RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in *Arabidopsis*

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A reference was omitted in *Development* 137, 3911-3920.

In the first sentence on p. 3912, a paper by Jeong et al. (Jeong et al., 1999) should have been cited.

Reference


The authors apologise to readers for this mistake.
RPK2 is an essential receptor-like kinase that transmits the CLV3 signal in Arabidopsis

Atsuko Kinoshita1, Shigeyuki Betsuyaku1,2, Yuriko Osakabe3, Shinji Mizuno4,5, Shingo Nagawa1,6, Yvonne Stahl7, Rüdiger Simon7, Kazuko Yamaguchi-Shinozaki3,5, Hiroo Fukuda1 and Shinichiro Sawa1,*

SUMMARY
The shoot apical meristem (SAM) is the fundamental structure that is located at the growing tip and gives rise to all aerial parts of plant tissues and organs, such as leaves, stems, and flowers. In Arabidopsis thaliana, the CLAVATA3 (CLV3) pathway regulates the stem cell pool in the SAM, in which a small peptide ligand derived from CLV3 is perceived by two major receptor complexes, CLV1 and CLV2-CORYNE (CRN)/SUPPRESSOR OF LPP1 2 (SOL2), to restrict WUSCHEL (WUS) expression. In this study, we used the functional, synthetic CLV3 peptide (MCLV3) to isolate CLV3-insensitive mutants and revealed that a receptor-like kinase, RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), also known as TOADSTOOL 2 (TOAD2), is another key regulator of meristem maintenance. Mutations in the RPK2 gene result in stem cell expansion and increased number of floral organs, as seen in the other clv mutants. These phenotypes are additive with both clv1 and clv2 mutations. Moreover, our biochemical analyses using Nicotiana benthamiana revealed that RPK2 forms homo-oligomers but does not associate with CLV1 or CLV2. These genetic and biochemical findings suggest that three major receptor complexes, RPK2 homomers, CLV1 homomers and CLV2-CRN/SOL2 heteromers, are likely to mediate three signalling pathways, mainly in parallel but with potential crosstalk, to regulate the SAM homeostasis.

KEY WORDS: Arabidopsis, Meristem, CLAVATA3 signalling pathway, Receptor-like kinase

INTRODUCTION
Multicellular organisms require the precise coordination of cell division and differentiation to ensure organised development. Plants have evolved a unique structure called the meristem, which consists of cells that divide continuously and renew themselves. Two major meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM), are located on the top and the bottom ends of the apical-basal axis, respectively, and are responsible for providing cells for postembryonic growth and development. Each meristem forms repetitive structures, and is maintained throughout the life of the plant. In Arabidopsis thaliana, the SAM is divided into three areas: the peripheral zone (PZ), the central zone (CZ) and the rib zone (RZ). In the CZ, undifferentiated stem cell resides just above the organising centre (OC), and their descendant cells displaced to PZ are recruited into organ differentiation. The RAM, on the other hand, consists of several cell files, which originate from the stem cells surrounding the quiescent centre (QC) (Miwa et al., 2009a; Tucker and Laux, 2007).

Genetic studies have revealed that members of a homeobox gene family play a pivotal role in meristem maintenance. In the SAM, the WUSCHEL (WUS) homeodomain transcription factor is expressed in the OC and its activity promotes stem cell fate in a non-cell autonomous manner (Mayer et al., 1998). By contrast, a small signalling peptide encoded by CLAVATA3 (CLV3) is secreted from stem cell to restrict WUS expression, thus establishing a negative feedback loop (Brand et al., 2000; Clark et al., 1995; Schoof et al., 2000). This CLV3-WUS negative feedback loop is represented by the opposite phenotype of these loss-of-function mutants: wus mutants exhibit SAM termination, whereas clv3 mutants produce an enlarged SAM (Clark et al., 1995; Laux et al., 1996). This model is also supported by the fact that CLV3 overexpression leads to wus-like phenotype (Brand et al., 2000). Recently, genetic and biochemical studies have demonstrated that the conserved C terminus of the CLV3 protein, the CLV3/ESR (CLE) domain, is necessary and sufficient for its function, and a series of exogenously applied synthetic peptides corresponding to the CLE domain is able to mimic the CLV3 overexpression phenotype (Fiers et al., 2006; Fiers et al., 2005; Kondo et al., 2006; Sawa et al., 2008; Sawa et al., 2006). Although the clv3 mutants develop normal roots, the synthetic CLV3 peptides, as well as CLV3 overexpression induce RAM consumption in a CLV2-dependent manner, suggesting that there is a CLV-like pathway for sensing CLV3 ligand in roots (Clark et al., 1995; Fiers et al., 2005).

In the SAM, at least two receptor complexes function for the CLV3 recognition. CLV1, which encodes a member of leucine-rich repeat (LRR) receptor-like kinase (RLK), is expressed at the centre of the SAM, including the WUS-expressing OC, and the extracellular domain of the protein has been proved to bind CLV3 peptides (Clark et al., 1993; Ogawa et al., 2008; Ohyama et al.,...
In the second pathway, CLV2, an LRR receptor-like protein lacking a cytoplasmic domain, acts together with a membrane-associated protein kinase, CORYNE (CRN)/SUPPRESSOR OF LLP1 2 (SOL2), to transmit the CLV3 signal (Casamitjana-Martínez et al., 2003; Kayes and Clark, 1998; Miwa et al., 2008; Miwa, 2009b; Müller et al., 2008). The CRN/SOL2-CLV2 interaction is shown to promote the plasma membrane localization of the complex, where the complex is supposed to perceive extracellular CLE ligand signal(s). The homo-oligomer of CLV1 is able to weakly interact with CRN/SOL2-CLV2 complex, presumably via CRN/SOL2, suggesting a potential crossstalk between two CLV3 signalling pathways (Bleckmann et al., 2010; Zhu et al., 2009).

