

# CLAVATA2 forms a distinct CLE-binding receptor complex regulating Arabidopsis stem cell specification

Yongfeng Guo, Linqi Han, Matthew Hymes<sup>†</sup>, Robert Denver and Steven E. Clark<sup>\*</sup>

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI 48109-1048, USA

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<sup>\*</sup>For correspondence (fax 734 647 0884; e-mail clarks@umich.edu).

<sup>†</sup>Present address: Pall Life Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48103, USA.

## SUMMARY

CLAVATA1 (CLV1), CLV2, CLV3, CORYNE (CRN), BAM1 and BAM2 are key regulators that function at the shoot apical meristem (SAM) of plants to promote differentiation by limiting the size of the organizing center that maintains stem cell identity in neighboring cells. Previous results have indicated that the extracellular domain of the receptor kinase CLV1 binds to the CLV3-derived CLE ligand. The biochemical role of the receptor-like protein CLV2 has remained largely unknown. Although genetic analysis suggested that CLV2, together with the membrane kinase CRN, acts in parallel with CLV1, recent studies using transient expression indicated that CLV2 and CRN form a complex with CLV1. Here, we report detection of distinct CLV2-CRN heteromultimeric and CLV1-BAM multimeric complexes in transient expression in tobacco and in Arabidopsis meristems. Weaker interactions between the two complexes were detectable in transient expression. We also find that CLV2 alone generates a membrane-localized CLE binding activity independent of CLV1. CLV2, CLV1 and the CLV1 homologs BAM1 and BAM2 all bind to the CLV3-derived CLE peptide with similar kinetics, but BAM receptors show a broader range of interactions with different CLE peptides. Finally, we show that BAM and CLV1 overexpression can compensate for the loss of CLV2 function *in vivo*. These results suggest two parallel ligand-binding receptor complexes controlling stem cell specification in Arabidopsis.

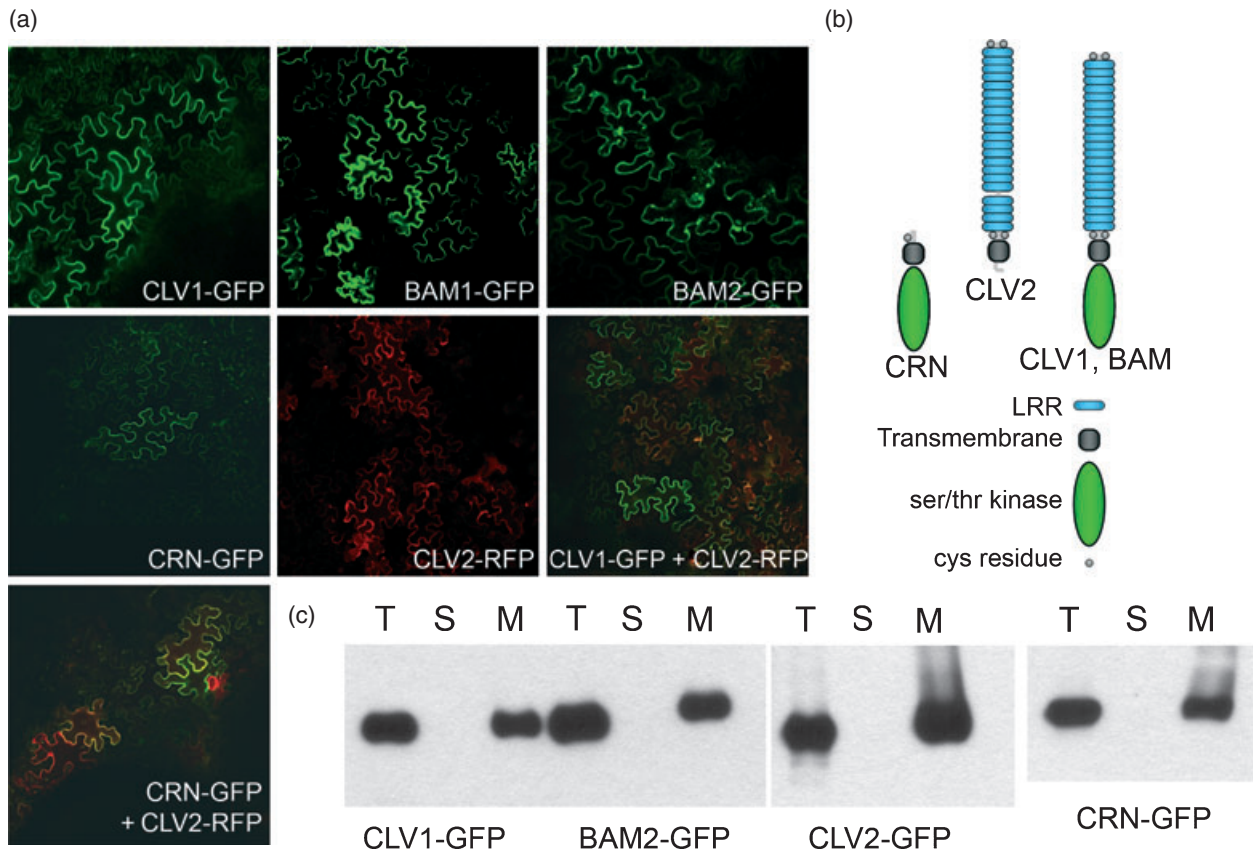
**Keywords:** CLAVATA2, CLE peptide, CORYNE, CLAVATA1, receptor complex, meristem.

## INTRODUCTION

In plants, aerial organs are developed from the shoot apical meristem (SAM) (Steeves and Sussex, 1989). To continuously generate new organs throughout the life of a plant, a small number of pluripotent stem cells must be maintained at the center of the SAM. As these stem cells divide, distal and basal progeny cells switch towards differentiation, and become competent to form organ primordia. A functional meristem is maintained through a delicate balance between the maintenance of stem cell identity and differentiation.

A key pathway regulating Arabidopsis stem cell specification is the CLAVATA (CLV) signaling pathway, which is essential to promote differentiation of lateral stem cell daughters (Clark *et al.*, 1993, 1995; Kaye and Clark, 1998). Loss-of-function mutations in any of the *CLV* genes result in plants with shoot and flower meristems that accumulate massive populations of stem cells. *CLV1* and *CLV2* encode plasma-membrane receptors, whereas *CLV3* encodes a small secreted proprotein that appears to undergo proteo-

lytic maturation to release a mature CLE peptide (Clark *et al.*, 1997; Jeong *et al.*, 1999; Rojo *et al.*, 2002) (Figure 1b). The CLV1 leucine-rich repeat receptor kinase (LRR-RK) binds to the CLV3 CLE peptide through its extracellular domain, providing direct evidence that CLV3–CLV1 function as a ligand–receptor pair to regulate stem cell specification (Ogawa *et al.*, 2008). The *CLV2* gene encodes a receptor-like protein with 21 extracellular LRRs, a single transmembrane domain and a short cytoplasmic tail. Unlike CLV1 and CLV3, which are expressed in very restricted regions in the center of the SAM, and function only in stem cell specification (Clark *et al.*, 1997; Fletcher *et al.*, 1999), CLV2 has a much wider expression pattern and is required for the proper development of many organ types (Jeong *et al.*, 1999). The CLV1-related receptors BAM1 and BAM2 act redundantly with CLV1 in the meristem center, but also play a poorly characterized role on the meristem periphery, and are broadly functional in many developing organs (DeYoung *et al.*, 2006; DeYoung and Clark, 2008). CLV1 and BAM



**Figure 1.** Localization of receptors expressed in *Nicotiana benthamiana* leaves.

(a) Confocal images of fluorescently-tagged receptors 2 days post-inoculation. The signal was observed primarily at the cell periphery, although some internal foci, perhaps corresponding to endocytosis, were also observed. All infiltrations were performed with the addition of the P19 co-suppression inhibitor (Voinnet *et al.*, 2003).

