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BAM 1 and RECEPTOR-LIKE PROTEIN KINASE 2 constitute a signaling pathway and modulate CLE peptide-triggered growth inhibition in *Arabidopsis* root

Noriko Shimizu[1]*, Takashi Ishida[1]*, Masashi Yamada[2], Shuji Shigenobu[3,4], Ryo Tabata[1], Atsuko Kinoshita[5], Katsushi Yamaguchi[3], Mitsuyasu Hasebe[4,6], Kanako Mitsumasu[1] and Shinichiro Sawa[1]

[1]Graduate School of Science and Technology, Kumamoto University, Kumamoto 860-8555, Japan; [2]Department of Biology and Institute for Genome Science and Policy Center for Systems Biology, Duke University, Durham, NC 27708, USA; [3]Functional Genomics Facility, National Institute for Basic Biology, Okazaki 444-8585, Japan; [4]School of Life Science, SOKENDAI (The Graduate University for Advanced Studies), Okazaki 444-8585, Japan; [5]RIKEN Center for Sustainable Resource Science, Tsurumi, Yokohama 230–0045, Japan; [6]Division of Evolutionary Biology, National Institute for Basic Biology, Okazaki 444-8585, Japan

Author for correspondence:
Takashi Ishida
Tel: +81 96 342 3436
Email: ishida@sci.kumamoto-u.ac.jp

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Summary

- Ligand receptor-based signaling is a means of cell-to-cell communication for coordinating developmental and physiological processes in multicellular organisms. In plants, cell-producing meristems utilize this signaling to regulate their activities and ensure for proper development. Shoot and root systems share common requirements for carrying out this process; however, its molecular basis is largely unclear. It has been suggested that synthetic CLV3/EMBRYO SURROUNDING REGION (CLE) peptide shrinks the root meristem through the actions of *CLAVATA2* (*CLV2*) and the *RECEPTOR-LIKE PROTEIN KINASE 2* (*RPK2*) pathway in *Arabidopsis thaliana*.
- Our genetic screening for mutations that resist CLE peptide signaling in roots determined that BAM1, which is a member of the leucine-rich repeat receptor-like kinase (LRR-RLK) family, is also involved in this pathway.
- BAM1 is preferentially expressed in the root tip, including the quiescent center and its surrounding stem cells. Our genetic analysis revealed that BAM1 functions together with RPK2. Using coimmunoprecipitation assay, we showed that BAM1 is capable of forming heteromeric complexes with RPK2.
- These findings suggest that the BAM1 and RPK2 receptors constitute a signaling pathway that modulates cell proliferation in the root meristem and that related molecules are employed in root and shoot meristems.

Introduction

The plant meristem is a pivotal tissue where cell production takes place to sustain organ growth and organogenesis. The shoot apical meristem (SAM) and root apical meristem (RAM) are well-studied meristems that are located at the tips of the aerial part and root, respectively. The behavior of meristems is controlled by a group of cells that are collectively called the stem cell niche. The stem cell niche is composed of specialized cells that function as a reserve of undifferentiated cells and is responsible for the regulation of cell division in multicellular organisms (Sablowski, 2007). The niche is composed of two types of cells: stem cells and niche cells. Stem cells are capable of self-renewing and producing daughter cells, while niche cells are responsible for the regulation of stem cells. In the SAM, stem cells are located just above the niche cells in a region termed the organizing center (OC), and daughter cells differentiate into various types of cells in the aerial part. By contrast, niche cells in the RAM, also called the quiescent center (QC), are located at the center of the niche, and stem cells surround the QC. The fates of these root stem cells are partially predetermined, and they are termed accordingly as columella initial, cortex/endodermal initial, stele initial, and epidermis/lateral root cap initial. Thus, the structures of the meristems in the root and shoot are different, although both meristems share the basic characteristics of stem cell niche systems.

To balance cell proliferation and differentiation in meristems, well-organized intercellular signaling is required. Historical genetic studies have identified molecules that function in cell-to-cell communication, particularly in the SAM of *Arabidopsis thaliana*. A homeobox transcription factor, WUSCHEL (WUS), is a positive regulator of cell proliferation and the expression of *CLAVATA3* (*CLV3*), which encodes a small signaling peptide, whereas CLV3 plays a role in the negative regulation of *WUS* expression (Clark *et al.*, 1995; Laux

*These authors contributed equally to this work.

et al., 1996; Fletcher *et al.*, 1999; Brand *et al.*, 2000; Schoof *et al.*, 2000). Thus, they form a feedback loop that sets the appropriate level of SAM activity (Brand *et al.*, 2000; Schoof *et al.*, 2000). CLV3 is expressed in and secreted from stem cells, and the extracellular ligand is perceived by transmembrane receptors. According to genetic and biochemical analyses, at least three receptor complexes – two leucine-rich repeat (LRR) receptor-like kinases (RLKs), CLAVATA1 (CLV1) and RECEPTOR-LIKE PROTEIN KINASE 2 (RPK2)/TOADSTOOL 2, and an LRR receptor-like protein that lacks a cytoplasmic domain, CLAVATA2 (CLV2), which acts together with a membrane-associated protein kinase, CORYNE (CRN)/SUPPRESSOR OF LLP1 2 (SOL2) – are responsible for the recognition of CLV3 in the SAM (Clark *et al.*, 1997; Jeong *et al.*, 1999; Miwa *et al.*, 2008; Müller *et al.*, 2008; Bleckmann *et al.*, 2010; Kinoshita *et al.*, 2010). Despite the presence of clear feedback modules that consist of positive and negative regulators in the SAM, it is unclear whether there are equivalent regulatory mechanisms in the RAM. Among the CLAVATA-related proteins, the CLV2-CRN/SOL2 and RPK2 receptor complexes are expressed in actively dividing regions of the RAM, whereas CLV1 is expressed in columella cells and is localized to the distal region of the RAM (Müller *et al.*, 2008; Kinoshita *et al.*, 2010; Replogle *et al.*, 2013; Stahl *et al.*, 2013). The ectopic stimulation of the CLV2-CRN/SOL2 and RPK2 pathways by the application of synthetic CLE peptide inhibits root elongation, indicating the involvement of CLAVATA-like signaling modules in the repressive regulation of RAM (Casamitjana-Martinez *et al.*, 2003; Hobe *et al.*, 2003; Fiers *et al.*, 2005; Kinoshita *et al.*, 2007; Meng & Feldman, 2010). In addition, CLE40 and CLV1 together with ARABIDOPSIS CRINKLY4 (ACR4), membrane-localized receptor kinase, control the activity of the distal side of the root stem cell niche (Stahl *et al.*, 2009, 2013). However, further analysis is needed to clarify the molecular basis of the repressive mechanism that is employed in root meristems.