Nevertheless, these two complexes can independently transmit the CLV3 signal, because a crn mutation exerts an additive effect on the clv1 phenotype. However, clv1 crn double mutants have slightly smaller inflorescence meristems and fewer carpels than single clv3 mutants, suggesting the presence of an additional receptor complex that allows CLV3 signalling (Müller et al., 2008).

In this study, we showed that an LRR-RLK, RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2), also known as TOADSTOOL 2 (TOAD2), is involved in maintenance of the SAM and the RAM downstream of a synthetic CLV3 peptide. A loss-of-function mutant of RPK2 exhibited a clv-like phenotype, and is additive to both clv1 and clv2. Furthermore, biochemical analysis using Nicotiana benthamiana demonstrated a biochemical interaction between CLV2-CRN/SOL2, but failed to detect an interaction between RPK2 and any of the other receptor proteins: CLV1, CLV2 and CRN/SOL2. These observations suggest that RPK2 is an essential component of an independent third pathway for CLV3 signalling.

**MATERIALS AND METHODS**

**Plant materials and growth conditions**

*Arabidopsis* wild-type and mutant lines were obtained as follows: Columbia-0 (Col-0), Wassilewskija-2 (Ws-2), clv1-101 (CS85348) in Col-2 background, clv1-3 8 ER in unknown background (CS3604) (Dievart et al., 2003), rpk2-1 in Col-0 background, rpk2-2 in Col-0 background and rpk2-3 (CS3960) in Ws-2 background (ABRC Stock Centre, Ohio, USA); clv2-101 (GK866A09) in Col-0 background (GABI-kat, Bielefeld, Germany); was-101 (GK870H12) in Col-0 background (Prof. Jan Lohmann, University of Heidelberg, Heidelberg, Germany); 35S::RPK2 in Col-0 background (Prof. Kazuko Yamaguchi Shinozaki, University of Tokyo, Tokyo, Japan) (Mizuno et al., 2007).

For mature plant materials, seeds were sown on soil. For root and seedling assays, 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. Seeds were then transferred to a growth room at 22°C under continuous white light (20-50 mmol m⁻²s⁻¹). MCLV3 was synthesized as described previously (Kondo et al., 2006).

**Screening and map-based cloning of clv1**

clv1/rpk2-4 was isolated from the ethylmethane-sulfonate (EMS)-mutagenized population generated previously (Sawa et al., 2005), and was backcrossed three times before further analyses. To map the CLV1 locus, the clv1 mutant in Columbia-0 (Col-0) background was crossed to Landsberg erecta (La-er) and homozygous mutants were selected in the F2 population for segregation analyses. For their male sterility, all rpk2 alleles were screened for their homozygosity using a PCR strategy.

**Construction of transgenic plants**

For the complementation test and expression analysis, the RPK2- and the GFP-coding region were combined using PCR with overlapping primers, and Smal and BamHI restriction sites were introduced at each end. The 1310 bp promoter and 983 bp terminator of RPK2 were amplified by PCR, with Smal and BamHI sites, respectively. Each fragment was cloned into the gateway entry vector pENTR-D-TOPO in accordance with the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA), and fused using corresponding restriction enzymes (TaKaRa, Shiga, Japan) and Ligation High enzyme (TOYOBO, Osaka, Japan). The entry vector possessing the full pRPK2::RPK2-GFP:ter fragment was verified by sequencing, and subsequently transferred to the gateway-compatible binary vector pGWB1 using LR clonase (Invitrogen). For the 35S::WUS construct, WUS-coding region was amplified from reverse-transcribed total RNA of wild-type inflorescence meristem, and was cloned into pENTR-D-TOPO, subsequently transferred to the gateway-compatible binary vector pH35G. These vectors were introduced into Agrobacterium tumefaciens strain GV3101::pMP90 and then into rpk2-2 plants using the floral dip method (Clough and Bent, 1998).

**Microscopic analyses**

Scanning electron microscopic (SEM) analysis was performed as described previously (Kinosita et al., 2007). In situ hybridization was performed according to the previous report (Hejakto et al., 2006) but using 8 μm sections. The RPK2 antisense probe was generated from the 1791 bp fragment amplified by PCR using the primers 5'-GCTAGCGTG-GGTGCAGAAGA-3' and 5'-GAACTGGCTGCCATTTCTCC-3'. The CLV3 antisense probe was obtained from the 721 bp fragment amplified by PCR using the primers 5'-TGGATCCGGAAGTTTCTG-3' and 5'-CTTGACGACAAAAGGTAAGT-3'. The WUS antisense probe was generated as described previously (Mayer et al., 1998).

For GFP analysis, aerial parts of plant materials were embedded in 5% agarose and sliced. Longitudinal sections (50 μm) were mounted in water and the GFP fluorescence was observed by confocal laser scanning microscopy (TCS SP, Leica Microsystems, Heidelberg, Germany). Root samples were directly mounted in water and the GFP signal was detected.

**Gene expression analyses**

Total RNA for quantitative RT-PCR was isolated from inflorescence meristems with stage 6 and earlier flowers using TRizol Reagent (Invitrogen). Dissolved RNA samples were subjected to on-column DNA digestion with the RNase-free DNAse set (QIAGEN, Hilden, Germany), and cleaned up with the RNasey spin column (QIAGEN). μg of total RNA were used for generating the first-strand cDNA using Superscript II (Invitrogen). Quantitative RT-PCR analysis was conducted on a Roche LightCycler using the LightCycler TaqMan Master (Roche Diagnostics, Basel, Switzerland). 1 μl out of 20 μl of RT reaction and the following primer-probe pairs: CLV3-155F (5'-AGCTTTTCAACCCGGCAAGATG-3') and CLV3-155R (5'-TCATGTAGTCCTAAACCCTTCGT-3') and probe #155 for CLV3; WUS-33F (5'-AAACCAAGACCATCTCATCTATC-3') and WUS-33R (5'-TCAGTACCTGAGCTTGCATGA-3') and probe #33 for WUS; and TUA4-22F (5'-CGTTTTCAGAGTGGTTA-3') and probe #22 for TUA4 (TUBULIN ALPHA-4 CHAIN) as a control (Universal Probe Library, Roche Diagnostics). Standard curves were constructed using purified PCR fragments of each gene-coding region.