(b) The domain organization of the proteins in this study.

(c) Total input (T) for each expressed protein was fractionated into soluble (S) and membrane (M) components, and detected by protein gel blot analysis with anti-GFP antibodies.

receptors can cross-complement, suggesting a shared mechanism of signaling (DeYoung *et al.*, 2006).

A prevalent model for CLV signaling suggests that a membrane-bound CLV1–CLV2 co-receptor complex is activated upon CLE binding (Clark, 2001; Becraft, 2002; Fletcher, 2002). Although this model is consistent with existing genetic interaction studies, identification of the transmembrane kinase CORYNE (CRN)/SOL2 as a factor in CLV signaling raised questions about this model (Miwa *et al.*, 2008; Muller *et al.*, 2008) (Figure 1b). The epistasis of *clv2* to *crn* led to the model that CLV2, which lacks a cytoplasmic signaling domain, acts with CRN, which lacks an extracellular receptor domain (Muller *et al.*, 2008). Two recent studies reported that when transiently expressed in tobacco leaves (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010) and in *Arabidopsis* mesophyll protoplasts (Zhu *et al.*, 2010), CLV2 interacted with CRN, providing evidence for this model. Both of these studies also suggested that the CLV2–CRN heterodimer formed a complex with CLV1 (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010), and one study reported CLV1–CRN inter-

action (Zhu *et al.*, 2010), suggesting the possibility that CLV1, CLV2 and CRN function together in one large receptor complex.

To address the mechanism of receptor activation, and the function of CLV2, we independently tested and quantified receptor associations in transient expression and in *Arabidopsis*. We also tested the ability of each receptor component to interact with the CLV3-derived CLE signal. Our findings lead us to propose a distinct model for CLV function *in vivo*.

## RESULTS

### Expression of the CLV receptors

To study the biochemical functions of the CLV pathway receptors, we generated a variety of transgenes driving expression of each receptor protein with a combination of GFP, FLAG and MYC epitope tags using their native promoter and/or the 35S cis elements. These transgenes were used both in transient expression of receptor proteins in *Nicotiana benthamiana* and in stable transformation of

*Arabidopsis thaliana*. We have shown both in previous studies (Diévert *et al.*, 2003; DeYoung *et al.*, 2006) and here (Figure S1) that each of these chimeric proteins can replace the endogenous protein *in vivo*.

When GFP- or RFP-tagged proteins were expressed in *N. benthamiana* leaves, all of the receptor proteins, including CLV1-GFP, CLV2-RFP, BAM1-GFP, BAM2-GFP and CRN-GFP, showed accumulation in the membrane fraction, and showed fluorescence primarily at the cell periphery in a significant portion of cells by 2 days post-inoculation (DPI) (Figure 1a,c). To avoid any artifacts caused by the long-term receptor accumulation that might occur at later time points, all assays used membrane fractions at 2 DPI. Co-infiltration of GFP- and RFP-fluorescent proteins revealed incomplete overlap in expression (Figure 1a). When co-expressed with GFP tag proteins, CLV2-RFP showed co-localization with CLV1 and CRN (Figure 1a).

### Evidence for two separate receptor complexes

Based on genetic data, it has been hypothesized that CLV2 and CRN act in parallel with CLV1 to perceive the CLV3 signal (Muller *et al.*, 2008). Using firefly luciferase complementation imaging (LCI) assays (Zhu *et al.*, 2010), and efficiency of fluorescence resonance energy transfer ( $E_{\text{FRET}}$ ) assays (Bleckmann *et al.*, 2010), two recent studies reported CLV2–CRN interaction (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010) and CLV1 homomultimerization (Bleckmann *et al.*, 2010) in transient expression systems, supporting the genetic model. Both of these studies indicated the presence of a CLV1–CRN–CLV2 complex (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010), raising the question of whether CLV1 and CLV2–CRN act as separate interacting complexes or as a single larger complex. One complication of both luciferase complementation and  $E_{\text{FRET}}$  is that the results can be strongly affected by the particular orientation of the fluorescent tags and expression level of proteins, making quantification of interactions difficult. We have addressed this issue through co-immunoprecipitation (co-IP) analysis of various receptor proteins co-expressed in tobacco leaves and *in vivo*.

We first tested interactions among the related receptor kinases CLV1, BAM1 and BAM2. We observed robust co-IP between CLV1–CLV1, CLV1–BAM1, CLV1–BAM2, BAM1–BAM1 and BAM1–BAM2 (Figures 2a,b,3a and S2). Interestingly, none of the *clv1* dominant-negative mis-sense mutant isoforms tested had any detectable effect on *clv1*–CLV1 or *clv1*–BAM1 co-IP (Figure 2a,b). To quantify the extent and stability of these receptor kinase interactions, we analyzed the efficiency of co-IP between CLV1-GFP and BAM2-FLAG (Figure 3a). We compared bound and unbound fractions from CLV1-GFP and BAM2-FLAG co-expression precipitated with anti-GFP antibodies. We estimate an efficiency of interaction of 20% [based on 10% co-IP of BAM2-FLAG in an experiment with 50% IP of CLV1-GFP (Figure 3a); see Experimental procedures]. The CLV1–BAM2 complex

appears to involve nearly all of the available CLV1 (given the possibility of CLV1 homodimerization and incomplete overlap in expression), and is quite stable.

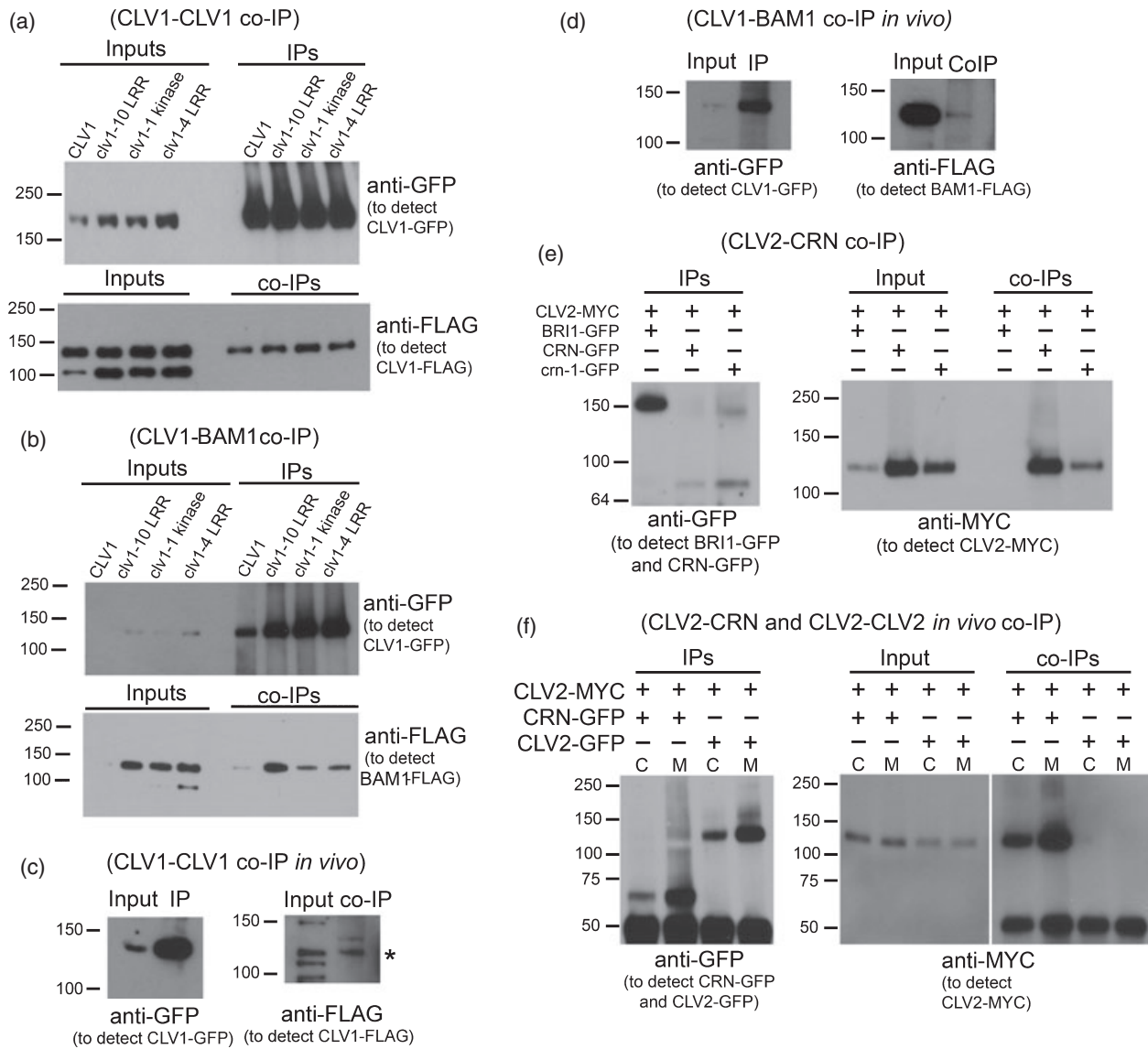
Controls to test for non-specific antibody interaction, non-specific tag–receptor interactions and interactions that might occur post-isolation during the immunoprecipitation procedure all showed that the interactions are specific, and require receptor co-expression (Figures S2,S3 and 2e).

