this protein (**Fig. 6A**, bottom and second from the bottom, lanes 3–4 and 7–8). It is most likely that SOL2/CRN requires other receptor-like proteins such as CLV2 to receive the extracellular signal of CLV3. CLV2-3FLAG overexpression caused massive activation of the 46 kDa protein in the absence of CLV3–YFP (**Fig. 6A**, bottom and second from the bottom, lane 5). However, this activation was repressed to the equivalent of the CLV3–YFP-activated 46 kDa protein under both CLV2-3FLAG and CLV3–YFP expression (**Fig. 6A**, bottom and second from the bottom, lane 6). Overexpression of RPK2/TOAD2-10Myc alone weakly induced the activity of the 46 kDa protein and this activation was further enhanced in a CLV3–YFP-dependent manner (**Fig. 6A**, bottom and second from the bottom, lanes 9–10). Thus, respective CLV receptors possess unique activities for the regulation of the 46 kDa protein. We then expressed all four CLV receptors, resembling the Arabidopsis SAM cells expressing all the receptors (**Fig. 6A**, bottom and second from the bottom, lanes 11–12). Intriguingly, co-expression of all the CLV receptors eliminated any effect on 46 kDa protein activity with or without CLV3–YFP (**Fig. 6A**, bottom and second from the bottom, lanes 11–12).

Next, we analyzed the function of endogenous CLV1 or CLV2 in MAPK activation using Arabidopsis null mutants. In-gel kinase assay and IP-kinase assay using anti-MPK6 revealed that the loss of CLV1 activity resulted in an elevation of MPK6 activity in the absence of exogenously applied MCLV3 (**Fig. 6B**, top, second from the top and bottom, lane 3). The MCLV3 treatment suppressed MPK6 activation in the *clv1-101* mutant to the level of the wild type without MCLV3 treatment, which might be exerted by the CLV3-dependent negative regulation of MPK6 by the remaining CLV receptors (**Fig. 6B**, top, second from the top and bottom, lane 4). The *clv2-101* mutants showed weak down-regulation of MPK6 activity compared with the wild type in the absence of MCLV3 (**Fig. 6B**, top, second from the top and bottom, lane 5). The up-regulation of MPK6 activity in the MCLV3-treated *clv2-101* mutants can be explained by the CLV3-triggered RPK2/TOAD2 positive regulation toward 46 kDa MAPK, namely MPK6 (**Fig. 6B**; top, second from the top and bottom, lane 6). The *clv1-101 clv2-101* double mutants did not exhibit any effect on MPK6 activity, regardless of MCLV3, which satisfies the idea that CLV1 is a negative and CLV2 is a positive regulator in MPK6 activation (**Fig. 4B**, top, second from the top and bottom, lanes 7–8). After the application of MCLV3, the activity of the 46 kDa protein kinase appeared to be enhanced in the *clv1-101 clv2-101* mutants, but IP-kinase assay suggested that it was not MPK6 activity. These results indicated that the respective CLV receptors have a different influence on MAPK activation. CLV1 and RPK2/TOAD2 are CLV3-dependent negative and positive regulators of MAPK, respectively. CLV2 potentially acts as an activator of MAPK in the absence of CLV3; however, this effect is repressed when it is supplied together with CLV3. In addition, our data obtained from an *N. benthamiana*-based assay suggested that the presence of all the CLV receptors may function to suppress the CLV3-triggered ectopic activation of MAPK, especially MPK6,

in the SAM or to maintain a certain balance or equilibrium of MAPK activity, which might be crucial to maintain SAM homeostasis.

MKK4-KN is able to complement the carpel phenotype of the *clv1-11* mutant

Our biochemical data suggested that CLV3 is capable of activating MAPK in *N. benthamiana* and also in Arabidopsis (**Fig. 6A, B**). Amongst the CLV receptors, CLV1 was found to act as a CLV3-dependent negative regulator of MPK6 activity (**Fig. 6A**, lanes 3 and 4, and **B**, lanes 3 and 4). These data raised the possibility that abnormal activation of MPK6 in *clv1-11* plants might contribute to the *clv* phenotype of the mutant. In order to assess this hypothesis, we generated conditional transgenic *clv1-11* plants expressing a kinase-negative (KN) form of MKK4 (MKK4-KN) under the control of a steroid-inducible promoter since it has been suggested that MKK4, Arabidospsis MAPKK4, is one of the direct upstream factors for MPK6 (Rodriguez et al. 2010). In addition, conditional expression of functional CLV receptor proteins has been established to complement the carpel phenotype of the *clv* mutants (Bleckmann et al. 2010). Following the published protocol, we induced the expression of MKK4-KN by spraying dexamethasone (DEX) onto the emerging inflorescence meristem of multiple independent *MKK4-KN clv1-11* T_2 transgenic lines, derived from independent T_1 plants. Our observation of the resulting siliques of the DEX-induced plants identified four independent *MKK4-KN clv1-11* lines carrying one or more siliques with the wild-type carpel number (**Fig. 7**). In contrast, DEX-induced expression of a constitutive active form of MKK4 (MKK4-DD) was unable to recover the *clv1-11* carpel phenotype in any siliques of 10 independent *clv1-11* transgenic plants (**Supplementary Fig. S1**). Therefore, we concluded that MKK4-KN is at least able to complement the carpel defect of the *clv1-11* mutants. Thus, our data provided the first experimental evidence to demonstrate the possible involvement of the MAPK cascade in the CLV signaling pathway.