**Transient expression in Nicotiana benthamiana**

Constructs were made using primers and vectors listed in Table S2 in the supplementary material. A. tumefaciens strains GV3101 MP90 or MP90RK carrying expression constructs were grown in YEB media with appropriate antibiotics, harvested by centrifugation at 2600 g for 10 minutes, and resuspended in infiltration buffer [10 mM MES (pH 5.7), 10 mM MgCl₂, 150 μM acetoxyringonone]. The cultures were adjusted to an OD600 of 1.0 and incubated at room temperature, at least, for 3 hours prior to infiltration. Equal volumes of cultures of different constructs were mixed for co-infiltration, and then mixed with agrobacterial cultures (OD600 of 1.0) carrying p19 silencing suppressor in a 1:1 ratio (Voinnet et al., 2003). The resulting cultures were infiltrated into leaves of 3- to 4-week-old *N. benthamiana*. The leaf samples were harvested 3 days after infiltration for subsequent protein extraction.
A third pathway for CLV3 signalling

RESULTS
The CLV3 peptide acts through the endogenous CLV3 pathway involved in SAM regulation
Previously, we have shown that the synthetic CLV3 dodecapeptide (MCLV3) is able to restrict the size of the SAM, however, the molecular basis for the MCLV3-induced SAM defect was yet unknown (Kondo et al., 2006). Here, our bioassay using the synthetic MCLV3 confirmed that the exogenously applied MCLV3 mimics endogenous CLV3 overexpression to restrict the size of the SAM after WUS depression through the functions of CLV1 and CLV2 (see Fig. S1 in the supplementary material). We have also observed a similar trend in the bolting phenotype, when growing plants on a solid medium supplied with MCLV3, enabling us to perform simple chemical genetics screenings on MCLV3 solid media (see Table S1 in the supplementary material).

In order to identify novel molecular components operating in the CLV3 signalling pathway, we have performed mutational screens for insensitivity to MCLV3 on ~10,000 M2 plants derived from EMS-mutagenised Arabidopsis seed pools. A total of 14 mutants, designated clv3 peptide insensitive (cli) mutants, were isolated for maintaining the SAM when grown on agar media containing 5 μM MCLV3. Among them, cli1 mutant showed the strongest resistance to the MCLV3. When grown on agar plate containing 5 μM MCLV3, cli1 plants were able to maintain the SAM activity and to restrict the meristem size both in the shoot and the root, similar to CLV2 and CRN/SOL2. This indicates that the cli1 phenotype was caused by a single recessive mutation.

CL11 encodes a leucine-rich repeat receptor like kinase
The cli1 mutation was mapped to a single locus on chromosome 3. Fine mapping located the gene between 74683 bp of BAC F28J7 and 55403 bp of BAC F14P3 (see Fig. S2A in the supplementary material). The DNA sequencing of this region identified a nucleotide exchange from G to A at 2656 bp from the start codon, which causes substitution of Gly886 with an arginine at the putative ATP-binding site of the predicted protein (see Fig. S2B in the supplementary material). At3g02130 encodes RPK2-RLK and has been reported as RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2) (Mizuno et al., 2007) or TOADSTOOL 2 (TOAD2) (Nodine et al., 2007). Previous reports have demonstrated that RPK2 is a key regulator of another development (Mizuno et al., 2007) or RPK2′ (designated as rpk2-4) (Fig. A-H) and rpk2-2 transformed with the pRPK2::RPK2:GFP (H); and 14-day-old seedlings of wild type (Ws) (C) and rpk2-3 (F). Plants were grown on agar medium with (B-H) or without (A) 5 μM MCLV3. Black arrows indicate the inflorescence meristem regions, and red arrowheads indicate no apical meristem phenotype. (I-R) Eight-day-old seedlings of wild type (Col; I, K; Ws; J, L), rpk2-1 (M), rpk2-2 (N), rpk2-3 (O), cli1 (rpk2-4, P), cli1/rpk2-2 (designated as cli1/rpk2-4) (Q) and rpk2-2 transformed with the pRPK2::RPK2:GFP (R). Plants were grown on agar medium with (K-R) or without (I, L) 5 μM MCLV3. Scale bars: 5 mm in A-H; 1 cm in I-R.

Co-immunoprecipitation
Total protein was extracted from 0.5 g of infiltrated N. benthamiana leaves with 1 ml of extraction buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 10 mM NaF, 0.1 mM Na3VO4, 1× Proteinase inhibitor cocktail SIGMA P9599 and 1 mM EDTA]. The lysates were centrifuged at 20,000 g for 20 minutes at 4°C and the supernatants were then centrifuged again at 20,000 g for 5 minutes at 4°C. The resulted supernatants were incubated with respective antibodies for 1 hour in a rotary shaker at 4°C. Protein G sepharose (Roche 17-0618-01) was then added to the samples and the samples were incubated in a rotary shaker at 4°C overnight. The sepharose beads were collected and washed four times with 1 ml of the extraction buffer. Immunoprecipitated proteins were eluted by boiling in SDS sample buffer at 95°C and analysed on western blot using corresponding antibodies. We used the following antibodies: anti-HA 3F10 (Roche, 1867423), anti-Myc 9E10 (Sigma, M4439) and anti-FLAG M2 (Sigma, F3165).