To examine if the CLV1–CLV1 interactions observed in transient expression represented endogenous interactions, we tested co-IP between CLV1-GFP and CLV1-FLAG in *Arabidopsis* meristem tissue. Despite the poor accumulation of CLV1 in transgenic *Arabidopsis* (DeYoung *et al.*, 2006), we could readily detect interaction (Figure 2c). We also detected the interaction between CLV1-GFP and BAM1-FLAG in stably transformed *Arabidopsis* (Figure 2d).

In transient expression we observed robust co-IP between CLV2-MYC and CRN-GFP (Figure 2e), with approximately 20% of the CLV2-MYC co-IPd by anti-GFP antibodies (Figure 3b). Thus, the CLV2–CRN complex must also be relatively abundant and stable. Control experiments demonstrated that the interactions were specific and dependent on co-expression (Figures 2e, S3).

Transgenic *Arabidopsis* plants stably expressing CLV2-MYC and CRN-GFP were used to assess interactions *in vivo*. Here, we also observed robust co-IP between the two proteins (Figure 2f), indicating this is a physiologically relevant complex. No homomultimerization of CLV2 was observed *in vivo* (Figure 2f). When *crn-1* was co-expressed with CLV2 in transient expression, and tested for co-immunoprecipitation, we found the mutation did not eliminate the CLV2–CRN interaction (Figure 2e), nor did the *crn-1* mutation affect CRN-FLAG–CRN-GFP interactions when transiently expressed (Figure S4a). Given that *crn-1* is a dominant-negative allele, it may act by sequestering CLV2 in a non-functional manner.

We also tested interactions between receptor protein pairs CLV1–CLV2, BAM1–CLV2, BAM2–CLV2 and CLV1–CRN, and were able to detect co-IP between each combination (Figures S4b, 4c). However, based on the co-IP signal, all of these interactions were much weaker than the ones observed between CLV2 and CRN, and between CLV1 and CLV1–BAM. In quantifying the interactions between CLV1 and CLV2 using the same approach that was used to quantify CLV1–BAM and CLV2–CRN associations, the interactions were nearly an order of magnitude lower, around 3% (2.5% co-IP of 75% IPd protein; Figure 3c). When all three proteins, CLV1, CLV2 and CRN were co-expressed, the level of CLV1–CLV2 interaction was similarly low, around 5% (2.5% co-IP of 50% IPd protein; Figure 3d). Perhaps related to the weak interaction between BAM1 and CLV2 in transient expression, repeated attempts to detect co-IP between BAM1 and CLV2 in *Arabidopsis* have been unsuccessful (B. DeYoung, L. Han and S.E. Clark, unpublished data).