In addition to CLV3 receptors, BAM family proteins are thought to be involved in shoot meristem development. BAM1, BAM2 and BAM3 are members of CLV1 class LRR-RLKs and are able to bind to the CLE peptide (Guo *et al.*, 2010, 2011; Shinohara *et al.*, 2012). DeYoung *et al.* (2006) have reported that *bam1 bam2* double mutants have smaller shoot meristems than those of the wild-type, suggesting that BAMs might have a role as a positive regulator of shoot meristem development. Furthermore, the combination of *bam* mutations with *clv3* suppresses the cell overproliferation phenotypes (DeYoung & Clark, 2008). However, when *bam* mutations are combined with a *clv1* mutation, the phenotypes are significantly enhanced, suggesting that BAMs act redundantly with CLV1 in SAM homeostasis as negative regulators of the shoot meristem (DeYoung & Clark, 2008). BAMs are involved in the development of not only the vegetative shoot meristem but also the floral meristem and anther (Hord *et al.*, 2006). The additive effects of the *bam1* and *clv1* mutations have been detected in the floral meristem, similar to what has been observed in the vegetative meristem (Durbak & Tax, 2011). The hypothesis that BAMs are negative regulators of cell proliferation is consistent with a previous report describing the rescuing of multiple *bam* phenotypes by the ectopic expression of CLV1 (DeYoung *et al.*, 2006).

To explain these discrepancies in results, a model in which BAM1 acts to sequester the CLE peptide(s) has been proposed (DeYoung & Clark, 2008; Kiyohara & Sawa, 2012). Furthermore, BAM1 has also been suggested to function in the root. Guo *et al.* (2011) reported that application of the synthetic CLE-like peptide of the nematode shows diminished root growth, and *bam1 clv2* mutants are resistant to synthetic peptide application. Based on these results, BAM1 and CLV2 are probably involved in CLV-like signaling in the root as well as in nematode infection (Guo *et al.*, 2011). However, little is known about the role of BAM1 in the regulation of RAM activity.

Recent genome-wide analyses have revealed that various CLE family small peptides are expressed in different tissues, and some are involved in similar processes, such as the regulation of cell production processes, even in different tissues (Jun *et al.*, 2010; Betsuyaku *et al.*, 2011a). Because the CLE peptide inhibits RAM activity and several proteins in the CLAVATA pathway are considered to be involved in this regulation, the presence of a CLV-like signaling pathway in the root meristem has been proposed (Casamitjana-Martinez *et al.*, 2003; Fiers *et al.*, 2005; Stahl *et al.*, 2009; Yamada & Sawa, 2013). To identify components of this pathway, we conducted an enhancer genetic screening to find mutations that diminish the CLE peptide-dependent signaling that inhibits root growth. This screening revealed that BAM1 was involved in CLE peptide-mediated root meristem consumption in a CLV2-independent manner. The characteristic expression of BAM1 in the central region of the root meristem was observed. Genetic analysis indicated that BAM1 functioned synergistically with RPK2. Furthermore, a protein–protein interaction assay demonstrated that BAM1 formed a heteromeric complex with RPK2. These observations suggest that the BAM1–RPK2 receptor complex may participate in a regulatory pathway that modulates cell production in the RAM and that shoot and root meristems utilize similar molecules in different ways to maintain their architecture.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L.) Heynh wild-type Columbia-0 (Col-0), *rpk2-2* and *clv2-101* were described previously (Kinoshita *et al.*, 2010), and *bam1-3* (SALK_015302) was provided by the Arabidopsis Biological Resource Center. Seeds were surface-sterilized and plated on growth medium containing Murashige and Skoog (MS) basal salts, 1% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7) and 1.5% (w/v) agar. After cold treatment in the dark for 2 d, seeds were transferred to a growth room at 22°C under continuous white light (20–50 mmol m^{-2} s^{-1}). CLV3 and CLE25 were synthesized as described previously (Kondo *et al.*, 2006).

Microscopic observations of the root meristem

The root meristem organization was visualized using seedlings stained with 10 mg ml^{-1} propidium iodide. Stained roots were observed using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Tokyo, Japan). The onset of cell expansion in epidermal cell layer was observed and the root meristem size was estimated by the distance from the QC to the expanding cell (Ishida *et al.*, 2010).

Whole-mount *in situ* hybridization

The BAM1 antisense or sense probe was generated from a PCR product that was amplified with the following primer sets: 5′-CCAAGCTTCTAATACGACTCACTAGGGAGACTCGCCGTCACGTGACTTCT-3′ and 5′-GAAGCTTCCCAATCTCCGGC-3′, or 5′-CCAAGCTTCTAATACGACTCACTAGGGAGAGAAGCTTCCCAATCTCCGGC-3′ and 5′-CTCGCCGTCACGTGACTTCT-3′, respectively.

Whole-mount *in situ* hybridization was performed as described previously (Hejatko *et al.*, 2006) with minor modifications. After hybridization, the samples were washed three times for 15 min in ×4 saline sodium citrate (SSC) at 55°C and three times for 20 min in ×0.1 SSC containing 0.1% (w/v) sodium dodecyl sulfate at 55°C and then incubated for 5 min in maleic acid buffer (MAB; 100 mM maleic acid, 150 mM NaCl, pH 7.5) at room temperature (RT). Preincubation was performed in blocking solution (2% Boeringer Blocking Reagent (Boehringer, 1096 176), heat-inactivated goat serum, 0.1% (w/v) Tween-20 in MAB) for 30 min at RT. The antibody reaction was performed using Anti-Digoxygenin-AP Fab fragments (Roche, 1093274; 1 : 5000) in blocking solution for 2 h at RT, and the samples were washed three times for 15 min in MAB containing 0.05% (w/v) Tween-20. After incubation for 5 min in 0.1 M Tris-HCl (pH 9.5), the samples were stained in alkaline phosphatase buffer containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Hejatko *et al.*, 2006) for 10 min in the dark at 37°C and observed using a Zeiss Axioimager M1 microscope.