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Fig. 1. The cli1/rpk2 mutants are insensitive to MCLV3 treatment. (A-H) Twenty-day-old seedlings of wild-type (Col; A, B), rpk2-1 (D), rpk2-2 (E), cli1 (designated as rpk2-4) (G) and rpk2-2 transformed with the pRPK2::RPK2:GFP (H); and 14-day-old seedlings of wild type (Ws) (C) and rpk2-3 (F). Plants were grown on agar medium with (B-H) or without (A) 5 μM MCLV3. Black arrows indicate the inflorescence meristem regions, and red arrowheads indicate no apical meristem phenotype. (I-R) Eight-day-old seedlings of wild type (Col; I, K; Ws; J, L), rpk2-1 (M), rpk2-2 (N), rpk2-3 (O), cli1 (rpk2-4, P), cli1/rpk2-2 (designated as cli1/rpk2-4) (Q) and rpk2-2 transformed with the pRPK2::RPK2:GFP (R). Plants were grown on agar medium with (K-R) or without (I, L) 5 μM MCLV3. Scale bars: 5 mm in A-H; 1 cm in I-R.
Although the size of the inflorescence meristem of rpk2 was not strongly affected (Fig. 2A-C), the size of the rpk2 vegetative SAM was slightly enlarged (see Fig. S9A-C in the supplementary material). In addition, the numbers of floral organ in all four whorls of rpk2-2 and rpk2-4 was occasionally increased, compared with the typical 4-4-6-2 pattern of the wild type (Fig. 2D-O). To quantify the effect of rpk2 on the floral meristem, we counted the number of carpels, which has been established as a good indicator for the clv phenotype (Ni and Clark, 2006). In contrast to the wild-type flowers producing invariably two carpels per flower, rpk2-1, rpk2-2 and rpk2-4 developed an increased number of carpels (Table 1). Comparing with clv1, clv2 or crn/sol2, the rpk2 mutation is weak and impenetrant but statistically distinct from the wild type. The phenotype was often observed in the flowers generated at the basal part of the inflorescence in rpk2-2, and rpk2-4 produced the abnormal flowers constantly (see Fig. S4A-D in the supplementary material). The severe phenotype was also observed just before the termination of the inflorescence meristem (see Fig. S4E-G in the supplementary material), and the rpk2-4 flowers often developed carpels interior to the whorl 4 gynoecium (see Fig. S4H in the supplementary material).

In the root, all the rpk2 alleles were resistant to MCLV3. As in the carpel number phenotypes, rpk2-4 showed the strongest resistance (see Fig. S4I in the supplementary material). In contrast to the rpk2 phenotypes in the floral organ, rpk2 mutants do not exhibit any obvious morphological alterations in the root when grown without the peptide (data not shown). This observation again led us to the idea that rpk2 shares similarities with clv2 and crn/sol2 that also do not show any morphological root phenotype per se (Kayes and Clark, 1998; Miwa et al., 2008; Müller et al., 2008). Taken together, our data indicate that RPK2 contributes to CLV3 signalling in the SAM and may compose a CLV-like pathway together with CLV2 and CRN/SOL2 acting in the RAM.

Expression of RPK2

Analyses of pRPK2::GUS transgenic plants have shown RPK2 expression in the root tips and the shoot meristems ( Mizuno et al., 2007). In order to dissect further the spatial pattern of the RPK2 expression, we performed in situ mRNA hybridization experiments. Expression of RPK2 was observed in the inflorescence meristem, floral meristem, floral organ primordium, the vascular bundles and the RAM region (Fig. 3A-E). The serial sections of the inflorescence meristem detected the RPK2 transcripts uniformly through the inflorescence meristem (Fig. 3B). In the RAM, RPK2 signal was detected through the RAM.

Table 1. Number of carpels in various mutants

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Carpels per flower</th>
<th>SE</th>
<th>n</th>
<th>Valveless siliques (%)*</th>
</tr>
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<tbody>
<tr>
<td>Col</td>
<td>2.0</td>
<td>0.0</td>
<td>100</td>
<td>N/D</td>
</tr>
<tr>
<td>rpk2-1</td>
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<td>0.0</td>
<td>150</td>
<td>N/D</td>
</tr>
<tr>
<td>rpk2-2</td>
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<td>0.1</td>
<td>90</td>
<td>N/D</td>
</tr>
<tr>
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<td>0.1</td>
<td>110</td>
<td>0.9</td>
</tr>
<tr>
<td>rpk2-2/rpk2-4</td>
<td>2.5</td>
<td>0.1</td>
<td>90</td>
<td>1.1</td>
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<tr>
<td>RPK2rpk2-4</td>
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<td>0.0</td>
<td>100</td>
<td>N/D</td>
</tr>
<tr>
<td>clv1-101</td>
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<td>0.1</td>
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<td>N/D</td>
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<td>0.1</td>
<td>50</td>
<td>N/D</td>
</tr>
<tr>
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<td>0.1</td>
<td>120</td>
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<td>190</td>
<td>32.1</td>
</tr>
</tbody>
</table>

*Percent of flowers with gynoecia in which the basal 25% completely lacked valves.

In order to decipher the functions of RPK2 in meristem and flowers in more detail, we observed the phenotypes of rpk2 plants grown under MCLV3-free condition. Although the size of the inflorescence meristem of rpk2 was not strongly affected (Fig. 2A-C), the size of the rpk2 vegetative SAM was slightly enlarged (see Fig. S9A-C in the supplementary material). In addition, the numbers of floral organ in all four whorls of rpk2-2 and rpk2-4 was occasionally increased, compared with the typical 4-4-6-2 pattern of the wild type (Fig. 2D-O). To quantify the effect of rpk2 on the floral meristem, we counted the number of carpels, which has been established as a good indicator for the clv phenotype (Ni and Clark, 2006). In contrast to the wild-type flowers producing invariably two carpels per flower, rpk2-1, rpk2-2 and rpk2-4 developed an increased number of carpels (Table 1). Comparing with clv1, clv2 or crn/sol2, the rpk2 mutation is weak and impenetrant but statistically distinct from the wild type. The phenotype was often observed in the flowers generated at the basal part of the inflorescence in rpk2-2, and rpk2-4 produced the abnormal flowers constantly (see Fig. S4A-D in the supplementary material). The severe phenotype was also observed just before the termination of the inflorescence meristem (see Fig. S4E-G in the supplementary material), and the rpk2-4 flowers often developed carpels interior to the whorl 4 gynoecium (see Fig. S4H in the supplementary material).