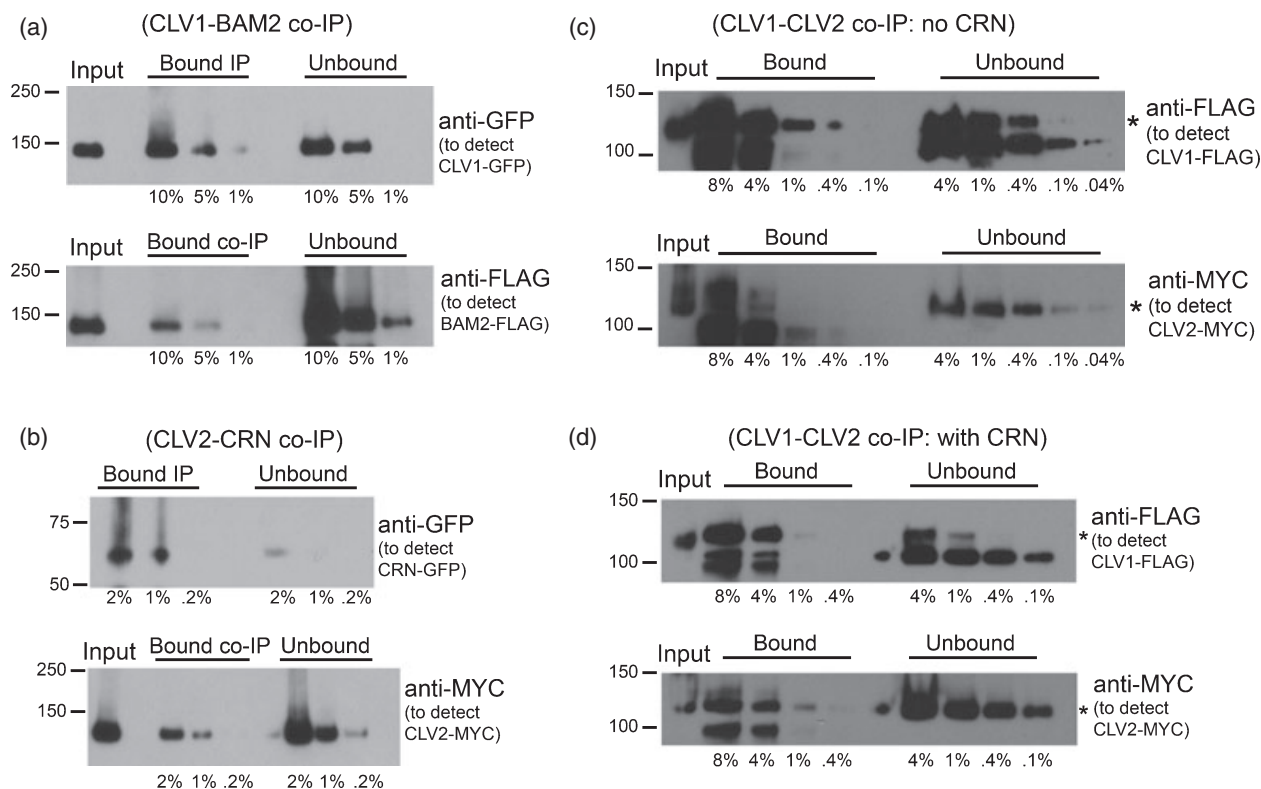


**Figure 2.** Detection of receptor interactions. (a) Solubilized membrane extracts from CLV1-FLAG co-expressed with wild-type and mutant versions of CLV1-GFP in transient expression were immunoprecipitated (IPd) with anti-GFP antibodies, and co-IPs were detected with anti-FLAG antibodies. Note, the anti-FLAG antibody can detect a non-specific band of ~100 kDa in input samples. (b) Wild-type and mutant versions of CLV1-GFP co-expressed with BAM1-FLAG in transient expression were IPd with anti-GFP antibodies, and co-IPs were detected with anti-FLAG antibodies. (c) Solubilized membrane extracts from Arabidopsis plants co-expressing CLV1-GFP and CLV1-FLAG were IPd with anti-GFP antibodies, and co-IPs were detected with anti-FLAG antibodies. (d) Solubilized membrane extracts from Arabidopsis plants co-expressing CLV1-GFP and BAM1-FLAG were IPd with anti-GFP antibodies, and co-IPs were detected with anti-FLAG antibodies. (e) CLV2-MYC co-expressed with BRI1-GFP, CRN-GFP or crn-1-GFP in transient expression were IPd with anti-GFP antibodies, and co-IPs were detected with anti-MYC antibodies. (f) Both crude extracts (C) and solubilized membrane extracts (M) were used to perform IPs using anti-GFP antibodies from Arabidopsis plants co-expressing CRN-GFP and CLV2-MYC or CLV2-MYC and CLV2-GFP. co-IPs were detected with anti-MYC antibodies. The cross-reacting signal below the 50-kDa marker was from IgG proteins.

**A CLV2 CLE-binding activity similar to that of CLV1**

If CLV1 and CLV2-CRN form distinct receptor complexes, what is the role of the CLV2-CRN complex? The BRI1 sig-

naling is relayed through BSKs, which are membrane-associated cytoplasmic kinases (Tang *et al.*, 2008). Thus, one hypothesis would be that CLV2-CRN functions analogously to BSKs by relaying the signal from the ligand-binding

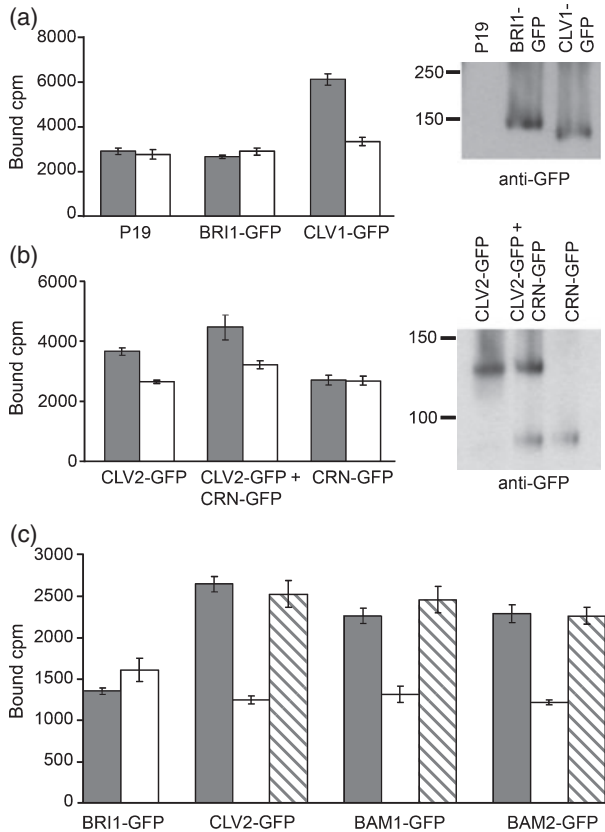


**Figure 3.** Quantification of receptor interactions.

- (a) Solubilized membrane extracts from CLV1-GFP co-expressed with BAM2-FLAG in transient expression were immunoprecipitated (IPd) with anti-GFP antibodies, and co-IPs were detected with anti-FLAG antibodies. A dilution series of 10, 5 and 1% of total bound and unbound fractions were assayed on protein gel blots to estimate the efficiency of IP and co-IP (see Experimental procedures).
- (b) CRN-GFP co-expressed with CLV2-MYC in transient expression was IPd with anti-GFP antibodies, and co-IPs were detected with anti-MYC antibodies. A dilution series of 2, 1 and 0.2% of total bound and unbound fractions were assayed on protein gel blots to estimate the efficiency of IP and co-IP.
- (c) CLV1-FLAG co-expressed with CLV2-MYC in transient expression was IPd with anti-FLAG antibodies, and co-IPs were detected with anti-MYC antibodies. A dilution series of 8, 4, 1, 0.4 and 0.1% of total bound, and a dilution series of 4, 1, 0.4, 0.1 and 0.04% of unbound fractions were assayed on protein gel blots to estimate the efficiency of IP and co-IP.
- (d) CLV1-FLAG co-expressed with CLV2-MYC and CRN-GFP in transient expression was IPd with anti-FLAG antibodies, and co-IPs were detected with anti-MYC. A dilution series of 8, 4, 1 and 0.4 of total bound, and a dilution series of 4, 1, 0.4 and 0.1 unbound, fractions were assayed on protein gel blots to estimate the efficiency of IP and co-IP.

receptor (in this case CLV1). However, CLV2-CRN together differ from BSKs in that CLV2 contains an extensive extracellular domain. What then is the role of the CLV2 extracellular LRR domain? Genetic evidence has suggested that the CLE domain processed from CLV3 is the ligand for the receptor-kinase CLV1 (Fletcher *et al.*, 1999; Trotochaud *et al.*, 1999; Lenhard and Laux, 2003; Fiers *et al.*, 2004, 2005; Ni and Clark, 2006). Recent reports showed that CLV3 CLE peptides bind to the truncated CLV1 LRR domain expressed in tobacco BY-2 cells, confirming an interaction between this ligand-receptor pair (Ogawa *et al.*, 2008). To address the function of the LRR domains of the various receptors, we have tested ligand binding activities of the CLV pathway receptors via radioiodination of the CLV3 CLE peptide (Mayers and Klostergaard, 1983). In this process, the two histidine residues in the CLE domain are targeted for  $^{125}\text{I}$  labeling, followed by HPLC purification of labeled, active peptides.

We found that expression of full-length CLV1 in *N. benthamiana* generated specific binding for  $^{125}\text{I}$ -radio-labeled CLV3 CLE peptides to membrane fractions (Figure 4a). CLV3 CLE-binding activity was also generated by expressing the CLV1-related BAM1 and BAM2 receptors (Figure 4c). Critically, CLV3 CLE-binding sites within membrane fractions were generated by expression of full-length CLV2 (Figure 4b). This suggests that CLV1 and CLV2 perceive the same CLV3 CLE ligand signal in controlling stem cell homeostasis. Although CRN acts with CLV2 as a separate receptor complex, as indicated by the co-IP analyses, the observed CLV2 ligand binding activity was not dependent on the co-expression of CRN, nor did CRN expression alone lead to CLV3 CLE binding (Figure 4b). Membrane fractions from *N. benthamiana* leaves in which no receptor protein was expressed or samples in which the brassinosteroid receptor BRI1 (Li and Chory, 1997) was expressed served as negative



**Figure 4.** CLV3 CLE binding activity.

(a) Detergent-washed membrane fractions from P19, BRI1-GFP and CLV1-GFP inoculations were tested for  $^{125}$ I-labeled CLV3 CLE peptide binding without (black bars) and with (white bars) excess unlabeled CLV3 CLE competitor. Means  $\pm$  standard errors over four replicates are shown. The fractions tested for CLE binding were assayed in a protein gel blot with anti-GFP antibodies to detect BRI1-GFP and CLV1-GFP accumulation.