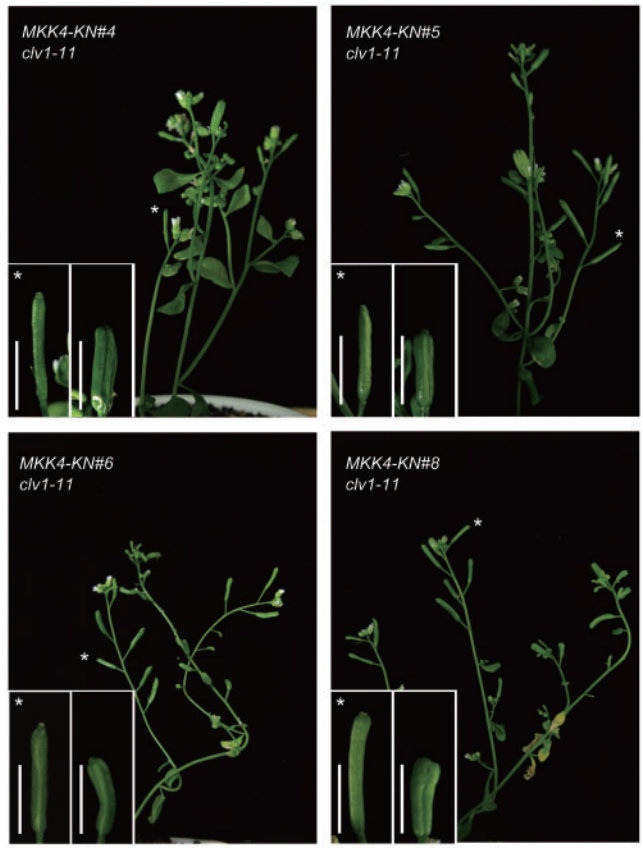

Fig. 7 MKK4-KN rescues the *clv1-11* defect. Right panels show the whole *clv1-11* T_2 transgenic plant with DEX-inducible *MKK4-KN*. Left insets show the carpels treated with or without DEX. Asterisks indicate the rescued siliques with two carpels, whereas older and younger siliques form abnormal *clv*-like carpels. White bars = 5 mm.

Discussion

In the CLV signaling pathway of Arabidopsis, the secreted CLV3 signal regulates the homeostasis of the SAM by restricting the *WUS* expression domain through the activities of the CLV receptor proteins, namely, CLV1, CLV2, SOL2/CRN and RPK2/TOAD2 (Schoof et al. 2000, Clark 2001, Müller et al. 2008, De Smet et al. 2009, Kinoshita et al. 2010). Since CLV2 and SOL2/CRN form an LRR kinase complex and CLV1 as well as RPK2/TOAD2 are LRR-RLKs, phosphorylation-based signaling including the MAPK cascade has been proposed to participate in the CLV signaling pathway (Clark 2001, Jun et al. 2008, Müller et al. 2008, Butenko et al. 2009, Kinoshita et al. 2010). However, only a few studies reported possible involvement of phosphorylation in CLV signaling, such as KAPP identified by a targeted approach and POL isolated by a genetic screen (Williams et al. 1997, Stone et al. 1998, Trotochaud et al. 1999, Yu et al. 2000, Yu et al. 2003). Perhaps genetic redundancy and/or lethality of such potential candidates might provide difficulties in identifying novel downstream components in the CLV pathway. Furthermore, the small size of the SAM tissues might also be a limitation to conducting any biochemical experiment. To overcome these points, we have adopted an *N. benthamiana*-based transient expression system of the CLV components. A number of studies including ours suggested that C-terminally epitope-tagged CLV receptors expressed in *N. benthamiana* seem to retain their protein characteristics (Zhu et al. 2009, Bleckmann et al. 2010, Kinoshita et al. 2010). Furthermore, addition of the specific ligand, CLV3, into the system also enabled us to analyse early downstream events of CLV3 perception. Our biochemistry-oriented approaches provided a series of novel findings on the CLV receptor activity, and some of them were further confirmed genetically in a targeted way in Arabidopsis. Taken together, our data suggested experimentally, for the first time, the involvement of MAPK activity in CLV signaling. Here, we discuss and update the possible regulatory machinery of the CLV signaling pathway.

CLV1 association with CLV2 and RPK2/TOAD2 via SOL2/CRN

In this study, we have shown that CLV1 was immunoprecipitated with CLV2, SOL2/CRN and RPK2/TOAD2 in a SOL2/CRN-dependent manner (**Fig. 2**). Previous studies by other groups have demonstrated that SOL2/CRN mediates a weak interaction between CLV1 and CLV2 (Zhu et al. 2009, Bleckmann et al. 2010). Our result presented here agrees with these previous data and further provides a new insight that RPK2/TOAD2 can also participate in this intermolecular association(s) among the four CLV-related receptors. However, in our previous study, we reported that SOL2/CRN immunoprecipitation detected only CLV2, but not CLV1 in *N. benthamiana*, which does not fit with our current observation (**Fig. 2**) (Kinoshita et al. 2010). This contradiction could be explained if we assume that the numbers of CLV2 and SOL2/CRN molecules are much higher than those of CLV1 in plant tissue. In this scenario, it is plausible that SOL2/CRN immunoprecipitation detects only the abundant interacting partner CLV2, but not the limited CLV1. However, the reciprocal immunoprecipitation of the limited CLV1 might be able to detect SOL2/CRN as well as CLV2 and RPK2/TOAD2. Supporting this idea, we were unable to detect CLV1 protein using anti-FLAG when we expressed *CaMV35S:CLV1-3xFLAG* in *N. benthamiana*, which has the same vector backbone that we successfully used for CLV2 expression and detection in this study (**Figs. 2, 4, 6**) (data not shown). This observation indicates that the abundance of CLV1 protein is likely to be substantially lower than that of CLV2 protein in *N. benthamiana*. In addition to this stoichiometry among the CLV receptors, the presence of RPK2/TOAD2, which we did not include in our previous immunoprecipitation, might influence CLV1 affinity for the other CLV receptors in addition to SOL2/CRN (Kinoshita et al. 2010).

In agreement with the previous studies, our data provided information on the function of SOL2/CRN as a molecular adaptor among the CLV receptors including a newly identified RPK2/TOAD2 (**Fig. 2**) (Zhu et al. 2009, Bleckmann et al. 2010). In addition, the kinase activity of SOL2/CRN was found to be dispensable for the CLV2–SOL2/CRN interaction and the relocation of CLV2–SOL2/CRN from the ER to the plasma membrane (Bleckmann et al. 2010). Although it is still possible that SOL2/CRN association with CLV1 or RPK2/TOAD2 might require SOL2/CRN kinase activity, our study revealed that the kinase activity of SOL2/CRN function is essential in CLV signaling, suggesting that SOL2/CRN is unlikely to function simply as an adaptor among the CLV receptors. Recent genetic studies also showed that *SOL2/CRN*, together with *CLV2*, comprises one branch of CLV signaling independently of the *CLV1* and *RPK2/TOAD2* pathways. Together with our finding, the *CLV2–SOL2/CRN* pathway of CLV signaling is most likely to be mediated by the kinase activity of SOL2/CRN (Müller et al. 2008, Kinoshita et al. 2010). Future studies revealing direct downstream targets of the respective CLV receptors should provide further insights into understanding the role of SOL2/CRN protein in CLV signaling.