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including initial cells and the QC but not the columella cells (Fig. 3D). We also assessed the localization of RPK2 protein using the stable transgenic Arabidopsis expressing functional RPK2-GFP fusion protein under the control of the own promoter. In contrast to the uniform distribution of the RPK2 transcripts, confocal microscopic analysis showed that the RPK2-GFP fusion protein is preferentially detected in the PZ and rather weakly in the CZ (Fig. 3F-I). In the RAM, however, the RPK2-GFP is detected similarly with the RPK2 transcript (Fig. 3J,K). Together with the rpk2 phenotypic data, these results suggest that RPK2 may function as a signalling molecule in both SAM and RAM maintenance mediated by endogenous CLV3 or a CLV3-related CLE peptide.

**Overexpression of RPK2 phenocopies CLV3 overexpressing plants and wus mutants**

We then investigated the roles of RPK2 activity in SAM and RAM maintenance using Arabidopsis stable transgenic lines constitutively overexpressing the RPK2 gene under the control of the cauliflower mosaic virus 35S promoter (RPK2 OX). The RPK2 OX plants occasionally showed developmental arrest of SAM growth, which resembles the MCLV3-treated plants, CLV3 OX plants and wus mutants (Fig. 4A,B; see Fig. S5A in the supplementary material). The RAM of the RPK2 OX transgenic lines was diminished compared with the wild type (Fig. 4I,J) and resulted in a short-root phenotype (Fig. 4C-H). Quantitative RT-PCR confirmed that the expression level of RPK2 is slightly upregulated in rpk2-2 (Fig. 5D,H). Quantitative RT-PCR confirmed that the expression level of CLV3 in rpk2-2 is slightly upregulated compared with wild type, but less strongly upregulated than in clv1-101 or clv2-101 (Fig. 5I). These results correlate with the severity of the mutant phenotype. Interestingly, although its expression regions are enlarged, WUS expression levels in clv1-101 and clv2-101 are only slightly affected (Fig. 5J).

**Expressions of shoot meristem marker genes in rpk2**

Given its identity as an LRR-RLK, we hypothesised that RPK2 functions closely together with other known receptors to transmit the CLV3 signal. To examine this hypothesis, we compared the phenotype of rpk2, clv1 and clv2. clv1-101 and clv2-101 are the presumed null alleles of CLV1 and CLV2, respectively, both in Col background, and these mutants show a weak clv phenotype (see Fig. S3A-C; Fig. S6 in the supplementary material). Histological analysis revealed that the expression regions of both CLV3 and WUS are enlarged in clv1-101 and clv2-101 (Fig. 5A-C,E-G), which is consistent with the previous reports for La-er alleles. By contrast, these expression regions are almost normal or slightly expanded in rpk2-2 (Fig. 5D,H). Quantitative RT-PCR confirmed that the expression level of CLV3 in rpk2-2 is slightly upregulated compared with wild type, but less strongly upregulated than in clv1-101 or clv2-101 (Fig. 5I). These results correlate with the severity of the mutant phenotype. Interestingly, although its expression regions are enlarged, WUS expression levels in clv1-101 and clv2-101 are only slightly affected (Fig. 5J).

**Genetic interaction between CLV1, CLV2, WUS and RPK2**

In order to examine the relationship among CLV1, CLV2 and RPK2, we constructed double and triple mutants of these three genes. To avoid any interfering effects as known for clv1 alleles (Dievart et al., 2003), null alleles in Col background were chosen for the crossing. First, we observed the inflorescence meristem of bolting plants. As reported for La-er alleles before, clv1-101 and clv2-101 produced

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**Fig. 3. RPK2 expression in the shoot and root apical meristems.** (A-E) RNA in situ hybridization performed using the RPK2 antisense probe. SAM sections of wild type (Col; A, serial sections: B1-B7) and rpk2-2 (C), and the whole-mount root meristems of wild type (Col; D) and rpk2-2 (E). (F-K) GFP signals of inflorescence meristem (G), stage 3 flower (H), stage 7 flower (I) and root meristems (J,K) in pRPK2::RPK2:GFP transgenic plant. The autofluorescence of chloroplasts is also detected in the Col wild type (F) with the same microscopic condition, and the signal at the plasma membrane is specifically detected in the transgenic plant (G-K). IM, inflorescence meristem; FM, floral meristem; asterisk, quiescent centre; arrows, columnella cells. White arrowheads indicate the central zone. Scale bars; 200 μm (A), 50 μm (B-K).
larger SAM than wild type (Fig. 6A-C), but rarely fasciated under long-day conditions. However, clv1-101 clv2-101, clv1-101 rpk2-2 and clv2-101 rpk2-2 double mutants exhibited larger SAM than any single mutants (Fig. 6B,C,E-J). Especially, four out of nine and three out of five SAM were fasciated in clv1-101 clv2-101 and clv1-101 rpk2-2, respectively (Fig. 6G,J). Furthermore, clv1-101 clv2-101 rpk2-2 triple mutant showed not only the fasciated SAM (3 out of 12; Fig. 6K), but also the massively overproliferated SAM, as seen in clv3 single mutants (eight out of 12; Fig. 6D,L). A similar trend was observed for 7-day-old vegetative SAMs of each mutant (see Fig. S7A-C in the supplementary material), and the SAM of clv1-101 clv2-101 rpk2-2 triple mutants is similar in size to that of clv3-8. We next examined the effect of the rpk2 mutation on the clv1 or clv2 floral meristems. Compared with each single mutant, carpel numbers were increased from 3.4 to 4.0 in clv1-101 rpk2-2, and from 2.3 to 3.0 in clv2-101 rpk2-2 (Table 1). In addition, as reported, a fifth whorl of organs was observed in clv1-101, clv2-101, clv1-101 rpk2-2 and clv2-101 rpk2-2. However, only the double mutant of clv1-101 rpk2-2 and clv2-101 rpk2-2 exhibited externally emerged, highly proliferated fifth whors (Fig. 6M-P), indicating that rpk2 is additive with clv1 or clv2 in the floral meristem phenotype. Unexpectedly, however, clv1-101 clv2-101 double mutant and clv1-101 clv2-101 rpk2-2 triple mutant had fewer carpels than the clv1-101 single mutant (Table 1). This may be due to the valveless phenotype (Fig. 6Q-S), which is often seen in clv2 and clv1 severe alleles (Diévart et al., 2003; Kayes and Clark, 1998). Alternatively, it is also possible that these receptors redundantly function in fruit development other than the floral meristem regulation. These data suggest that RPK2 functions independently of CLV1 or CLV2 in both inflorescence and floral meristems.