(b) Assays identical to those in (a) performed for CLV2-GFP and CRN-GFP in independent and co-inoculations.

(c) GFP-fused CLV2, BRI1, BAM1 and BAM2 were immunoprecipitated with anti-GFP antibodies and then tested for CLV3 CLE binding activity without (black bars) and with excess unlabeled CLV3 CLE (white bars) or the non-functional CLV3S peptide (gray bars) as competitors. Means  $\pm$  standard errors over three replicates are shown.

controls, and showed no specific binding to radiolabeled CLV3 CLE (Figure 4a).

We next tested immunopurified CLV2, BRI1, BAM1 and BAM2 for CLV3 CLE binding activity (Figure 4c). Specific binding was observed from immunoprecipitated CLV2, BAM1 and BAM2, but not from BRI1. As a further control, the non-functional CLV3S peptide was also used as a cold competitor to radiolabeled CLV3 CLE, and, as previously reported for CLV1, was unable to compete (Kondo *et al.*, 2008; Ogawa *et al.*, 2008) (Figure 4c).

To compare the CLV3 CLE binding affinities of CLV1 and CLV2, we assayed the binding kinetics (Figure 5). Binding was saturable for all receptors, with a very similar  $K_d$  for each receptor: 30 nm for CLV1, 32 nm for CLV2, 26 nm for

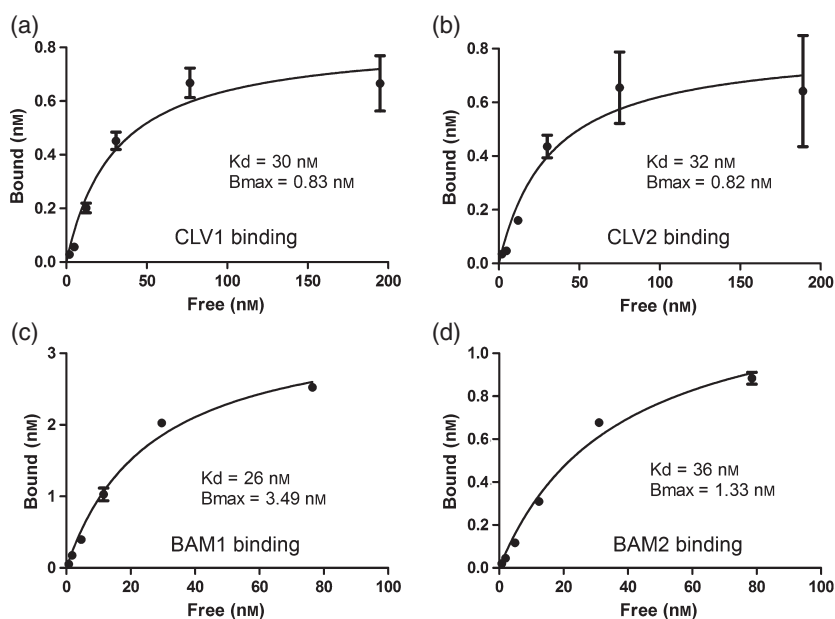
BAM1 and 36 nm for BAM2 (Figures 5 and S5). Thus, CLV1 and CLV2 binding activities have very similar affinities for the same CLV3 CLE peptide, suggesting that they each perceive the CLV3 signal *in vivo* in a similar manner. Interestingly, *clv2* mutants are resistant to CLE peptide treatment in seedlings, both at the root and shoot meristem (Fiers *et al.*, 2005). This had been hypothesized to be the result of the requirement for CLV2 for the function of unknown receptor-kinases, but our results suggest that this resistance is a result of the loss of the CLV2 CLE-binding activity in the *clv2* mutant.

There are a large number of CLE-containing proteins that can differ significantly in their CLE domain sequences (Cock and McCormick, 2001; DeYoung and Clark, 2001; Oelkers *et al.*, 2008). We have shown previously that the different CLE-containing proteins can differ dramatically in their ability to replace CLV3 function *in vivo* (Ni and Clark, 2006). In addition, the different CLE-containing proteins can drive divergent overexpression phenotypes (Strabala *et al.*, 2006). These studies suggest that the different CLE peptides differ in receptor specificity. Indeed, other CLE peptides have been shown to bind to different receptors (Hirakawa *et al.*, 2008). To determine if CLV1 and CLV2 exhibited differential specificity to individual CLEs, we tested a variety of CLE peptides. These included CLE1, CLE5, CLE11, CLE18, CLE19, CLE22, CLE25, CLE26, CLE40, CLE41 and CLE42. Other peptides included for comparison were: CLV3S (Thr71–His81), which only contains a portion of the CLV3 CLE domain, and is non-functional; CLV3L (Arg70–Pro96), which includes the full CLV3 CLE domain plus the extended C-terminal tail, and is partially functional; the *clv3-1* mutant CLE isoform (Gly75Arg); the *clv3-G75A* (Gly75Ala) mutant isoform; and another proline-rich putative peptide ligand IDA (Pro278–Asn289) as a negative control (Stenvik *et al.*, 2006, 2008). It should be noted that the original annotation for the *clv3-1* mutation and the subsequent interpretation of *clv3-1* as a glycine to alanine substitution at residue 75 was inaccurate. We sequenced *clv3-1* and determined the GGA codon for glycine75 is mutated to AGA, leading to a glycine to arginine substitution.

When the various CLE peptides were used as cold competitors for radiolabeled CLV3 CLE binding to CLV1, CLV2, BAM1 and BAM2 (Figure 6), CLE peptides that bind effectively to the receptors should compete with the radiolabeled CLV3 CLE peptide, whereas those with no or reduced binding should be unable to fully displace the bound radiolabeled CLV3 CLE peptide. Figure 6 presents the level of bound radiolabeled CLV3 displaced by the various cold CLE competitors.

For CLV1 and CLV2, we observed a wide variation in the extent of competition (Figure 6). Several CLE peptides were unable to effectively compete with CLV3 CLE, suggesting that they bind poorly to CLV1 and CLV2. Interestingly, CLV1 and CLV2 displayed similar binding affinities for a number of

**Figure 5.** Kinetics of CLV3 CLE binding. Saturation curves for CLV3 CLE binding from detergent-washed membrane fractions expressing CLV1-GFP (a), CLV2-MYC (b), BAM1-GFP (c) and BAM2-GFP (d) are shown. The means  $\pm$  standard errors over three replicates are shown, as are the equilibrium dissociation constant ( $K_d$ ) and total binding ( $B_{max}$ ).



CLEs, including CLE1, CLE5, CLE18, CLE22, CLE26, CLE41 and CLE42 (Figure 6a,b). *clv3-1* exhibited poor binding to both CLV1 and CLV2, consistent with the partial loss-of-function for the *clv3-1* allele (Clark *et al.*, 1995). Differences were observed, however, and these were generally in CLEs that retained good competition for CLV1, but reduced competition for CLV2, including CLE11, CLE19 and CLE25.

A very different result was obtained with binding to BAM1 and BAM2 (Figure 6c,d). When 10  $\mu$ M of cold peptide was used, nearly every CLE provided full competition to CLV3 CLE binding, with the exception of CLE1 and CLE5. Even the mutant *clv3-1* peptide was largely functional in replacing CLV3 CLE binding to BAM1. This suggests that BAM receptors have a wider range of specificity for CLE binding than either CLV1 or CLV2, perhaps related to their broad developmental roles.