Microscopic observations of transiently expressed fluorescent proteins

Transformation of *Arabidopsis* protoplasts was performed as described previously (Yoo *et al.*, 2007). BAM1 complementary (c)DNA was PCR-amplified from the wild-type cDNA using the following primer set: BAM1left 5′-CACCATGAAACTTTTTCTTCTCCTTCTTTTTCTTCTCCAC-3′ and BAM1Right 5′-TGATAGATTGAGTAGATCCGGCGGACT-3′. The PCR product was then cloned using the pENTR/D-TOPO Cloning Kit (Life Technologies Japan, Tokyo, Japan). The entry clone containing the RPK2 cDNA was previously described (Betsuyaku *et al.*, 2011b). The entry clones were integrated into pGWB505 (green fluorescent protein (GFP) fusion) or pGWB660 (TagRFP fusion) vectors using LR clonase (Life Technologies Japan) and prepared using the PureLink HiPure Plasmid Mixiprep Kit (Life Technologies Japan). For transient expression analysis, 5 μg of the expression vectors were introduced into 200 μl of protoplast suspension at a concentration of 2×10^7 protoplasts ml^{-1} using the polyethylene glycol (PEG)-mediated transformation method. The protoplasts were then incubated for 12–24 h at 23°C, and observed using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems).

Coimmunoprecipitation analysis

Entry clones containing BAM1 or RPK2 cDNA were integrated into the expression vectors using LR clonase (Life Technologies Japan) and prepared using the PureLink HiPure Plasmid Maxiprep Kit (Life Technologies Japan) (Akamatsu *et al.*, 2013). For transient expression analysis, 60 μg of the expression vectors were introduced into 1000 μl of protoplast suspension at a concentration of 2×10^7 protoplasts ml^{-1} using the PEG-mediated transformation method. The protoplasts were then incubated for 12–24 h at 23°C. Protein extraction and immunoprecipitation using the anti-GFP antibody and the μMACS GFP isolation kit (Miltenyi Biotec K. K., Tokyo, Japan) were performed as previously described (Akamatsu *et al.*, 2013).

Western blotting

Western blotting was performed as described previously (Ishida *et al.*, 2014). Extracted samples were resuspended in NuPAGE LDS sample buffer (Life Technologies Japan) and sample reducing agent (Life Technologies Japan) and then incubated at 98°C for 2 min. Protein was fractionated on NuPAGE Novex 4–12% Bis-Tris gels (Life Technologies Japan) and then transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories K. K., Tokyo, Japan). The membrane was blocked in TBST (10 mM Tris-Cl, pH 8.0, 150 mM NaCl and 0.05% Tween 20) containing 5% nonfat dried milk at RT for 1 h and incubated with anti-FLAG M2-HRP (Sigma-Aldrich, A8592; 1 : 1000) or anti-GFP-HRP (Miltenyi Biotec K.K. 130-091-833; 1 : 2500) for 1 h at RT. The membrane was treated with Western BLoT Hyper HRP Substrate (TaKaRa Bio, Shiga, Japan), and luminescence was detected using the ChemiDoc XRS system (Bio-Rad Laboratories K.K.).

Results

Isolation and identification of *clv2* enhancer mutants

Ligand-receptor systems represent an indispensable molecular basis for perceiving extracellular signals in cells. In plants, LRR-containing receptors play important roles in intercellular signaling, and some are involved in meristem maintenance. Previously, we have shown that various synthetic CLE peptide treatments mimic endogenous *CLE* gene overexpression and induce RAM consumption, resulting in the short-root phenotype (Kinoshita *et al.*, 2007; Tamaki *et al.*, 2013). The *clv2* mutant shows partial resistance to CLE peptide-mediated growth inhibition and produces longer roots compared with wild-type seedlings grown on an agar plate containing synthetic CLE peptides (Fiers *et al.*,

2005). However, root growth is inhibited at higher concentrations of CLE peptide, even in the *clv2* mutant, indicating the presence of an additional signaling pathway (Fiers *et al.*, 2005). Similarly, we have found that CLE25, which is a strong inhibitor among the CLE peptides and is expressed in the RAM, affects root growth in the *clv2* mutant (Miwa *et al.*, 2008; Jun *et al.*, 2010). To decipher the molecular basis of CLE peptide signaling in the root, we isolated 26 recessive *clv2* enhancer mutants (*clen101-clen126*) that produced longer roots than *clv2* on an agar plate containing 10 μM of synthetic CLE25 peptide.

Prediction and identification of the causal gene of the *clen101-clen103* mutants

To identify the mutations responsible for CLE peptide resistance, we performed whole-genome sequencing of the *clen101-clen126* mutants using the SOLiD platform. We constructed sequence libraries from the 26 mutant lines using the SOLiD barcoding system to distinguish the samples, and the multiplex libraries were sequenced on a single SOLiD slide (Tabata *et al.*, 2013). The resulting sequence reads were mapped to the *A. thaliana* Col-0 genome, and single nucleotide polymorphisms (SNPs) were called for each library. On average, 1076 ± 73 SNPs were found per line that were not identified in the Col-0 reference. After subtracting the SNPs that were shared among the majority (> 70%) of the 26 mutants and were present in the parental transgenic line, 752 ± 73 SNPs remained. When we focused on the protein-coding genes in each mutant line, we found that 51 ± 20 genes contained nonsynonymous mutations. Interestingly, three lines, *clen101*, *102* and *103*, had nonsynonymous mutations in the same gene, *AT5G65700*, which encodes BAM1, a member of the LRR-RLK family (Fig. 1a).

Previously, Shinohara *et al.* (2012) showed that several CLE peptides can bind to the LRR domain of BAM1 *in vitro*. In addition, phylogenetic analysis revealed that BAM1 is a CLV1-type LRR-RLK with relatively high sequence similarity (Fig. 1b) (DeYoung *et al.*, 2006). The identity of the amino acid sequences of CLV1 and BAM1 is 54%. CLV1 is well known to perceive the CLV3 peptide hormone signal in the SAM (Ogawa *et al.*, 2008; Shinohara *et al.*, 2012). Although BAM1 has been shown to play a role in shoot meristem development, its function in the root meristem has been rarely studied.