Although we have detected that CLV1 is capable of associating with CLV2, SOL2/CRN and RPK2/TOAD2 in the presence of SOL2/CRN, we were unable to dissect whether CLV1 forms a huge complex containing all the CLV receptors or several independent complexes with distinct receptors (**Fig. 2**). We also did not reveal the stoichiometry in the complex(es). However, our data suggested that CLV1 forms either a huge CLV receptor complex containing CLV1, CLV2, SOL2/CRN and RPK2/TOAD2, or two complexes: CLV1–SOL2/CRN–CLV2 and CLV1–SOL2/CRN–RPK2/TOAD2. Future studies with rapidly improving biochemical techniques would answer these questions.

CLV3-dependent CLV1 phosphorylation

The activation of RLK is often coupled with its autophosphorylation upon ligand perception (Wang et al. 2001). The kinase domain of CLV1 (CLV1KD) produced in bacterial cells has been shown to possess autophosphorylation activity, and only the phosphorylated form of CLV1KD was demonstrated to bind to KAPP (Williams et al. 1997, Stone et al. 1998). Together with the fact that CLV1 was immunoprecipitated with KAPP from Arabidopsis lysates, CLV1 has been expected to be constitutively autophosphorylated by CLV3 in planta (Stone et al. 1998). However, it has been unclear how CLV1 autophosphorylation is regulated in planta in response to CLV3. Our data presented in this study established that, indeed, CLV3 specifically stimulates CLV1 phosphorylation in planta, which can be detected as a phosphatase-sensitive mobility shift of CLV1 on SDS–PAGE (**Fig. 5**). Due to the difficulty in expressing a kinase-dead version of CLV1 in *N. benthamiana*, we could not discriminate whether CLV1 is autophosphorylated or transphosphorylated by other *N. benthamiana* protein kinases activated by CLV3. However, considering a previous report had shown that the synthetic CLV3 peptide is capable of binding directly to the CLV1 ectodomain, CLV3 is most likely to promote autophosphorylation of CLV1 through its direct binding to the ectodomain of CLV1 (Ogawa et al. 2008, Ohyama et al. 2009). Thus, our finding provides a piece in the puzzle of the mode of activation of CLV1 receptor kinase upon specific CLV3 ligand recognition.

We also observed a CLV2 mobility shift, suggesting the possibility of CLV3-induced phosphorylation of CLV2 (**Fig. 4B**). However, we failed to detect phosphatase sensitivity of the mobility shift of the immunoprecipitated CLV2 co-expressed with CLV3 due to banding of the immunoprecipitated CLV2 on SDS–PAGE not being very sharp (data not shown). It would be meaningful to unravel the molecular basis of this CLV3-dependent modification of CLV2 protein.

Effect of CLV2, SOL2/CRN and RPK2/TOAD2 on CLV1 phosphorylation

In addition to CLE ligands, the presence of the other CLV receptors, namely CLV2, SOL2/CRN and RPK2/TOAD2, was also revealed to affect the electrophoretic mobility of CLV1, presumably the degree of CLV1 phosphorylation (**Fig. 5C**). Our finding of CLV1 association with CLV2, SOL2/CRN and

RPK2/TOAD2 in *N. benthamiana* raises the possibility that these receptors might modify the CLV1 phosphorylation status through direct interactions. It is still unclear whether the degree of CLV1 mobility shift correlates with the degree of CLV1 phosphorylation; however, CLV1 co-expressed with CLV2, SOL2/CRN and RPK2/TOAD2 migrated faster than CLV1 expressed alone (**Fig. 5C**). In this context, the presence of other CLV receptors might function in protecting CLV1 from irregular activation. It is plausible that direct association of CLV2, SOL2/CRN and RPK2/TOAD2 might tighten the CLV1 tertiary structure so that it is only responsive to the specific CLV3 signal. Although we were unable to observe phosphorylation-dependent mobility shifts of SOL2/CRN and RPK2/TOAD2 on SDS–PAGE, their kinase activity might also be regulated by the other CLV receptors to be sensitive only to the specific CLV3 ligand (**Fig. 4**). A number of examples that a ligand promotes an association between specific receptor pairs are known; however, in the case of CLV signaling, the association among specific receptors might direct all receptors to be responsive to a specific ligand signal (Chinchilla et al. 2007). BRI1-associated kinase 1 (BAK1) is known to amplify, upon brassinosteroid perception, the downstream signal by transphosphorylation with brassinosteroid insensitive 1 (BRI1) (Li et al. 2002, Nam and Li 2002, Wang et al. 2008). Although the MAPK pathway is not known to be involved in signaling downstream of BRI1–BAK1 thus far, BAK1 has also been shown to interact with flagellin sensing 2 (FLS2), independent of BRI1, to activate the MAPK pathway upon flg22 recognition (Chinchilla et al. 2007, Kemmerling and Nürnberger 2008). It is still unclear what regulates this BAK1 specificity to trigger appropriate downstream signaling upon a certain signal; however, it is tempting to assume that BAK1 interaction with a specific receptor partner, in addition to the binding of a specific ligand to BAK1, is also a specificity determinant to direct the activity of a BAK1 complex into the appropriate downstream signaling. This specificity determination of the downstream target, promoted by the specific ligand and the cognate receptors, might be a common strategy to fine-tune developmental and environmental signaling for plants, which have an increased number of receptor-like protein in their genomes compared with other organisms (Shiu and Bleecker 2001).

MAPK functions in the CLV signalling

Several reports suggest that RLKs positively regulates MAPK cascades as downstream signaling in stomata development, floral organ abscission and immune responses (Heese et al. 2007, Wang et al. 2007, Cho et al. 2008). In this study, we have demonstrated that the MAPK pathway is possibly involved in CLV signaling. Ectopic overexpression of CLV3 and exogenous application of the synthetic MCLV3 peptide were revealed to trigger activation of NbSIPK-like kinase in *N. benthamiana* and MPK6 in Arabidopsis, respectively (**Fig. 6A, B**). This MPK6 activation might be the molecular basis of the SAM termination phenotype triggered by application of MCLV3 and CLV3 overexpression. On the other hand, we have also shown that the respective CLV receptors affect MAPK activation (**Fig. 6A, B**). This regulation of MAPK activation surprisingly differs among these receptors. CLV1 functions as a negative regulator of CLV3-dependent activation of MPK6, CLV2 as a positive factor of MPK6 activation, and RPK2 as a CLV3-dependent MPK6 activator (**Fig. 6A**). The negative regulatory function of CLV1 and the positive role of CLV2 in MPK6 activation were also confirmed using Arabidopsis young seedlings (**Fig. 6B**). SOL2/CRN expressed in *N. benthamiana* did not display any CLV3-dependent regulation of MPK6 activity, probably due to the lack of an extracellular domain providing the ability to respond to the specific ligand. However, it is tempting to speculate that SOL2/CRN should share the same activity with CLV2 downstream, since it has been established that CRN/SOL2 acts together with CLV2, at least in the regulation of the inflorescence and the floral meristems (Miwa et al. 2008, Müller et al. 2008). Thus, we assume that SOL2/CRN, together with CLV2, acts as a positive regulator of MPK6 activity. Intriguingly, the presence of all the CLV receptors eradicated any effect on MPK6 activity (**Fig. 6A**, lanes 11 and 12). Thus, MPK6 activity levels do not simply explain the molecular basis of the *clv-wus* mutational effects on SAM size. However, our successful complementation of *clv1-11* using *MKK4-KN* strongly supports the possibility that the MAPK pathway is involved in CLV signaling.