#### BAM and CLV1 can replace CLV2 function *in vivo*

If CLV1–BAM and CLV2–CRN form distinct complexes *in vivo* (as opposed to a single larger complex), it might be possible to bypass the requirement for one of the complexes through overexpression of the other. To this end, we crossed the receptor overexpression constructs  $P_{35S}:CLV1-GFP$ ,  $P_{ER}:BAM1-GFP$  and  $P_{ER}:BAM2-GFP$  with *clv2* mutants. We observed that both the BAM1 and BAM2 receptors could provide a complete rescue of the *clv2* mutant phenotype (Figure 7a). The effect of CLV1 overexpression on *clv2* was weaker, with only a barely significant change in carpel number, but a clear reduction in valve-lessness and gynocelia defects (Figure 7b): 65% of *clv2-3* flowers exhibited valve-lessness ( $n = 60$ ) (Kayes and Clark, 1998), whereas none of the flowers of the  $P_{35S}:CLV1-GFP$  *clv2-3* plants

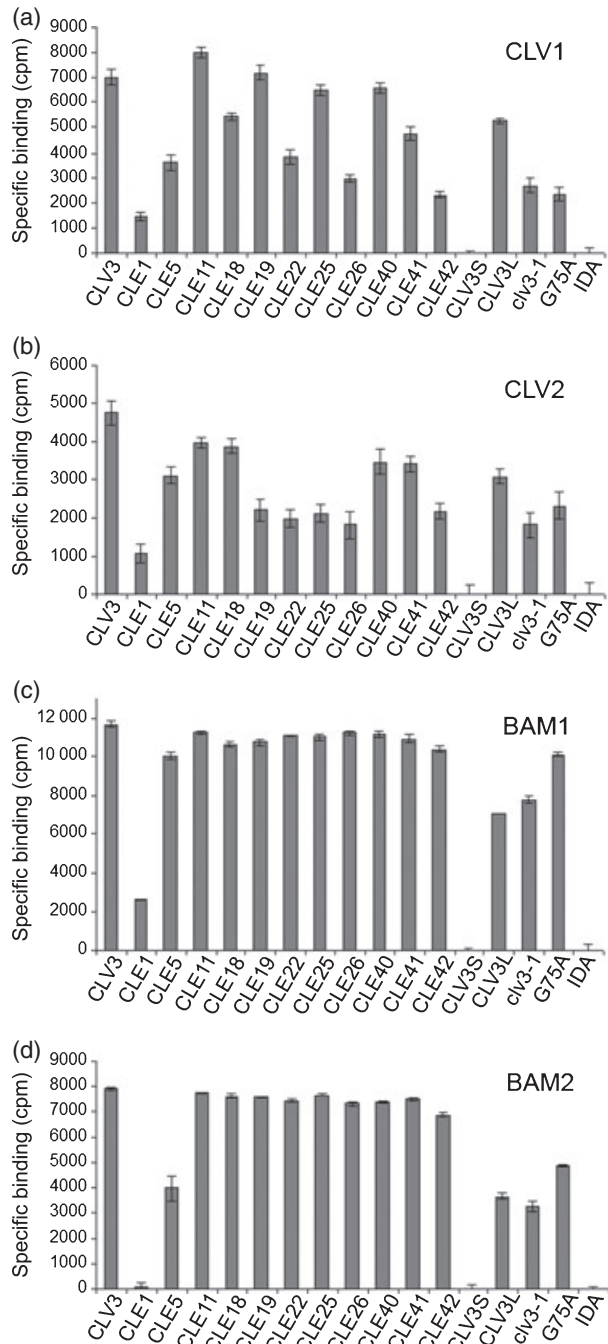
displayed valve-lessness ( $n = 60$ ). Thus, exogenous BAM1 and BAM2 can completely, and CLV1 can partially, bypass the function of CLV2 in meristem development.

#### DISCUSSION

In this study we find evidence for two distinct receptor complexes involved in stem cell regulation in Arabidopsis: CLV2–CRN and CLV1–BAM. Both CLV1 and CLV2 bind to the CLV3-derived CLE ligand. The presence of two ligand-binding complexes suggests a unique mode of receptor activation of the CLV pathway.

The biochemical function of CLV2 has remained unknown. Although originally viewed as a co-receptor for CLV3, two recent studies relying on fluorescent-based association assays have suggested that CLV2 forms a complex with CRN (Bleckmann *et al.*, 2010; Zhu *et al.*, 2010). Our results here have not only extended these findings, but also noted significant differences. First, we have tested aspects of recent studies, which were based on co-fluorescence, with the direct co-immunoprecipitation of proteins with entirely different epitope tags. In doing so, we have directly quantified the number of associated receptor proteins in each complex. Furthermore, we have tested the formation of each complex *in vivo* in Arabidopsis. Finally, we have assayed the ability of each receptor protein to bind to the peptide ligand derived from CLV3.

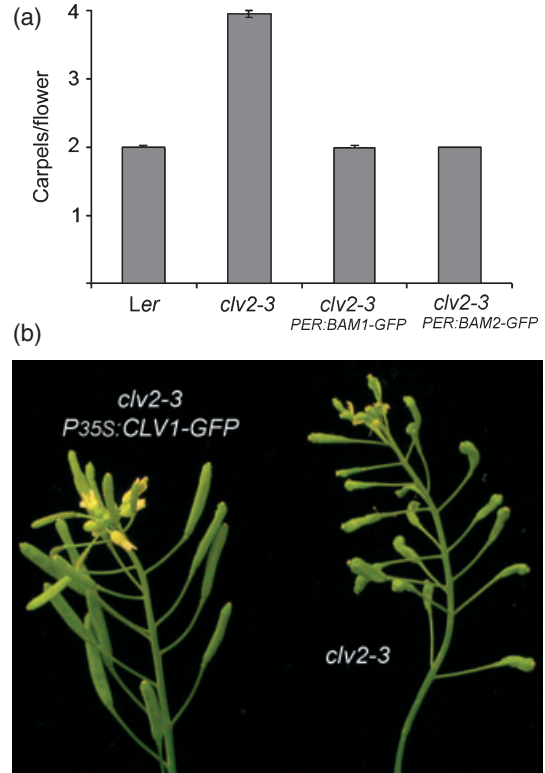
Consistent with previous reports, we observed the formation of the CLV2–CRN complex, and our quantification and *in vivo* analyses indicate that this complex is both robust and of significance *in vivo*. The presence of a robust CLV1–CLV1 complex in transient expression and *in vivo* in this study confirms data from fluorescent assays in transient *N. bethamiana* expression (Bleckmann *et al.*, 2010), but is



**Figure 6.** Receptor specificity for different CLEs. Specific binding in counts per minute (cpm) (total binding minus binding activity with competitor peptides) of different CLE peptides by detergent-washed membrane fractions expressing CLV1-GFP (a), CLV2-MYC (b), BAM1-GFP (c) and BAM2-GFP (d) are shown. See text for a description of the peptides. The means  $\pm$  standard errors over three replicates are shown.

inconsistent with earlier results from fluorescent assays in protoplasts (Zhu *et al.*, 2010).