Next-generation sequence analyses identified that *clen101* has a mutation that changes the 706th codon from GGT to GAT, resulting in an amino acid substitution of Gly to Asp, while *clen102* and *clen103* have the same point mutation that changes the 828th codon from CTT to TTT, resulting in an amino acid substitution of Leu to Phe (Fig. 1a). Because *clen102* and *clen103* were isolated from the same batch from the ethyl methanesulfonate-treated seed pool, it is possible that they originated from the same plant. The G-loop, with the sequence GxGxxG, is a crucial part of the structure of protein kinases, and the 706th Gly is positioned within this loop (Fig. 1c) (Scheeff *et al.*, 2009). In the *clen101* mutant, this sequence is converted to GxGxxD, which causes the deformation of the G-loop. Although the relevance of the 828th Leu is yet to be determined, it is positioned close to the catalytic domain (HRD) and the Mg-binding site (DFG) (Fig. 1d) (Scheeff *et al.*, 2009), and the SNP in the 828th codon may affect the BAM1 kinase activity.

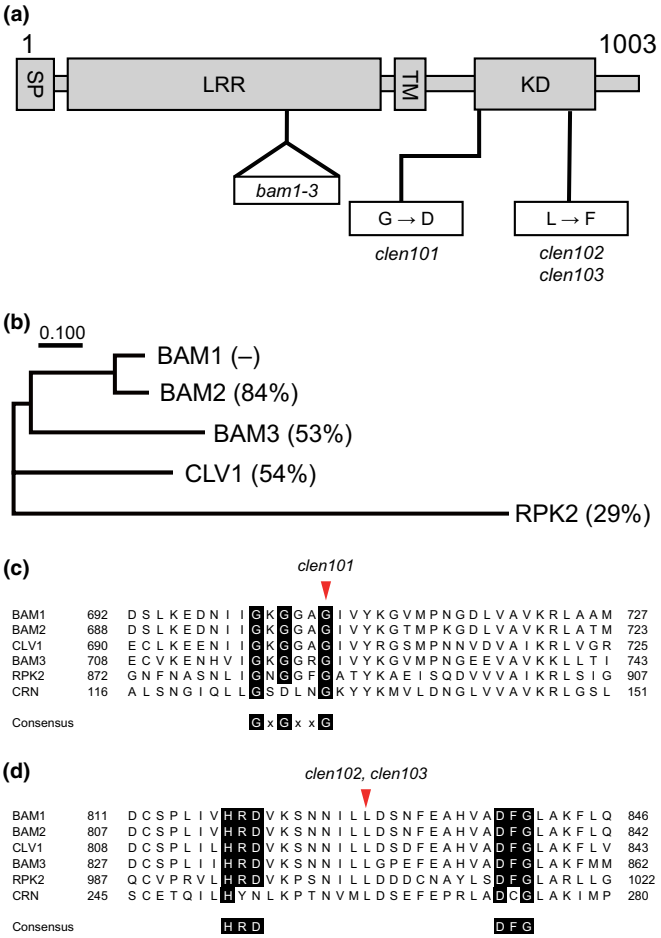

Fig. 1 (a) Schematic representation of the Arabidopsis BAM1 structure, illustrating the position of the signal peptide (SP), leucine-rich repeat domain (LRR), transmembrane domain (TM), and kinase domain (KD). The *clen101* mutation converts Gly (G706) to Asp. The *clen102* mutation substitutes Leu (L828) for Phe. The *bam1-3* allele (SALK_015302) contains a T-DNA insertion in the LRR domain. (b) Phylogenetic tree of related LRR receptor-like kinases (LRR-RLKs). The sequence identities relative to BAM1 are shown in brackets. The scale bar indicates the number of amino acid substitutions per site. (c) Sequence alignment of the G-loop sequence in CLV-like pathway-related kinases. (d) Sequence alignment of the catalytic domain and Mg-binding site in CLV-like pathway-related kinases.

clv2 clen101 and *clv2 clen102* show obvious peptide resistance

We selected *clen101* and *clen102* for further analysis because we suspected that the causal gene and *clen103* have the same mutation as that of *clen102* in the *BAM1* gene. Wild-type seedlings produced shorter roots when they were grown on MS media containing CLE25 compared with those produced when they were grown on nontreated media (0.95 vs 5.50 cm, respectively; Fig. 2), and *clv2-101* was partially insensitive to this peptide, growing roots of 2.79 cm in length, although the nontreated

roots were similar to the wild-type roots at 10 d after germination. By contrast, the *clv2-101 clen101* and *clv2-101 clen102* double mutants produced longer roots (5.41 and 4.35 cm, respectively) even when they were grown on media with the CLE25 peptide (Fig. 2).

The loss of function of BAM1 is responsible for the effects of the *clen101-103* mutants

To examine whether the *clen101* and *clen102* mutations disrupt BAM1 function and are truly responsible for CLE peptide resistance in the *clv2 clen101* and *clv2 clen102* mutants, we crossed the *bam1-3* mutant, which is a loss-of-function allele that contains a T-DNA insertion in the first exon of the *BAM1* gene (Fig. 1a), with *clv2-101* to generate the *clv2-101 bam1-3* double mutant. Similar to *clv2-101 clen102*, the *clv2-101 bam1-3* double mutant could produce longer roots on the CLE25 peptide-containing media; therefore, the *clv2 clen102* mutant was termed *clv2 bam1*^{clen102} (Fig. 3). We then crossed *clv2 bam1*^{clen102} with *clv2 bam1-3*, and the *clv2 bam1*^{clen102}*/bam1-3* seedlings were subjected to the CLE25 treatment (Fig. 3a). A comparison of the *clv2 bam1*^{clen102}, *clv2 bam1*^{clen102}*/bam1-3*, and *clv2 bam1-3* plants revealed similar root lengths (4.18, 4.86, and 3.98 cm, respectively), indicating that *BAM1* is the causal gene for the *clen102* phenotypes (Fig. 3b). Based on these results, we hereafter utilized *bam1-3* as the representative *bam1* mutant genotype.