In order to position RPK2 genetically in the CLV3-WUS signalling, we tested the effect of WUS in the rpk2-2 background. As observed in the wus-101 single mutant, the wus-101 rpk2-2 double mutant exhibited a terminated SAM (see Fig. S8A-C in the
supplementary material). Furthermore, 35S::WUS in rpk2-2 transgenic plants produced the larger SAMs, resulting in stem fasciation (see Fig. S8D-G in the supplementary material). Thus, these genetic data strongly suggest that WUS is epistatic to RPK2.

Biochemical interaction of RPK2 with CLV1 and CLV2

Our genetic data suggested that RPK2 functions in parallel with CLV1 and CLV2 to restrict WUS expression in the SAM. To further provide an evidence for our genetic data on RPK2 function, we next investigated biochemically whether RPK2 associates with CLV1 or CLV2-CRN/SOL2 complexes using the Agrobacterium tumefaciens-mediated transient expression system in Nicotiana benthamiana leaf epidermis. The N. benthamiana system has been established to demonstrate that CLV1 homo-oligomer and CLV2-CRN/SOL2 hetero-oligomer co-exist at the plasma membrane by FRET analysis, consistent with genetically proposed two independent CLV3 signalling pathways. These two complexes are capable of interacting weakly with each other, suggesting a potential crosstalk (Bleckmann et al., 2010; Zhu et al., 2009).

First, we co-expressed CLV1 C-terminally fused to triple HAs-single StrepII (CLV1-3HS), CLV2 with triple FLAGs (CLV2-3FLAG) and CRN/SOL2 with 10 times Myc (SOL2-10Myc), under the control of the 35S promoter. Co-immunoprecipitation using anti-Myc antibody detected CLV2-FLAG, but failed to detect CLV1-3HS, showing that CLV1 complex and CLV2-CRN/SOL2 complex co-exists in N. benthamiana (Fig. 7A). This is consistent with previous genetic data using A. thaliana and microscopic data in N. benthamiana, and validates our biochemical interaction assay in N. benthamiana.

Then, we co-expressed RPK2 fused to 10 times Myc with CLV1-3HS and RPK2 fused to triple HA (3HA) with CLV2-3FLAG. In co-immunoprecipitation experiments using anti-HA, we did not detect interaction of RPK2-10Myc with CLV1-3HS (Fig. 7B). Interaction between RPK2-3HA and CLV2-3FLAG was also undetectable in co-immunoprecipitation using anti-HA (Fig. 7C). The biochemical data suggests that RPK2 is not included in the previously shown CLV1 complex or CRN/SOL2-CLV2 complex, at least, under our experimental conditions. However, when RPK2-10Myc was co-expressed with RPK2-3HA, RPK2-10Myc was co-immunoprecipitated with RPK2-3HA (Fig. 7D). Taken together, our biochemical study on RPK2 interactions in N. benthamiana demonstrated that RPK2 can form homo-oligomers, independently of CLV1 or CRN/SOL2-CLV2 complexes.

DISCUSSION

Via mutational screening using synthetic MCLV3 peptide, we have identified RPK2 as a novel regulator of plant meristem maintenance. Although previous studies have shown that RPK2 operates in anther and embryo development, our findings have uncovered additional roles for RPK/TOAD2 in SAM and RAM maintenance. Furthermore, our genetic studies indicate that RPK2 transmits the CLV3 signal in the SAM, independently of the two previously known CLV3 signalling pathways: the CLV1 and the CLV2-CRN/SOL2 pathways. The existence of these three major CLV3 receptor complexes was further supported by our transient in planta interaction assay using N. benthamiana.

RPK2 function in the CLV3 signalling

The rpk2 mutant shows defects both in fertility and floral organ number. Previous studies, however, have not mentioned the latter phenotype (Mizuno et al., 2007; Nodine et al., 2007). The null alleles of rpk2 by T-DNA insertions exhibit a weak and impenetrating phenotype and, furthermore, the male sterile nature of the rpk2 plants results in relatively small siliques, which makes it difficult to observe the typical club-shaped siliques. However, in this screening, we have isolated a new strong rpk2 allele, rpk2-4, which exhibits a consistently severe phenotype in floral organ number. The abnormal club-shaped siliques of this mutant then enabled us to notice the more subtle phenotypes of the null alleles. Previous reports suggest that specific clv1 alleles act semidominantly and interfere with other RLK(s) function, resulting in
This experiment was repeated at least twice with similar results. Myc, validating our biochemical interaction assay in CLV1-3HS, was specifically co-immunoprecipitated with CRN/SOL2-blot with anti-Myc, anti-HA or anti-FLAG antibody. CLV2-3FLAG, but antibody. The resultant immuno-complexes were analysed on western extracts were subjected to immunoprecipitation using anti-Myc CLV1-3HS, CLV2-3FLAG and CRN/SOL2-10Myc in 2003). Considering that has a point mutation at the putative ATP binding site of RPK2 protein, it could interfere with other functional protein kinase such as CLV1 or CRN/SOL2, and could interfere with the downstream signal in a similar fashion to the strong clv1 alleles.