Recently, it has been reported that the MKK4/MKK5 pathway including MPK6 mediates developmental signaling as well as stress signaling in Arabidopsis (Heese et al. 2007, Wang et al. 2007, Cho et al. 2008). The eFP browser (http://bar.utoronto.ca/) and promoter–GUS (β-glucuronidase) analysis revealed that MKK4 and MPK6 are expressed in the SAM and floral organs (Wang et al. 2007, Cho et al. 2008). Therefore, it is plausible that the MKK4 pathway mediates SAM homeostasis and morphogenesis of the floral organ. In addition, our study revealed that the constitutive activation of MPK6 was observed in the *clv1-101* mutant in the absence of MCLV3 (**Fig. 6B**, lane 3). The *MKK4-KN clv1-11* plants showed the wild-type carpel phenotype compared with the *MKK4-DD clv1-11* plants in a DEX-dependent manner (**Fig. 7**, and **Supplementary Fig. S1**). Overexpression of the *MAPKK-KN* form is known to have an inhibitory influence on the primary signaling in planta (Nishihama et al. 2001). These results suggest that in *MKK4-KN clv1-11* plants, the MKK4-KN activity possibly counteracts the unbalanced CLV signaling pathway of the *clv1-11* plants, resulting in complementation of the *clv1-11* carpel phenotype. However, it has not been reported that loss or gain of function of MPK6 exhibits the *clv*-like abnormal phenotype in floral organs (Rodriguez et al. 2010). Our in-gel kinase assay revealed that other MAPKs in addition to MPK6 are also activated in a CLV3-dependent manner in both *N. benthamiana* and Arabidopsis (data not shown). Protein microarray indicated that MAPKK–MAPK phosphorylation pathways are likely to constitute a very complex network (Popescu et al. 2009), suggesting that several MAPK pathways might mediate a part of SAM homeostasis in plant. More detailed analysis will be

valuable for further understanding of the role of the MAPK pathway in the regulation of the *WUS* expression domain controlling SAM homeostasis.

Based on our results, we propose that the CLV receptors function in maintaining appropriate activity of MAPKs including MPK6 to control the homeostasis of the SAM through limiting the *WUS* expression domain (**Fig. 8A**). As suggested by our analysis of the loss-of function *clv* mutants in the absence of MCLV3, CLV1 functions as a negative regulator; CLV2, presumably together with SOL2/CRN, acts as a positive regulator (**Fig. 6B**). We did not examine MAPK activity of the *rpk2*/*toad2* mutant due to a technical difficulty regarding its male sterility nature, although RPK2/TOAD2 was shown to be a CLV3-dependent positive regulator of MPK6 activity in *N. benthamiana* (**Fig. 6A**). These CLV receptors may regulate the MAPK cascade indirectly via effectors, as proposed by the study on ROP–CLV1 association, or they may directly control the MAPK activity. It would also be necessary to position POL and PLLs, the known downstream components of CLV1 and CLV2–SOL2/CRN pathways, in relation to RPK2/TOAD2 activity and the MAPK activity in the SAM for further understanding of the CLV system (**Fig. 8A**) (Yu et al. 2000, Yu et al. 2003, Song et al. 2006, Müller et al. 2008).

However, in our model, these unique activities of the respective CLV receptors would control a fine-tuning of MAPK activity in the SAM in a CLV3-dependent manner (**Fig. 8A**). This balanced regulation of MAPKs was, indeed, observed when all the four CLV receptors were co-expressed in *N. benthamiana* (**Fig. 6A**). This equilibrium effect among the activities of the CLV receptors can be achieved either by them regulating each other directly through their direct association shown in **Fig. 2** or by modifying common downstream targets of the CLV receptors which influence MAPK activity, such as ROP (**Fig. 8A**). Thus, loss of a component in this CLV3–CLV receptor system or exogenous application of MCLV3 might perturb this well-tuned balance of MAPK activity, resulting in the deregulation of the *WUS* expression domain (**Fig. 8A**).

In addition, it should also be considered that the expression domains of the respective CLV components in the SAM display distinct patterns (summarized in **Fig. 8B**). *CLV1* transcripts were exclusively detected in the center of the SAM, overlapping with the *WUS* domain, and a broad ubiquitous expression pattern over the SAM was detected for *CLV2* and *SOL2*/*CRN* expression (**Fig. 8B**) (Clark et al. 1997, Mayer et al. 1998, Fletcher et al. 1999, Jeong et al. 1999). Our recent study on RPK2/TOAD2 localization using the fully functional RPK2/TOAD2–GFP under the control of its own promoter revealed that RPK2/TOAD2 protein distributes preferentially in the PZ and is very weakly detected in the center of the CZ in the SAM (**Fig. 8B**) (Kinoshita et al. 2010). A merged view of the expression domains of the CLV components displays several distinct domains expressing a certain set of the components in the SAM (**Fig. 8B**). Every combination of the CLV receptors in every domain might provide different activity on downstream MAPK regulation, as we have shown that the respective CLV receptors exert