Furthermore, we found that BAM receptors have a similar complex affinity for CLV1 as CLV1 itself does. CLV1-CLV1



**Figure 7.** Ectopic BAM1, BAM2 and CLV1 can replace CLV2 function *in vivo*. (a) The mean number of carpels per flower from wild-type Ler ( $n = 70$ ), *clv2-3* ( $n = 90$ ), *P<sub>ER</sub>:BAM1-GFP clv2-3* ( $n = 90$ ) and *P<sub>ER</sub>:BAM2-GFP clv2-3* ( $n = 90$ ) plants. (b) Inflorescences from *clv2-3* and *P<sub>35S</sub>:CLV1-GFP clv2-3* plants. Note that the distortion in *clv2-3* gynoecia shape resulting from fifth whorl growth and valve-lessness (Kayes and Clark, 1998) are reduced by the *P<sub>35S</sub>:CLV1-GFP* transgene.

and CLV1-BAM multimers, and the insensitivity of the CLV1-CLV1 interaction to *clv1* mis-sense mutations in both the LRR and kinase domains, are consistent with our genetic analyses: *clv1* strong alleles are all dominant-negative, and act in part by interfering with BAM function in the meristem center (DeYoung *et al.*, 2006; DeYoung and Clark, 2008).

Similarly, the dominant-negative *crn-1* isoform also retained the ability to interact with CLV2. In both of these cases, the dominant-negative isoforms interact with their partner proteins, which presumably poisons the function of the receptor complex *in vivo*.

What then is the relationship between the CLV1-CLV1 and CLV2-CRN complexes? The ability of CLV2 to independently bind CLE indicates that CLV2-CRN does not function analogously to the BSK proteins that relay BRI1 signaling (Tang *et al.*, 2008). One might speculate that each complex is activated by CLE binding, and the two complexes then come together to form a larger signaling assembly. The formation of a CLV1-CRN-CLV2 complex in transient expression, as indicated by two recent studies (Bleckmann *et al.*, 2010; Zhu



*et al.*, 2010), as well as the weaker interaction between CLV1–BAM and CLV2–CRN that we show in this study, might reflect such a higher order complex (Figure 8). Alternatively, each complex may control the activity of a common, or even different, effector protein(s). The ability of BAM overexpression to bypass the *clv2* mutation supports a model in which the two complexes act on common intermediates. Genetic analysis indicates that CLV1 and CLV2 signaling converge to repress the activity of the phosphatases POL and PLL1 (Figure 8; Song *et al.*, 2006). Whether POL–PLL1 repression is the function of the two complexes acting independently, or the function of a larger CLV1–CLV2–CRN complex, is unclear. Identification of the factors directly interacting with the CLV1 and CRN kinase domains is a critical next step in further understanding the mechanism of receptor activation.

Several other receptor systems also involve multiple receptor complexes. TGF $\beta$  signaling, for example, features two receptor complexes, each a homodimer (Heldin *et al.*, 1997; Gilboa *et al.*, 1998). The type-II dimer binds the ligand TGF $\beta$  and then interacts with a type-I dimer to facilitate transphosphorylation and signaling. Wnt signaling involves two different receptors, LRP and Frizzled, that appear to bind to a single ligand simultaneously (Cong *et al.*, 2004; Cadigan and Liu, 2006). Our results indicating separate CLV3 CLE binding activity for CLV1 and CLV2 suggest that CLV signaling is distinct from both of these models. That is, two separable receptor complexes bind independently to the same ligand.

We measured the specificity of the CLV1, BAM1, BAM2 and CLV2 binding activity for different CLE peptides by measuring their ability to compete with radiolabeled CLV3 CLE binding. Interestingly, the CLV1 and CLV2 binding specificities behaved in a similar manner, with different CLEs showing a range of competition from complete to negligible. Furthermore, effective competitors for the CLV1 binding activity were generally effective competitors for the CLV2 binding activity, whereas poor competitors for CLV1

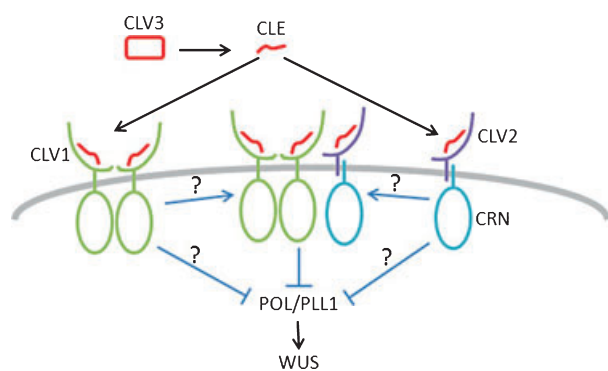
were generally poor competitors for CLV2. This similarity suggests that the two binding activities have similar binding pockets, with a related CLE specificity. The BAM1 and BAM2 receptors, on the other hand, exhibited a very different result in that nearly all of the CLE peptides tested were full competitors for the radiolabeled CLV3 CLE. This suggests that BAM1 and BAM2 have a broader range of CLE binding specificity. This may be explained by the broad expression and function of BAM receptors throughout plant development, where they regulate leaf, stem, vascular, floral organ and gamete developmental patterning (DeYoung *et al.*, 2006; Hord *et al.*, 2006). On the other hand, the more limited role of CLV1 and CLV2 in the meristem explains their more restrictive pattern of CLE binding.

For many of the CLE peptides, their CLV1 and CLV2 binding activity correlated well with their function *in vivo*. For example, the non-functional CLV3S peptide showed no binding, the partial loss-of-function *clv3-1* peptide showed reduced binding and the root meristem regulator CLE40 (Stahl *et al.*, 2009) showed strong competition. The binding activity of several peptides, including CLE1 and CLE25, however, is not consistent with earlier genetic data showing that *CLE1* can rescue the *clv3* mutant (Ni and Clark, 2006) and cause a *wus*-like phenotype when overexpressed (Strabala *et al.*, 2006), whereas *CLE25* can not (Ni and Clark, 2006; Strabala *et al.*, 2006). The CLV1 binding activity of CLE1 and CLE25 peptides, however, is consistent with earlier results when synthetic peptides were used in treating Arabidopsis plants, in which the CLE25 peptide, among others, caused the SAM to be reduced in size, whereas CLE1 did not (Kinoshita *et al.*, 2007). By using nano LC-MS/MS analysis of the apoplastic peptides of transgenic Arabidopsis, a recent study showed that the mature form of several CLE peptides, including CLV3, were glycosylated, and the glycosylation affected receptor binding (Ohya *et al.*, 2009). Post-translational modifications have been observed in several other peptide ligands in plants (Matsubayashi and Sakagami, 2006). The inconsistency between our binding results and genetic data suggests that several of the CLE peptide ligands require post-translational modification for their function *in vivo*.

## EXPERIMENTAL PROCEDURES

### Co-immunoprecipitation assays and protein gel blot analysis

Transient co-expressions of proteins in *N. benthamiana* (Kim *et al.*, 2007) and Arabidopsis transformation (Clough and Bent, 1998) were performed as described. For all membrane protein extractions, leaf discs were harvested 2 DPI by grinding in cold extraction buffer without triton [50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 10 mM NaF, 10 mM NaVO<sub>3</sub>, 2% plant specific protease inhibitor cocktail (Sigma-Aldrich, <http://www.sigmaaldrich.com>), 10  $\mu$ g ml<sup>-1</sup> chymostatin and 2  $\mu$ g ml<sup>-1</sup> aprotinin]. The homogenates were centrifuged at 3300 *g* at 4°C for 10 min. Supernatants were harvested and subjected to ultracentrifugation at 100 000 *g* for



**Figure 8.** A model for CLV signaling. CLV3 is proteolytically processed to a CLE signaling peptide. The CLV3 CLE peptide binds to both a CLV1 homodimer as well as a CLV2–CRN complex. The two activated receptor complexes either interact with each other or signal independently to repress POL–PLL1 activity.