Our genetic analysis indicated that RPK2 comprises the third pathway to transmit the CLV3 signal along with the CLV1 and CLV2-CRN/SOL2 complexes. We did not include CRN/SOL2 in our genetic study; however, it has been established that CRN/SOL2 acts together with CLV2, at least, in the regulation of the inflorescence and the floral meristems (Müller et al., 2008). Based on these established data, we used the clv2 mutant as a genetic marker of the CLV2-CRN/SOL2 pathway. Additionally, we analysed the phenotype of the double mutant of the clv1 null and clv2 null alleles, and we confirmed, for the first time, the additivity of the double mutant phenotype as suggested previously (Müller et al., 2008). Notably, our quantitative data revealed that any double mutant combinations among clv1, clv2 and rpk2 are additive; however, the triple mutant appears to be synergistic (see Fig. S7 in the supplementary material). Considering that the triple mutant is almost equivalent to clv3-8, the remaining receptor(s) in the single and double mutants might functions to over-activate "CLV3" expression via increased WUS expression (Fig. 5), resulting in masking the effect of the loss of the receptor(s). The rpk2 mutant exhibits the weaker clv-like phenotype compared with the clv1 or clv2 mutants; however, RPK2 should play a minor but indispensable role in the SAM homeostasis along with CLV1, CLV2 and CRN/SOL2.

Recent study suggested that CLV1-related LRR-RLKs, BAM1 (for BARELY ANY MERISTEM) and BAM2 exert a function to sequester the CLE in the PZ of the SAM (DeYoung and Clark, 2008). The bam1 bam2 double mutant shows male sterility and enhances the clv1 phenotype (DeYoung and Clark, 2008; Hord et al., 2006), both of which are also observed in rpk2. However, rpk2 interacts additively with clv2 for the floral meristem phenotypes, while bam1 bam2 double mutant does not. CLV1 shares high sequence similarities with BAM1 and BAM2, whereas RPK2 belongs to a distinct subfamily of LRR-RLKs in the Arabidopsis genome (DeYoung and Clark, 2008; Shiu and Bleecker, 2001). With these points, we conclude that RPK2 function in the SAM is unlikely to overlap with BAM1 and BAM2 activities but is similar to CLV1, CLV2 and CRN/SOL2 functions.

We have revealed that RPK2 is required for exogenously applied MCLV3 mediated-reduction of the SAM size and the root length. However, we do not know exactly whether RPK2 participates in CLV3 recognition. Owing to the technical difficulties in synthesizing arabinosylated CLV3, the most likely mature form of CLV3 recognition. Owing to the technical difficulties in synthesizing arabinosylated CLV3, the most likely mature form of CLV3 in planta, it is still unclear whether RPK2 directly binds to the endogenous CLV3, as shown for CLV1 (Ogawa et al., 2008; Ohyama et al., 2009). It is possible that RPK2 perceives, instead of CLV3, other CLE(s) expressed in the SAM to regulate the meristem (see Fig. S7 in the supplementary material). Considering that the triple mutant is a distinct subfamily of LRR-RLKs in the Arabidopsis genome (DeYoung and Clark, 2008; Shiu and Bleecker, 2001). With these points, we conclude that RPK2 function in the SAM is unlikely to overlap with BAM1 and BAM2 activities but is similar to CLV1, CLV2 and CRN/SOL2 functions.

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Fig. 7. Biochemical interactions of RPK2. (A) Interaction assay using CLV1-3HS, CLV2-3FLAG and CRN/SOL2-10Myc in N. benthamiana. These constructs co-expressed in N. benthamiana, and total protein extracts were subjected to immunoprecipitation using anti-Myc antibody. The resultant immuno-complexes were analysed on western blot with anti-Myc, anti-HA or anti-FLAG antibody. CLV2-3FLAG, but not CLV1-3HS, was specifically co-immunoprecipitated with CRN/SOL2-Myc, validating our biochemical interaction assay in N. benthamiana. This experiment was repeated at least twice with similar results. (B-D) Interaction assay of RPK2. RPK2-10Myc was co-expressed with CLV1-3HS (B), RPK2-3HA was co-expressed with CLV2-3FLAG (C) and RPK2-3HA (D) in N. benthamiana. This experiment was repeated at least three times with similar results.
A third pathway for CLV3 signalling

Consistent with our genetic data, our biochemical data revealed that RPK2 does not associate with CLV1 or CLV2, instead, forms a homodimer (oligomer) in *N. benthamiana*, indicating that RPK2 is likely to comprise the third receptor complex in the SAM of *A. thaliana*, independently of CLV1 and CLV2-CRN/SOL2 complexes. RPK2 homo-oligomerization occurs independently of the MCLV3 application; however, we cannot rule out the possibility that RPK2 homo-oligomerization might be triggered upon recognition of CLV3-like CLE peptide derived from *N. benthamiana* leaf tissue. Recent studies using fluorescent-tagged CLV receptors revealed that CLV1 homo-oligomerization might be very transient and dynamic. This type of interaction might be very difficult to detect with co-immunoprecipitation. Moreover, our co-immunoprecipitation using anti-myc did not detect a clear strong interaction between CLV1-3HS and CLV2-3FLAG-CRN/SOL2-10Myc complexes, probably owing to the sensitivity limit of co-immunoprecipitation experiment in our buffer condition, compared with fluorescence-based cell biology techniques. It is also possible that the association between CLV1 and CLV2-CRN/SOL2 homo-oligomers might participate in such a formation of the larger complex. The *rpk2*-4 alleles, which carries a point mutation at the ATP-binding site of RPK2, might reflect such a crosstalk of RPK2 complex with the CLV1, CLV2-CRN/SOL2 complexes.