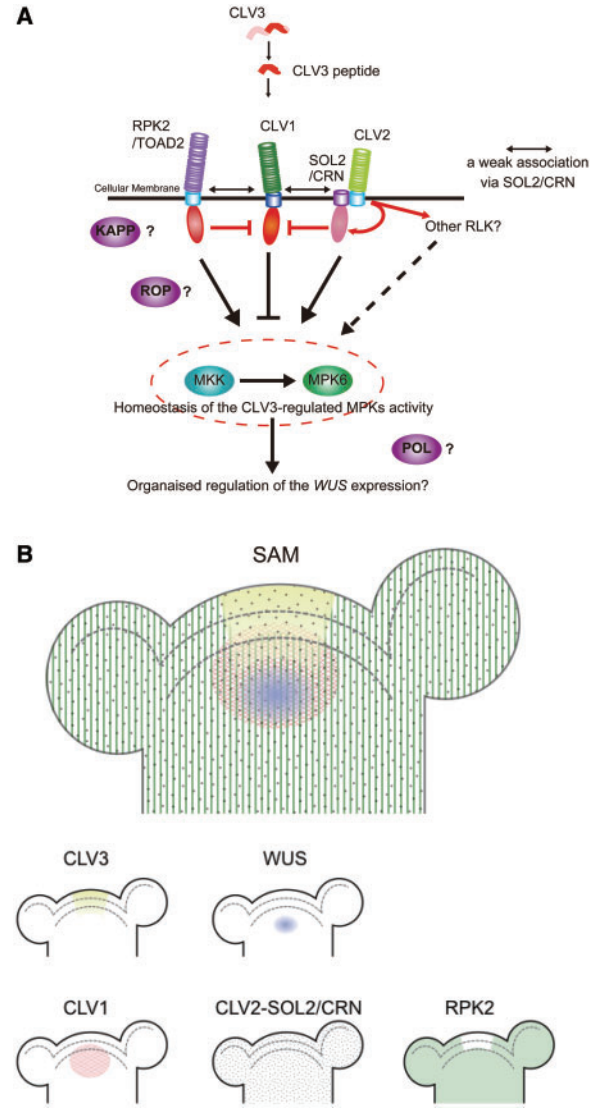

Fig. 8 A schematic model of the CLV receptor activities in MAPK regulation and the expression domains of the CLV components. (A) The activities of the CLV receptors on MAPK regulation and their association specificities detected in this study are schematically shown as discussed in the text. Important CLV signaling components, which might be involved in MAPK regulation, are also shown at the position where they possibly play roles (KAPP, ROP and POL shown in a purple circle). (B) The expression domains of the CLV components in the SAM are schematically shown (Clark et al. 1997, Mayer et al. 1998, Fletcher et al. 1999, Jeong et al. 1999, Kinoshita et al. 2010). The expression domains of *CLV3*, *WUS*, *CLV1*, *CLV2-SOL2/CRN* and RPK2/TOAD2 are indicated as a merged picture on the top and are also shown individually at the bottom.

a unique activity. The *CLV3* domain and the *WUS* domain should also be considered in relation to the spatial positions of the domains of the sets of receptors. This suggests that the specific spatial distribution of the CLV receptors possibly contributes to shaping and maintaining the SAM structure. The orchestrated activity of these different domains may be

critical to restrict the *WUS* domain in the SAM (**Fig. 8B**). Thus, the spatial regulation of the MAPK activity by the CLV receptors through their ability to interact within the CLV receptors and to regulate each other may maintain a sophisticated fine-tuned balance and timing of MAPK activation, and the coordinated regulation of MAPK activity may be required for SAM homeostasis to control the organized cell differentiation in the SAM.

Materials and Methods

Plant materials, growth conditions and MCLV3 assay

Nicotiana benthamiana seeds were grown on a 1:1 mixture of vermiculite and PRO-MIX BX (Premier Horticulture) in a growth room at 22°C under continuous white light (20–50 mmol m^{-2} s^{-1}) after a 2 d incubation at 4°C.

Arabidopsis wild-type and mutant lines have been published (Kinoshita et al. 2010). The *clv1-11* seeds were kindly provided by Professor Rüdiger Simon (Bleckmann et al. 2010). For the selection of transgenic plant expressing *SOL2:10Myc* or *SOL2*$_{K146E}$*:10Myc*, surface-sterilized seeds were plated on growth medium containing Murashige and Skoog basal salts, 1% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) and 1.5% (w/v) agar containing hygromycin (50 mg ml^{-1}). Seeds were then transferred to a growth room at 22°C under continuous white light (20–50 mmol m^{-2} s^{-1}). The selected transgenic plants were transferred onto soil. For the MCLV3 treatment of seedlings, 20 surface-sterilized seeds were incubated in 5 ml of growth medium containing 1/2× Murashige and Skoog basal salts, 1% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) on a rotary shaker (110 min^{-1}) at 22°C under continuous white light (20–50 mmol m^{-2} s^{-1}). After 6 d incubation, the seeds were shifted to a growth room at 22°C under continuous white light (20–50 mmol m^{-2} s^{-1}) and kept without shaking for 20 h before MCLV3 treatment. MCLV3 (10 mM) prepared in 0.1% trifluoroacetic acid (TFA) was added to a final concentration of 10 µM and incubated further for 30 min in the same growing conditions. Mock treatment was performed using the same volume of 0.1% TFA. MCLV3 was synthesized as described previously.

Construction of transgenic plants

All the constructs used for *N. benthamiana* transient expression have been published except *p35S:CLV3-YFP*, *p35S:CLE44-YFP* and *p35S:YFP-YFP* (Kinoshita et al. 2010). The *CLV3* and *CLE44* cDNAs were PCR-amplified from Col-0 cDNA library into pENTR-D/TOPO (Invitrogen). Then, the cDNAs were transferred into pH35GY (Kubo et al. 2005) via LR reaction (Invitrogen). *YFP* sequence was PCR-amplified from pH35GY and cloned into pH35GY as above. For the complementation test, the *CaMV35S:SOL2-10Myc* or *CaMV35S:SOL2*$_{K146E}$*-10Myc* constructs were generated as below. The *CaMV35S:SOL2-10Myc* construct has been reported previously; briefly it was cloned by conjugating the PCR-amplified *SOL2/CRN* full-length cDNA without the stop codon and pGWB20 vector with the aid of Gateway technology. For the kinase-dead version, a point mutation causing the K146E mutation was introduced into the pENTR clone of the *SOL2/CRN* full-length cDNA, which was used for the generation of *CaMV35S:SOL2-10Myc*, using the primers SOL2k146eFW (5′-GGTCTAGTGGTTGCAGTCgAGAGACTAG GCTCAC-3′) and SOL2k146eREV (5′-GTGAGCCTAGTCTCTcG ACTGCAACCACTAGACC-3′) by PCR. PCR was carried out under the following conditions: 95°C for 30 s, followed by 14 cycles of 95°C for 30 s, 55°C for 1 min and 68°C for 7 min, and then incubated at 68°C for 10 min. The amplicon was then treated with *Dpn*I at 37°C for 90 min, subsequently denatured by 65°C incubation for 20 min, and transformed into *Escherichia coli* TOP10 cells (Invitrogen). The resulting clones were confirmed for the introduced mutation by sequencing, and the confirmed *SOL2*$_{K146E}$*-10Myc* sequence was transferred into the Gateway-compatible binary vector pGWB20. The constitutively active mutant MAPKKs were generated by QuickChange site-directed mutagenesis (Stratagene) and confirmed by sequencing. The serine or threonine residues in the activation loop domain [S/T] xxxxx [S/T] were replaced by the acidic glutamate amino acids: MKK4DD (T224D S230D). The kinase-negative form of MKK4 was also generated by site-specific mutations, replacing the conserved lysine residues in the kinase ATP-binding loop by a methionine: MKK4-KN (K108M). All full-length fragments were introduced into the pTA7002 vector (Aoyama and Chua 1997). These plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101::pMP90 and then into *sol2-1* or *clv1-11* plants using the floral dip method (Clough and Bent 1998).