Although the *bam1-3* mutation significantly enhanced the CLE25 resistance of the *clv2* mutant, the single *bam1-3* mutant was indistinguishable from the wild-type seedling, at least with regard to growth on MS or CLE25 media. We found that *bam1-3* had weak but significant resistance to a synthetic CLV3 peptide treatment that had a milder inhibitory effect than CLE25 (Kinoshita *et al.*, 2007). The exogenous application of synthetic CLV3 peptide diminished root growth at concentrations ranging from 3 to 100 nM in both the wild-type seedlings and the *bam1-3* mutant (Fig. 4). Remarkably, when the wild-type seedlings were incubated on an agar plate containing 10 nM CLV3 peptide (Fig. 4a), the root length was reduced by 57.0% compared with a plant grown under normal conditions (Fig. 4b). Conversely, the inhibitory effect of CLV3 was diminished in the *bam1-3* mutant because only 15.6% growth inhibition was observed (Fig. 4b). Under these conditions, the *clv2-101* and *rpk2-2* mutants could grow roots similar to those of the MS mock-treated seedlings (Fig. 4b). Thus, the *bam1-3* mutant was more resistant to the synthetic CLV3 peptide than the wild-type seedlings and was relatively more sensitive to this peptide than *clv2-101* and *rpk2-2*. Considering that the inhibitory effect of CLV3 against root growth was relatively milder than that of CLE25, the level of BAM1 activity that is involved in the perception of CLE peptide in the root seems to be weaker than those of CLV2 or RPK2.

BAM1 is involved in CLE peptide-dependent meristem shrinkage

In vitro application of the CLE peptide triggered root growth inhibition and the shrinkage of the root meristem (Fiers *et al.*,

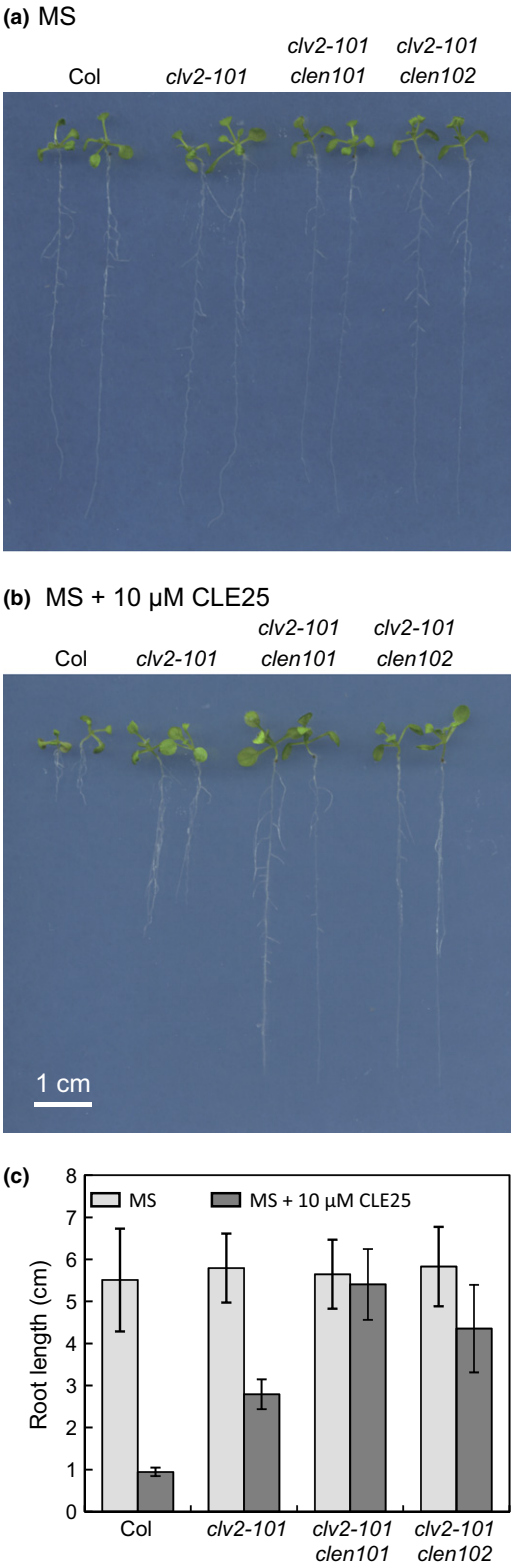

Fig. 2 CLE25-mediated root growth inhibition was rescued by the Arabidopsis *clen* mutations. Ten-day-old wild-type Columbia (Col), *clv2-101*, *clv2-101 clen101*, and *clv2-101 clen102* seedlings were grown on Murashige and Skoog (MS) plates with (b) or without (a) 10 μM CLE25 peptide. (c) Quantification of the root lengths of the 10-d-old seedlings grown on MS media with or without 10 μM CLE25. Data are shown as means ± SD.

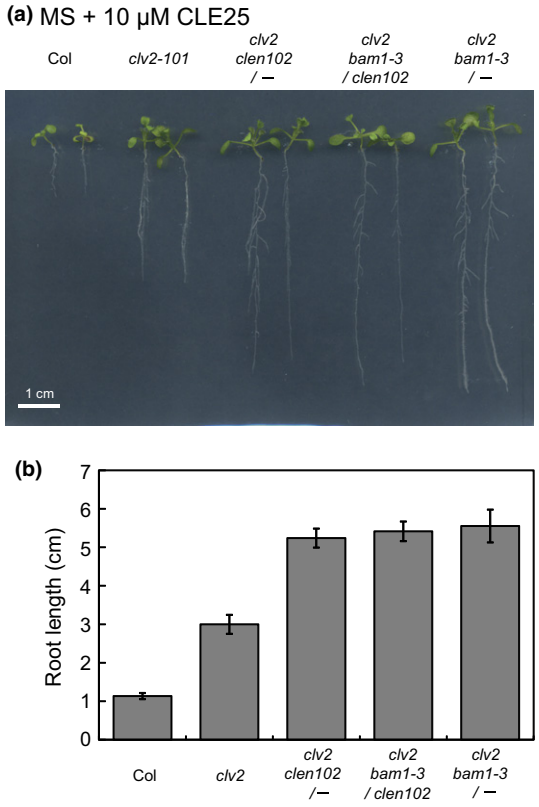

Fig. 3 Allelism test between Arabidopsis *clen102* and *bam1-3*. (a) Ten-day-old wild-type (Col), *clv2-101*, *clv2-101 clen102/-* homozygote, *clv2-101 bam1-3/clen102* heterozygote and *clv2-101 bam1-3* homozygote seedlings were grown on Murashige and Skoog (MS) medium containing 10 μM CLE25. (b) Quantification of the root lengths. Data are shown as means ± SD.