One interesting observation is that overexpression of RPK2 causes the *CLV3*-overexpression phenotype, which is distinct from the situation for CRN/SOL2. This observation possibly suggests that elevated levels of RPK2 protein accumulation might trigger dose-dependent auto-activation of the RPK2 pathway and/or the other pathways. Notably, our microscopic analysis of fluorescent-tagged RPK2 revealed that accumulation of RPK2 protein is relatively low in the CZ (Fig. 3G), whereas RPK2 transcript distributes almost uniformly in the SAM (Fig. 3B). These data led us to speculate that the lower RPK2-GFP signal in the CZ might reflect highly active internalization and subsequent degradation of RPK2 protein in the CZ, presumably triggered by CLE ligands recognition, as demonstrated for BRI1, BAK1, BOR1 and FLS2 (Chinchilla et al., 2007; Geldner et al., 2007; Robatzek et al., 2006; Takano et al., 2005). Alternative explanation might be that the amount of RPK2 protein is limited in the SAM in ratio to CLE ligands. However, our MCLV3 and the previous CLV3-overexpressing experiments resulted in the termination of the SAM, suggesting that the amount of RPK2 protein is not rate-limiting. Thus, the molecular basis of the RPK2 overexpression phenotype is still under discussion at this point. A future challenge will be a detailed analysis of the molecular function of RPK2 to gain insights into this interesting phenotype.

Here, we have shown our genetic, biochemical and histological analyses of RPK2 function. However, all the interaction data concerning the CLV receptors, including our data, have been obtained using transient and ectopic overexpression system. These data lack the information on the distribution of these receptor proteins and CLE ligands in the SAM, except for RPK2 shown in this study. Furthermore, downstream direct targets of these receptor complexes are yet unknown. Future detailed analysis of these aspects should enable us to understand precisely the complex signalling machinery comprising these multiple receptors and CLEs in the regulation of the SAM homeostasis.

Additional aspects of RPK2 function

Phenotypic analyses suggest that the RPK2 function is not be restricted in the SAM. Interestingly, the *rpk2* mutants do not exhibit MCLV3-induced short root phenotype. This is also observed in the *clv2* and *crn/sol2* mutants (Fiers et al., 2005; Miwa et al., 2008; Müller et al., 2008). Although none of these single mutants show any morphological phenotypes in the root, the application of CLE peptides apparently affects root architecture in CLV2- and CRN/SOL2-dependent manner. Further detailed genetic analyses would shed some light on CLE signalling in the proximal RAM. Furthermore, besides the MCLV3 resistance, *rpk2* shows multiple phenotypes, including male sterility, smaller rosette leaves, increased branching and spindly shoots (Mizuno et al., 2007; Nomine et al., 2007). Therefore, it would also be interesting to dissect involvement of CLEs in these phenotypes.

Many of the identified and proposed receptors for CLE ligands, so far, fall in the LRR-RLK subclass XI (Clark et al., 1993; DeYoung et al., 2006; Hirakawa et al., 2008; Shiu and Bleecker, 2001), whereas RPK2, CRN/SOL2 and CLV2 do not belong to this class. Interestingly, so far, none of these three receptors has been shown to bind directly to CLE peptides. Therefore, the alternative hypothesis is that RPK2, as well as CLV2 and CRN/SOL2, might contribute to the CLV3-dependent WUS suppression signalling by a non-CLE mediated manner. Recent study revealed involvement of cytokinin signalling in addition to CLE40-mediated regulation of the RAM homeostasis (Dello Ilio et al., 2008; Ruzicka et al., 2009; Stahl and Simón, 2009). Cytokinin signalling is known to function in the regulation of the SAM homeostasis (Gordon et al., 2009; Kurakawa et al., 2007; Leibfried et al., 2005). It is possible that RPK2 and, perhaps CLV2 and CRN/SOL2, might transmit non-CLE signal(s), such as cytokinin, to the *CLV3*-WUS signal flow. In this context, the RPK2-containing receptor complex might be a convergent point of different hormone signals, not only CLE but also others, in the regulation of the WUS expression levels to achieve an organized and orchestrated development of the SAM. A future challenge would be to identify a direct ligand for RPK2, which should provide further insights in understanding precise signalling machinery in the regulation of the SAM homeostasis.

Acknowledgements

We thank Prof. David Baulcombe for Agrobacterium strain carrying the p19 silencing suppressor; Prof. Jane Parker for Agrobacterium strains GV3101 pMP90RK, pXCSG-3HS and pXCSG-3FLAG; Prof. Tsuyoshi Nakagawa for pGWB binary vectors; and Prof. Taku Demura for pH35G vector. We also thank Prof. Masayoshi Kawaguchi, Dr Masashi Yamada and Mr Hikota Miyazawa for valuable discussion. We appreciate TAIR and GABI-kat for their valuable resources. A.K. was supported by Research Fellow of the Japan Society for the Promotion of Science. This work was supported by: Grant-in-Aid for Creative Scientific Research; Grant-in-Aid for Young Scientists (S (19677001) from Japan Society of the Promotion of Science; Grant-in-Aid for Scientific Research for Priority Areas from the Ministry of Education, Culture, Sports, Science, Technology (19060009 to H.F., 20060014 and 19060016); and a Program of Basic Research Activities for Innovative Biosciences from Bio-oriented Technology Research Advancement Institution.

Competing interests statement

The authors declare no competing financial interests.