Transient expression in *N. benthamiana*

Agrobacterium tumefaciens strains GV3101 MP90 or MP90RK carrying expression constructs were grown in YEB medium with appropriate antiboiotics, harvested by centrifugation at 4,500 rpm for 10 min, and resuspended in infiltration buffer [10 mM MES (pH 5.7), 10 mM $MgCl_2$, 150 µM acetosyringone]. The cultures were adjusted to an OD_{600} of 1.0 and incubated at room temperature for at least 3 h prior to infiltration. Equal volumes of cultures of different constructs were mixed for co-infiltration, and then mixed with agrobacterial cultures (OD_{600} of 1.0) carrying the *p19* silencing suppressor in a 1:1 ratio. The resulting cultures were infiltrated into leaves of 3- to 4-week-old *N. benthamiana*. The leaf samples were harvested 3 d after infiltration for subsequent protein extraction.

Semi-quantitative RT–PCR analysis

Total RNA was extracted from 7-day-old Arabidopsis seedlings using TRIzol (Invitrogen) following the manufacturer's protocol. SuperscriptIII (Invitrogen) was used for the cDNA synthesis according to the manufacturer's instructions. PCR was carried out in the following conditions: 96°C for 3 min, and the indicated number cycles of 96°C for 15 s, 55°C

for 30 s and 72°C for 1 min. The primer set SOL2Fw, caccC GTAGCATTGTCAAATTCATGAAACAG; and SOL2Rv, GTT ATCAACCACTTTGTACAAGAAAGCTGG, amplifying 996 bp amplicons was used for *SOL2:10Myc*, and the set TUB1Fw, TGCTGTTCTCATGGATCTCG; and TUB1Rv, CCATGCATTCA TCAGCATTC, amplifying 416 bp products was use for TUB1.

Protein extraction

Total protein was extracted from the infiltrated *N. benthamiana* leaves with IP extraction buffer (1:1 w/v, 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM NaF, 0.1 mM Na_3VO_4, 1× Proteinase inhibitor cocktail SIGMA P9599 and 1 mM EDTA). The lysates were centrifuged at 20,000×*g* for 20 min at 4°C and the supernatants were then centrifuged again at 20,000×*g* for 5 min at 4°C. The resultant supernatants were used as total protein lysates.

Co-immunoprecipitation

For immunoprecipitaion, 1 ml of the lysates prepared with IP extracation buffer from 0.5 g of leaves was incubated with the respective antibodies for 1 h in a rotary shaker at 4°C. Then, protein G–Sepharose (Roche 17-0618-01) was added to the samples and the samples were incubated in a rotary shaker at 4°C overnight. The Sepharose beads were washed, collected and washed six times with 1 ml of the extraction buffer. Immunopricipitaed proteins were eluted by boiling in SDS sample buffer at 95°C and analyzed by Western blot using the corresponding antibodies. We used the following antibodies; anti-HA 3F10 Roche 1867423, anti-Myc 9E10 SIGMA M4439 and anti-FLAG M2 SIGMA F3165.

Phosphatase treatment

Immunoprecipitated Sepharose beads were washed twice with 500 µl of IP buffer, once with 500 µl of IP buffer without phosphatase inhibitors (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1% Triton X-100) and once with 400 µl of alkaline phosphatase buffer (TAKARA BIO INC.). The beads were dissolved in 400 µl of alkaline phosphatase buffer and aliquoted into 3× 100 µl. An aliquot was saved as non-treated samples. Either 10 U of alkaline phosphatase [calf intestinal alkaline phosphatase (CIP); TAKARA BIO INC., 2250A] or 10 U of CIP, 50 mM NaF and 1 mM Na_3VO_4 were added to an aliquot and incubated at 37°C for 20 min. All aliquoted beads were boiled in 75 µl of 2× SDS sample buffer and analyzed by Western blotting.

IP-kinase assay

Nicotiana benthamiana lysates and Arabidopsis lysates were prepared using IP extraction buffer and immunoprecipitated as above using 4 µl of α-MPK6 (Ab6NT1) in the presence of protein A–Sepharose beads (Ichimura et al. 2000). After three washing steps with IP extraction buffer, immunoprecipitates were analyzed by IP-kinase assay. After immunoprecipitates were washed with 1 ml of reaction buffer without ATP (25 mM Tris–HCl, pH 7.5, 12 mM $MgCl_2$, 1 mM $CaCl_2$ or 5 mM EGTA, 1 mM dithiothreitol and 0.1 mM orthovanadate), kinase assays were performed in 20 µl of the same buffer containing 5 µM ATP, 1 mCi of [γ-^{32}P]ATP with MBP as a substrate at 30°C for 30 min. The reaction was stopped by the addition of sample buffer. After electrophoresis on a 12% SDS gel, the phosphorylated MBP was visualized by autoradiography.

In-gel kinase assay

The in-gel kinase assay was performed as previously described (Takahashi et al. 2007).

Complementation test using by *MKK4 clv1-11* plants

The seeds of transgenic plants were grown on GM agar plates containing hygromycin and 1% sucrose under long-day conditions (16 h light/8 h dark) at 22°C. Fourteen-day-old seedlings of each transgenic plant were transferred onto soil. Two weeks later, the flower bud clusters were dipped into 20 µM DEX dissolved in 0.05% aqueous Tween-20.

Supplementary data

Supplementary data are available at PCP online.

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References

Aoyama, T. and Chua, N.H. (1997) A glucocorticoid-mediated transcriptional induction system in transgenic plants. *Plant J.* 11: 605–612.

Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.-L., Gomez-Gomez, L., et al. (2002) MAP kinase signalling cascade in Arabidopsis innate immunity. *Nature* 415: 977–983.

Bleckmann, A., Weidtkamp-Peters, S., Seidel, C. and Simon, R. (2010) Stem cell signaling in Arabidopsis requires CRN to localize CLV2 to the plasma membrane. *Plant Physiol.* 152: 166–176.