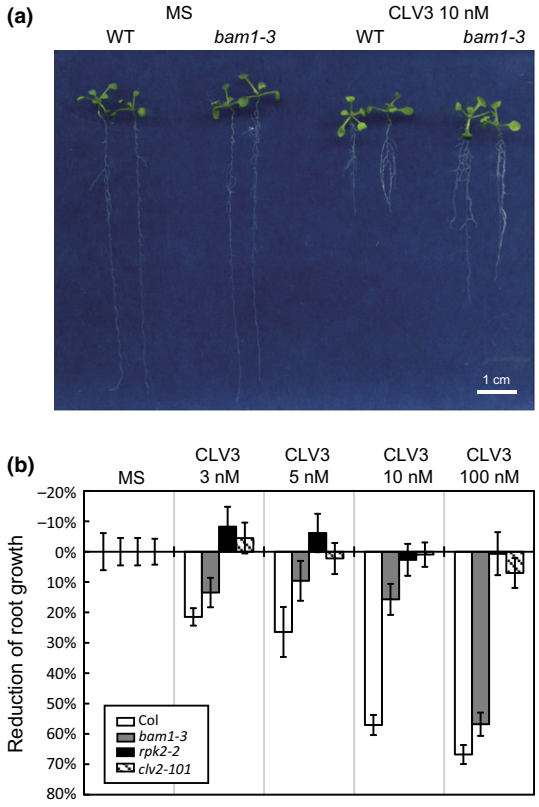

Fig. 4 Arabidopsis *bam1-3* single mutant was weakly resistant to the CLV3 peptide. (a) Ten-day-old wild-type (WT) and *bam1-3* mutant seedlings were grown on Murashige and Skoog (MS) medium with or without 10 nM CLV3 peptide. (b) Quantification of the inhibitive effects of the CLV3 peptide to root lengths of the 10-d-old WT, *bam1-3*, *clv2-101* and *rpk2-2* seedlings. The root length of 10-d-old seedlings grown on MS medium containing 10 μM CLE25 were measured and calculated relative to the mock treatment. Data are shown as means ± SD. Bar, 1 cm.

2005). CLV2 appeared to perceive the signals, and the loss of this protein led to the recovery of peptide-mediated meristem consumption (Fiers *et al.*, 2005). We then examined whether the *bam1* mutation in combination with the *clv2* mutation affected the structure of the RAM. Seven-day-old *clv2* and *clv2 bam1* seedlings grown on MS media displayed root tips with comparable meristem sizes (Fig. S1). Furthermore, the application of 10 μM CLE25 decreased the root meristems of *clv2*, whereas *clv2 bam1* was clearly resistant to this treatment, indicating that BAM1 and CLV2 independently modulate CLE peptide-dependent meristem consumption (Fig. S1). These results also suggest that CLV2-CRN/SOL2 and BAM1 receptors are involved in the regulation of RAM homeostasis under these conditions.

BAM1 is expressed in the RAM

BAM1 is expressed in the shoot meristem, which is consistent with its participation in shoot meristem development (DeYoung *et al.*, 2006). Because the *bam1-3* mutation affected CLE peptide-mediated meristem consumption in the roots, we expected that *BAM1* would be expressed in the RAM. mRNA *in situ* hybridization performed using 7-d-old wild-type plants detected signals in the QC and surrounding initial cells in the central region of the root tip (Fig. 5). We could not detect any signals in the cell elongation or differentiation zones in the root. The *BAM1* gene expression levels in the QC and columella cells were in agreement with the results of a digital *in situ* experiment (Nawy *et al.*, 2005) and an evaluation of the eFP browser database (Fig. S2).

Genetic interaction between BAM1 and RPK2

In the SAM, three receptor complexes, CLV1, CLV2-CRN/SOL2 and RPK2, are known to perceive CLV3 signaling (Kinoshita *et al.*, 2010), but this situation is slightly different in the RAM. Among the receptor complexes, CLV2- CRN/SOL2 and RPK2 might behave similarly, as in the SAM, whereas the contribution of CLV1 to signaling is limited in the RAM (Fiers *et al.*, 2005; Miwa *et al.*, 2008). Our results revealed the presence of a novel receptor; thus, three receptors, CLV2-SOL2/CRN, RPK2, and BAM1, play considerable roles in CLV-like signaling in the RAM.

BAM1 and CLV2-SOL2/CRN might function independently because their double mutant exhibited an additive phenotype in terms of CLE peptide sensitivity. To examine the genetic relationship between BAM1 and RPK2, we generated a double

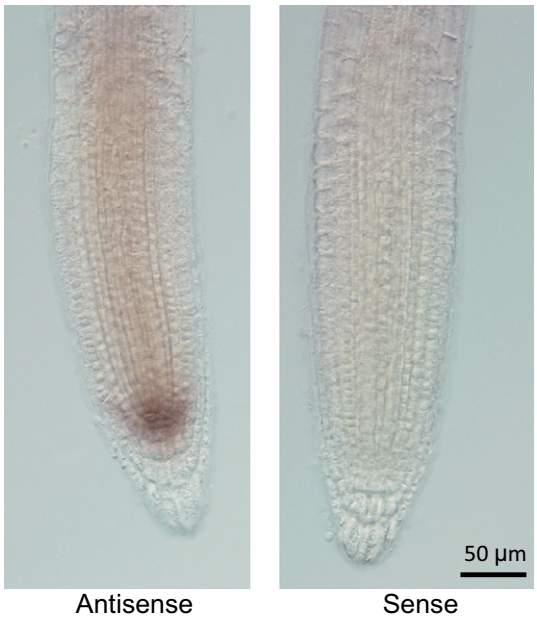

Fig. 5 *BAM1* expression in Arabidopsis root apical meristems. mRNA *in situ* hybridization was performed on 7-d-old seedlings using a *BAM1* antisense or sense probe.

mutant of *bam1* and *rpk2* and incubated it on MS media containing 10 µM CLE25 peptide. As a result, *bam1 rpk2* produced roots that were *c.* 50% shorter than the nontreated roots (Fig. 6). This finding clearly differs from what was observed with *clv2 bam1* and *clv2 rpk2*, which were able to grow roots that were > 80% of the length of the nontreated roots (Fig. 6). In addition, the *clv2 bam1 rpk2* triple mutant behaved as the *clv2 bam1* or *clv2 rpk2* double mutants (Fig. 6). Among the mutation combinations, the additive effects of *bam1 rpk2* were mild, suggesting that BAM1 and RPK2 act, at least partially, in the same pathway. Therefore, the presence of two independent signaling pathways that are BAM1-RPK2-dependent and CLV2-dependent and function to transmit CLE peptide signals is expected.