Brand, U., Fletcher, J.C., Hobe, M., Meyerowitz, E.M. and Simon, R. (2000) Dependence of stem cell fate in Arabidopsis on a feedback loop regulated by CLV3 activity. *Science* 289: 617–619.

Butenko, M.A., Vie, A.K., Brembu, T., Aalen, R.B. and Bones, A.M. (2009) Plant peptides in signalling: looking for new partners. *Trends Plant Sci.* 14: 255–263.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nurnberger, T., Jones, J.D., et al. (2007) A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature* 448: 497–500.

Cho, S.K., Larue, C.T., Chevalier, D., Wang, H., Jinn, T.L., Zhang, S., et al. (2008) Regulation of floral organ abscission in Arabidopsis thaliana. *Proc. Natl Acad. Sci. USA* 105: 15629–15634.

Clark, S.E. (2001) Cell signalling at the shoot meristem. *Nat. Rev. Mol. Cell Biol.* 2: 276–284.

Clark, S.E., Running, M.P. and Meyerowitz, E.M. (1993) *CLAVATA1*, a regulator of meristem and flower development in *Arabidopsis*. *Development* 119: 397–418.

Clark, S.E., Running, M.P. and Meyerowitz, E.M. (1995) *CLAVATA3* is a specific regulator of shoot and floral meristem development affecting the same processes as *CLAVATA1*. *Development* 121: 2057–2067.

Clark, S.E., Williams, R.W. and Meyerowitz, E.M. (1997) The CLAVATA1 gene encodes a putative receptor kinase that controls shoot and floral meristem size in Arabidopsis. *Cell* 89: 575–585.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. *Plant J.* 16: 735–743.

De Smet, I., Voss, U., Jürgens, G. and Beeckman, T. (2009) Receptor-like kinases shape the plant. *Nat. Cell Biol.* 11: 1166–1173.

Fiers, M., Golemiec, E., van der Schors, R., van der Geest, L., Li, K.W., Stiekema, W.J., et al. (2006) The CLAVATA3/ESR motif of CLAVATA3 is functionally independent from the nonconserved flanking sequences. *Plant Physiol.* 141: 1284–1292.

Fiers, M., Golemiec, E., Xu, J., van der Geest, L., Heidstra, R., Stiekema, W., et al. (2005) The 14-amino acid CLV3, CLE19, and CLE40 peptides trigger consumption of the root meristem in Arabidopsis through a CLAVATA2-dependent pathway. *Plant Cell* 17: 2542–2553.

Fletcher, J.C., Brand, U., Running, M.P., Simon, R. and Meyerowitz, E.M. (1999) Signaling of cell fate decisions by CLAVATA3 in Arabidopsis shoot meristems. *Science* 283: 1911–1914.

Fukuda, H., Hirakawa, Y. and Sawa, S. (2007) Peptide signaling in vascular development. *Curr. Opin. Plant Biol.* 10: 477–482.

Heese, A., Hann, D.R., Gimenez-Ibanez, S., Jones, A.M., He, K., Li, J., et al. (2007) The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proc. Natl Acad. Sci. USA* 104: 12217–12222.

Hirakawa, Y., Shinohara, H., Kondo, Y., Inoue, A., Nakanomyo, I., Ogawa, M., et al. (2008) Non-cell-autonomous control of vascular stem cell fate by a CLE peptide/receptor system. *Proc. Natl Acad. Sci. USA* 105: 15208–15213.

Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T. and Shinozaki, K. (2000) Various abiotic stresses rapidly activate Arabidopsis MAP kinases ATMPK4 and ATMPK6. *Plant J.* 24: 655–665.

Ito, Y., Nakanomyo, I., Motose, H., Iwamoto, K., Sawa, S., Dohmae, N., et al. (2006) Dodeca-CLE peptides as suppressors of plant stem cell differentiation. *Science* 313: 842–845.

Jeong, S., Trotochaud, A.E. and Clark, S.E. (1999) The Arabidopsis CLAVATA2 gene encodes a receptor-like protein required for the stability of the CLAVATA1 receptor-like kinase. *Plant Cell* 11: 1925–1934.

Jun, J.H., Fiume, E. and Fletcher, J.C. (2008) The CLE family of plant polypeptide signaling molecules. *Cell Mol. Life Sci.* 65: 743–755.

Kayes, J.M. and Clark, S.E. (1998) *CLAVATA2*, a regulator of meristem and organ development in *Arabidopsis*. *Development* 125: 3843–3851.

Kemmerling, B. and Nürnberger, T. (2008) Brassinosteroid-independent functions of the BRI1-associated kinase BAK1/SERK3. *Plant Signal. Behav.* 3: 116–118.

Kinoshita, A., Betsuyaku, S., Osakabe, Y., Mizuno, S., Nagawa, S., Stahl, Y., et al. (2010) RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis. *Development* 137: 3911–3920.

Kinoshita, A., Nakamura, Y., Sasaki, E., Kyozuka, J., Fukuda, H. and Sawa, S. (2007) Gain-of-function phenotypes of chemically synthetic CLAVATA3/ESR-related (CLE) peptides in *Arabidopsis thaliana* and *Oryza sativa*. *Plant Cell Physiol.* 48: 1821–1825.

Kondo, T., Sawa, S., Kinoshita, A., Mizuno, S., Kakimoto, T., Fukuda, H., et al. (2006) A plant peptide encoded by CLV3 identified by in situ MALDI-TOF MS analysis. *Science* 313: 845–848.

Kubo, M., Udagawa, M., Nishikubo, N., Horiguchi, G., Yamaguchi, M., Ito, J., et al. (2005) Transcription switches for protoxylem and metaxylem vessel formation. *Genes Dev.* 19: 1855–1860.

Laux, T., Mayer, K.F., Berger, J. and Jurgens, G. (1996) The WUSCHEL gene is required for shoot and floral meristem integrity in Arabidopsis. *Development* 122: 87–96.

Lenhard, M. and Laux, T. (2003) Stem cell homeostasis in the Arabidopsis shoot meristem is regulated by intercellular movement of CLAVATA3 and its sequestration by CLAVATA1. *Development* 130: 3163–3173.

Lewis, T.S., Shapiro, P.S. and Ahn, N.G. (1998) Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* 74: 49–139.

Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E. and Walker, J.C. (2002) BAK1, an Arabidopsis LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* 110: 213–222.

Madhani, H.D. and Fink, G.R. (1998) The riddle of MAP kinase signaling specificity. *Trends Genet.* 14: 151–155.

Mayer, K.F., Schoof, H., Haecker, A., Lenhard, M., Jurgens, G. and Laux, T. (1998) Role of WUSCHEL in regulating stem cell fate in the Arabidopsis shoot meristem. *Cell* 95: 805–815.

Miwa, H., Betsuyaku, S., Iwamoto, K., Kinoshita, A., Fukuda, H. and Sawa, S. (2008) The receptor-like kinase SOL2 mediates CLE signaling in Arabidopsis. *Plant Cell Physiol.* 49: 1752–1757.

Müller, R., Bleckmann, A. and Simon, R. (2008) The receptor kinase CORYNE of Arabidopsis transmits the stem cell-limiting signal CLAVATA3 independently of CLAVATA1. *Plant Cell* 20: 934–946.

Nam, K.H. and Li, J. (2002) BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* 110: 203–212.

Nishihama, R., Ishikawa, M., Araki, S., Soyano, T., Asada, T. and Machida, Y. (2001) The NPK1 mitogen-activated protein kinase

kinase kinase is a regulator of cell-plate formation in plant cytokinesis. *Genes Dev.* 15: 352–363.

Ogawa, M., Shinohara, H., Sakagami, Y. and Matsubayashi, Y. (2008) Arabidopsis CLV3 peptide directly binds CLV1 ectodomain. *Science* 319: 294.

Ohyama, K., Shinohara, H., Ogawa-Ohnishi, M. and Matsubayashi, Y. (2009) A glycopeptide regulating stem cell fate in *Arabidopsis thaliana*. *Nat. Chem. Biol.* 5: 578–580.

Popescu, S.C., Popescu, G.V., Bachan, S., Zhang, Z., Gerstein, M., Snyder, M., et al. (2009) MAPK target networks in Arabidopsis thaliana revealed using functional protein microarrays. *Genes Dev.* 23: 80–92.

Rodriguez, M.C., Petersen, M. and Mundy, J. (2010) Mitogen-activated protein kinase signaling in plants. *Annu. Rev. Plant Biol.* 61: 621–649.

Rojo, E., Sharma, V.K., Kovaleva, V., Raikhel, N.V. and Fletcher, J.C. (2002) CLV3 is localized to the extracellular space, where it activates the Arabidopsis CLAVATA stem cell signaling pathway. *Plant Cell* 14: 969–977.

Sawa, S., Kinoshita, A., Betsuyaku, S. and Fukuda, H. (2008) A large family of genes that share homology with CLE domain in Arabidopsis and rice. *Plant Signal. Behav.* 3: 337–339.

Sawa, S., Kinoshita, A., Nakanomyo, I. and Fukuda, H. (2006) CLV3/ESR-related (CLE) peptides as intercellular signaling molecules in plant. *Chem. Rec.* 6: 303–310.

Schoof, H., Lenhard, M., Haecker, A., Mayer, K.F., Jurgens, G. and Laux, T. (2000) The stem cell population of Arabidopsis shoot meristems in maintained by a regulatory loop between the CLAVATA and WUSCHEL genes. *Cell* 100: 635–644.

Sharma, P.C., Ito, A., Shimizu, T., Terauchi, R., Kamoun, S. and Saitoh, H. (2003) Virus-induced silencing of WIPK and SIPK genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in Nicotiana benthamiana. *Mol. Genet. Genomics* 269: 583–591.

Shiu, S.H. and Bleecker, A.B. (2001) Plant receptor-like kinase gene family: diversity, function, and signaling. *Sci. STKE* 2001(113): RE22.

Song, S.-K., Lee, M.M. and Clark, S.E. (2006) POL and PLL1 phosphatases are CLAVATA1 signaling intermediates required for Arabidopsis shoot and floral stem cells. *Development* 133: 4691–4698.

Stone, J.M., Trotochaud, A.E., Walker, J.C. and Clark, S.E. (1998) Control of meristem development by CLAVATA1 receptor kinase and kinase-associated protein phosphatase interactions. *Plant Physiol.* 117: 1217–1225.

Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seo, S., Yonezawa, M., et al. (2007) The mitogen-activated protein kinase cascade MKK3–MPK6 is an important part of the jasmonate signal transduction pathway in Arabidopsis. *Plant Cell* 19: 805–818.

Teige, M., Scheikl, E., Eulgem, T., Dóczi, R., Ichimura, K., Shinozaki, K., et al. (2004) The MKK2 pathway mediates cold and salt stress signaling in Arabidopsis. *Mol. Cell* 15: 141–152.

Tor, M., Lotze, M.T. and Holton, N. (2009) Receptor-mediated signalling in plants: molecular patterns and programmes. *J. Exp. Bot.* 60: 3645–3654.

Trotochaud, A.E., Hao, T., Wu, G., Yang, Z. and Clark, S.E. (1999) The CLAVATA1 receptor-like kinase requires CLAVATA3 for its assembly into a signaling complex that includes KAPP and a Rho-related protein. *Plant Cell* 11: 393–406.

Tucker, M.R. and Laux, T. (2007) Connecting the paths in plant stem cell regulation. *Trends Cell Biol.* 17: 403–410.

Wang, H., Ngwenyama, N., Liu, Y., Walker, J.C. and Zhang, S. (2007) Stomatal development and patterning are regulated by environmentally responsive mitogen-activated protein kinases in Arabidopsis. *Plant Cell* 19: 63–73.

Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M.B., et al. (2008) Sequential transphosphorylation of the BRI1/BAK1 receptor kinase complex impacts early events in brassinosteroid signaling. *Dev. Cell* 15: 220–235.

Wang, Z.Y., Seto, H., Fujioka, S., Yoshida, S. and Chory, J. (2001) BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature* 410: 380–383.

Williams, R.W., Wilson, J.M. and Meyerowitz, E.M. (1997) A possible role for kinase-associated protein phosphatase in the Arabidopsis CLAVATA1 signaling pathway. *Proc. Natl Acad. Sci. USA* 94: 10467–10472.

Yu, L.P., Miller, A.K. and Clark, S.E. (2003) POLTERGEIST encodes a protein phosphatase 2C that regulates CLAVATA pathways controlling stem cell identity at Arabidopsis shoot and flower meristems. *Curr. Biol.* 13: 179–188.

Yu, L.P., Simon, E.J., Trotochaud, A.E. and Clark, S.E. (2000) POLTERGEIST functions to regulate meristem development downstream of the CLAVATA loci. *Development* 127: 1661–1670.

Zhu, Y., Wang, Y., Li, R., Song, X., Wang, Q., Huang, S., et al. (2009) Analysis of interactions among the CLAVATA3 receptors reveals a direct interaction between CLAVATA2 and CORYNE in Arabidopsis. *Plant J.* 61: 223–233.