BAM1 and RPK2 form heteromeric complexes

To obtain further molecular insights into the function of receptor kinases, we analyzed the subcellular localization of BAM1 using a transient expression approach. It is suggested that the BAM1 localizes at plasma membrane region (Guo & Clark, 2010). In order to test the BAM1 subcellular localization, confocal microscopy analysis using BAM1 and RPK2 tagged with either GFP or TagRFP was conducted. As a result, their characteristic localizations to the surfaces of protoplasts was observed (Fig. S3). By contrast, neither GFP-tagged BAM1 nor RPK2 overlapped with free mCherry, which has been suggested to label the cytosol (shown in the merged image 'Merge' and as relative intensities in Fig. S3a,b). Further imaging analyses revealed that the localization patterns of the fluorescent protein-tagged BAM1 and RPK2 were largely overlapped at the cell surface (merged images in Fig. S3c,d), whereas those of the GFP-tagged BAM1 and RPK2 differed from that of mCherry (merged images in Fig. S3a,b),

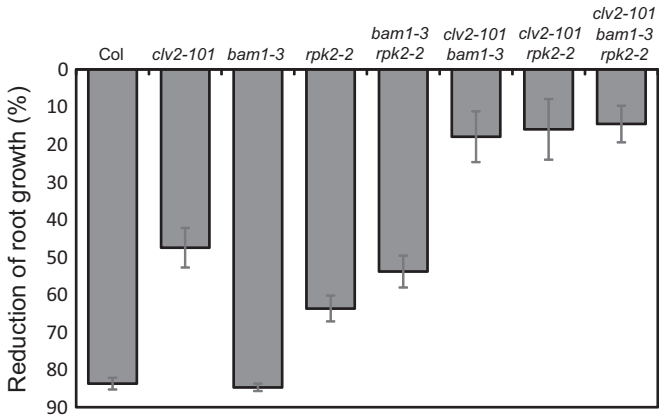

Fig. 6 The genetic relationships among Arabidopsis CLV receptors. The root lengths of 10-d-old seedlings grown on Murashige and Skoog (MS) medium containing 10 µM CLE25 were measured and calculated relative to the mock treatment. Data are shown as means ± SD.

probably because of mCherry subcellular localization in the cytosol. These results are consistent with their predicted functions and suggest their similar subcellular localizations at the cell surface.

Because BAM1 and RPK2 interact genetically, we then evaluated the possibility that they can form a multimer complex to participate in signaling. The physical interaction between BAM1 and RPK2 was confirmed by coimmunoprecipitation assays using *Arabidopsis* leaf mesophyll protoplast. BAM1-FLAG coexpressed with RPK2-VENUS was immunoprecipitated using an anti-GFP antibody that was conjugated to magnetic beads. Western blot analysis of the resulting samples revealed that BAM1-FLAG was efficiently precipitated with RPK2-VENUS (Fig. 7). This clearly differs from those of the samples expressing BAM1-FLAG alone. Taken together, our microscopic and biochemical studies demonstrate that BAM1 interacts with RPK2.

Discussion

BAM1 participates in CLV-like signaling in the RAM

Continuous cell production in the SAM and RAM is a fundamental mechanism that occurs during plant development.

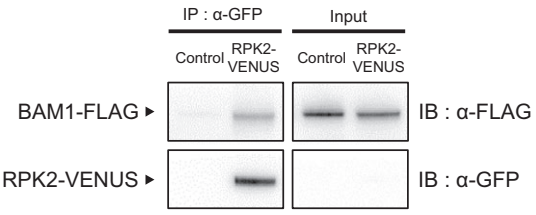

Fig. 7 Physical interaction between Arabidopsis BAM1 and RPK2. The BAM1-FLAG construct was introduced into protoplasts with or without RPK2-VENUS. Total protein extract was subjected to immunoprecipitation with an anti-green fluorescent protein (anti-GFP) antibody. The presence of BAM1-FLAG (upper panels) and RPK2-VENUS (lower panels) was determined by western blotting. The experiments were repeated three times with similar results.

Although the structures of the SAM and RAM are different, they both seem to possess similar basic mechanisms to balance cell proliferation and differentiation. The evolutionarily conserved LRR-RLK- and CLE peptide-mediated signaling systems are known to be responsible for maintaining cell production at appropriate levels; in particular, the CLV pathway and WUS comprise a feedback-regulating module in the SAM (Brand *et al.*, 2000; Betsuyaku *et al.*, 2011a; Miyawaki *et al.*, 2013). By contrast, equivalent repressive molecules in the RAM have not been identified. Ectopic expression of CLV3 or CLE ligands induces meristem consumption by repressing cell proliferation in the shoot and root, suggesting that the CLE peptide can act as an analogous signaling molecule to activate LRR-RLK-mediated signaling in the root meristem (Casamitjana-Martinez *et al.*, 2003; Hobe *et al.*, 2003; Fiers *et al.*, 2004, 2005; Kinoshita *et al.*, 2007; Jun *et al.*, 2010; Meng & Feldman, 2010). Furthermore, we have shown that RPK2 is involved in the regulation of meristem homeostasis in the RAM (Kinoshita *et al.*, 2010). Similar to RPK2, the dysfunction of CRN/SOL2 diminishes CLV-like signaling in the root (Miwa *et al.*, 2008; Müller *et al.*, 2008). However, CLV1 seems to have limited functioning in the RAM (Clark *et al.*, 1993; Fiers *et al.*, 2005; Miwa *et al.*, 2008).

BAM1, which is an LRR-RLK that strongly resembles CLV1, is capable of binding to the CLE peptide *in vitro*, and the ectopic expression of BAM1 as well as CLV1 compensates for the alterations of the *clv2* mutant *in vivo*, suggesting that BAM1 is involved in CLE peptide-mediated signaling in the root (DeYoung *et al.*, 2006; Guo *et al.*, 2010; Shinohara *et al.*, 2012). Furthermore, BAM1 has also been suggested to be involved in nematode infection in the root (Guo *et al.*, 2011). However, there is no critical evidence in support of the participation of BAM1 in the regulation of RAM activity.

In this report, we isolated many *clen* mutants showing resistance to the CLE peptide, indicating that the causal genes functioned in CLV-like signaling in the root. Our allelism test showed that the *clen102* mutation was comparable to the loss-of-function *bam1-3* mutation. Furthermore, we showed that the single *bam1-3* mutant was clearly resistant to the CLV3 peptide treatment (Fig. 4). Together with the results from whole-mount *in situ* hybridization analysis, which revealed the clear expression of *BAM1* mRNA in root stem cell niche regions (Fig. 5), we suggest that BAM1 participates in CLV-like signaling in the regulation of RAM activity.

BAM1 kinase activity may regulate BAM1 function in the RAM

The *clen101* mutant contains an amino acid substitution in the G-loop of the kinase domain, and the degree of peptide resistance is similar to that of the *clen102* mutant when combined with the *clv2-101* mutation. The G-loop (GxGxxG sequence) is responsible for binding to ATP, which is a crucial step in the functioning of kinases, and the *clen101* mutation generates the GxGxxD sequence. An equivalent amino acid substitution has been identified in the human protein STRADα, which shows lower levels of ATP binding activity, suggesting that it is a pseudokinase (Boudeau *et al.*, 2004). In addition, Arabidopsis CRN/SOL2 also contains an altered G-loop with the GxDxxG sequence. Nimchuk *et al.* (2011) showed that CRN/SOL2 does not exert kinase activity. These findings suggest that the *clen101* mutation disturbs kinase activity. Further, the responsible residues of *clen102* and *clen103* have been mapped to a region between the catalytic domain and the Mg-binding domain. Although the function of this loop has not been elucidated, kinase activity is expected to play a critical role.

BAM1, RPK2 and CLV2-CRN/SOL2 restrict cell proliferation in both the SAM and RAM

In the SAM, a single *bam1* mutation does not result in obvious abnormalities, whereas multiple mutants with *clv1* or other BAM family LRR-RLK mutants (i.e. *bam2* or *bam3*) exhibit abnormally shaped SAMs (DeYoung *et al.*, 2006). In particular, BAM1 and CLV1 are expected to act on related processes in aerial tissue; however, their activities do not seem to be similar in roots. The *clv1* mutant does not exhibit any visible phenotypic abnormalities in root growth or CLE peptide resistance, in contrast with other receptor mutants (Clark *et al.*, 1993; Fiers *et al.*, 2005; Miwa *et al.*, 2008). In addition, the *CLV1* spatial expression pattern differs from those of other receptors involved in CLE peptide-mediated growth inhibition in the root, suggesting the limited contribution of CLV1 in this pathway (Replogle *et al.*, 2013; Stahl *et al.*, 2013). RPK2 and CRN/SOL2 are also expressed in root tip regions containing *BAM1*-expressing cells (Müller *et al.*, 2008; Kinoshita *et al.*, 2010). Thus, the three receptors preferentially act on the CLV-like pathway in roots because their loss-of-function mutants show resistance to CLE peptide treatment. The biological relevance of the BAM family LRR-RLKs has aroused controversy. Based on the phenotypes of multiple BAM mutants, BAM1 has been labeled as a positive factor of cell proliferation in the SAM (DeYoung *et al.*, 2006). However, more recent studies have demonstrated that BAM1 can negatively influence SAM activity because the *bam1* mutation can enhance *clv1* abnormalities (DeYoung & Clark, 2008). To elucidate these complicated genetic interactions, a hypothesis has been proposed that BAM family LRR-RLKs act as decoys to insulate the OC. Remarkably, our analysis of the root system has provided an attractive possibility that BAM1 functions as a negative regulator of cell proliferation in CLE peptide-mediated root growth inhibition. Taken together, the three receptors, BAM1, RPK2 and CLV2-CRN/SOL2, are involved in analogous signaling processes to restrict cell proliferation in the RAM.

BAM1 restricts cell proliferation synergistically with RPK2 in the RAM

Based on genetic analysis, we determined that the *clv2 bam1* and *clv2 rpk2* double mutants and their triple mutant strongly resist growth inhibition when exposed to the CLE25 peptide (Fig. 6). By contrast, the *bam1 rpk2* mutant was sensitive to CLE25, which indicated that intact CLV2 can transmit the peptide signal at reliable levels and that the ability of CLV2 to perceive this

signal is independent of BAM1 or RPK2. Because BAM1 and RPK2 are LRR-RLK proteins, they may have several modes of action to form homo- or heteromeric receptor complexes (Bleckmann *et al.*, 2010; Stahl *et al.*, 2013). Our protein–protein interaction analysis showed that BAM1 and RPK2 form heteromeric complexes (Fig. 7). They can also form a multimer complex to perceive the extracellular CLV3 peptide via other CLV receptors, CLV1-CLV2-CRN/SOL2 or CLV1-BAM1 receptor complexes (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010). Our results provided additional possibilities for receptor complex formation, suggesting the flexible but complicated nature of the receptors and the presence of divergent signaling pathways. Although our results do not reveal the precise CLE peptide receptor in roots, the involvement of BAM1 in the perception of the CLE peptide is considerable. Because the impact of the *bam1* mutation is relatively small compared with that of *rpk2*, BAM1 might have a supportive role to RPK2 in signal transduction in the RAM, and it most probably functions in homomeric and/or heteromeric complexes.

The next steps to elucidate BAM1 function

The identification of the ligand(s) for BAM1 will be the next step in understanding the molecular mechanism of root meristem development. A previous study has shown that 11 *CLE* genes are expressed in the RAM (Jun *et al.*, 2010); however, it is still unknown which CLE peptide is responsible for RAM development.

By contrast, if nonCLE ligands are perceived by a specific LRR-RLK that can stimulate BAM1 or RPK2 through heteromultimerization, the LRR-RLK multimer could represent an alternative regulatory unit in the RAM. Although BAM1 and RPK2 are classified into distinct clades of the large LRR-RLK protein family (Shiu & Bleecker, 2003), they are able to form heteromeric complexes, suggesting that the wide variety of LRR-RLK proteins could be their potential binding partners (Fig. 2b). Previous studies have revealed that CLE peptides and other small peptides or compounds can stimulate LRR-RLK-mediated signaling. For example, brassinosteroids can bind to BRI1, and bacterial flagellin is sensed by FLS2 (Kinoshita *et al.*, 2005; Chinchilla *et al.*, 2006). Elucidating the specific or common ligands for BAM1 and RPK2 in the root will be one of the most important tasks in future studies and might help in the understanding of the similarities and diversity of shoot and root development.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Effect of the CLE25 peptide on the architecture of the RAM.

Fig. S2 *BAM1* is expressed in the center of the root meristem.

Fig. S3 The subcellular localizations of BAM1 and RPK2 in the *Arabidopsis* leaf mesophyll protoplast.

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