1 h at 4°C. The microsomal membrane pellets were resuspended in extraction buffer supplemented with 1% Triton X-100, and solubilization was conducted at 4°C with gentle agitation for 30 min. The crude membrane homogenates were centrifuged at 100 000 *g* for 1 h at 4°C, and the supernatants (solubilized membrane extracts) were recovered.

For all co-immunoprecipitation assays using GFP antibodies, solubilized membrane extracts were incubated with antibodies (see below) at 4°C for 2 h, and subsequently equilibrated protein A agarose (Invitrogen, <http://www.invitrogen.com>) was added to the protein-antibody mixture for an additional 2 h. For co-IP with FLAG antibodies, solubilized membrane extracts were incubated overnight with anti-FLAG M2-Agarose (Sigma-Aldrich). The beads were washed three times with wash buffer and protein complexes were eluted from beads with 1x SDS loading buffer.

Eluted proteins were resolved on 4–15% gradient SDS-PAGE gel and blotted onto polyvinylidene fluoride (PVDF) membrane by tank immunoblotting. For all western blot analyses the blotted membranes were blocked with 5% non-fat milk prepared in 1x Tris-buffered saline Tween-20 (TBST) buffer at room temperature (22°C) for 1 h. Primary antibody incubations were conducted at 4°C overnight, whereas secondary antibody incubation occurred at room temperature for 1 h. Following primary and secondary antibody incubation, membranes were washed for 10 min with 1x TBST (0.05% Tween) three times. Table S1 lists all antibodies used to detect the fusion proteins. The membranes were developed using enhanced chemiluminescence SuperSignal West Pico Substrates (Pierce, <http://www.piercenet.com>) or Immobilon Western Chemiluminescent HRP Substrates (Millipore, <http://www.millipore.com>).

Arabidopsis protein extracts were collected from inflorescence meristem tissue, focusing on the shoot and flower meristems and young developing flowers. Tissue samples were processed in a manner identical to that for samples from *N. benthamiana* leaves, as described above.

### Quantifying co-IP efficiency

To quantify the efficiency of co-IP, both the bound and unbound fractions in the first immunoprecipitation pull-down were collected. Dilutions series of each of the bound and unbound samples were then run simultaneously on duplicate protein gel blots. Blots were probed separately with antibodies to detect the immunoprecipitated protein (to test the efficiency of IP) and the co-IP protein (to test the efficiency of co-IP). The co-IP efficiency was then estimated by determining the portion of protein in the co-IP bound fraction versus the unbound fraction. In the case of CLV1-BAM2 interactions, for example, this estimate was adjusted to account for the fact that only 50% of the CLV1 protein was originally immunoprecipitated.

### Expression constructs

For CLV1-FLAG protein expression, a CLV1-FLAG fragment was obtained from the previously described  $P_{ER}$ :CLV1-FLAG (DeYoung *et al.*, 2006) by digestion with *Bam*HI and *Sal*I, and was cloned into the binary vector pCHF1 at the same restriction sites, resulting in an  $P_{35S}$ :CLV1-FLAG expression cassette with the pea RBCS-E9 terminator. CLV1 coding sequences were replaced with those from BAM1 and BAM2 using *Spe*I to generate the  $P_{35S}$ :BAM-FLAG constructs.

For CLV1-GFP, BAM1-GFP and BAM2-GFP, an mGFP5 fragment was PCR amplified with engineered *Spe*I and *Sal*I sites, and then cloned into the  $P_{35S}$ :CLV1-FLAG cassette, replacing the FLAG sequences. BAM1-GFP and BAM2-GFP were generated by replacing the CLV coding sequences in  $P_{35S}$ :CLV1-GFP using *Spe*I. The  $P_{35S}$ :BRI1-GFP plasmid was a gift from Jianming Li (Hong *et al.*,

2008). To generate  $P_{ER}$ :BAM1-GFP and  $P_{ER}$ :BAM2-GFP, BAM1-GFP and BAM2-GFP were put downstream of the *ERECTA* promoter (Diévar *et al.*, 2003) in the binary vector pGreen0029.

For CLV2-GFP and CRN-GFP, we used the Gateway (Invitrogen) system. The CLV2 and CRN coding sequences were PCR amplified and cloned into pENTR/D-TOPO to generate pCLV2 and pCRN entry vectors. These entry vectors were used in the LR-Gateway reaction with pMDC83 destination vector (Curtis and Grossniklaus, 2003) to generate  $P_{35S}$ :CLV2-GFP and  $P_{35S}$ :CRN-GFP. The GFP-fused *crn-1* mutant isoform was generated via site-directed mutagenesis of the  $P_{35S}$ :CRN-GFP cassette.  $P_{35S}$ :CRN-FLAG was generated by cloning the *CRN* coding sequence into  $P_{35S}$ :BAM2-FLAG to replace the BAM2 sequence at *Spe*I and *Sma*I.

*clv1* mutant isoforms were generated by PCR amplification of the coding sequences of different *clv* mutants. An engineered *Spe*I and the *Brs*GI site within the CLV1 sequence were used to clone the LRR mutant isoforms into the CLV1 overexpression vectors, in which the 3' end *Spe*I was destroyed. For the kinase domain mutations, the *Aat*II and *Sac*II sites from the CLV1 sequence were used to clone the mutant kinase domain into the  $P_{35S}$ :CLV1-FLAG and  $P_{35S}$ :CLV1-GFP cassettes. Note that the *clv1-10* allele consists of the original *clv1-1* kinase domain allele, plus an LRR domain intragenic enhancer (Diévar and Clark, 2004). For this study, only the LRR domain lesion was introduced for the *clv1-10* LRR isoform.

MYC-tagged CLV2 was generated by cloning the CLV2 genomic sequence, including the CLV2 promoter and coding sequence, into a binary vector pPZP221 to fuse with a 5X MYC tag at the C terminus. RFP-tagged CLV2 was constructed by cloning the CLV2 coding sequence into the vector pSAT6-RFP-N (Tzfira *et al.*, 2005) at *Sal*I and *Bam*HI (filled-in) and the 35S cis element-driven CLV2-RFP expression cassette was subsequently cloned into the binary vector pPZP211.

### Confocal microscopy

Two days after infiltration, the subcellular localizations of the GFP-RFP fused proteins were examined with a Leica TCS-SP5 confocal microscope (Leica, <http://www.leica.com>). The abaxial side of tobacco leaves was viewed at 20× magnification; 488- and 543-nm laser lines were used for excitation of GFP and RFP, respectively.

### Radioiodination of CLV3 and binding assays

The CLV3 peptide contains two histidine residues, so it can be radioiodinated using the Iodogen method (Pierce) (Mayers and Klostergaard, 1983). The tracer was purified by reversed-phase HPLC. We used a 25 cm × 4.6 mm, 5 m Supelcosil LC-318 HPLC column (Supelco, Bellefonte, PA, USA) with a 10–90% gradient of acetonitrile +0.1% trifluoroacetic acid (TFA) over 30 min (flow rate 1.5 ml min<sup>-1</sup>), and collected fractions every 0.5 min. Specific activity of the radiolabeled peptide was determined by measuring the peptide concentration of the tracer with a Micro BCA™ Protein Assay Kit (Pierce).

The receptor proteins were transiently expressed in tobacco leaves, and as described above, and proteins were extracted 2 days after infiltration. The microsomal membrane pellets were washed in protein extraction buffer containing 1% Triton X-100 for 3 h at 4°C with rotation. A significant proportion of protein of each of the CLV pathway receptors remained in the resulting detergent-washed membrane pellet (Figure 4a,b), and this fraction was used in binding assays. The binding assays were carried out as described by Ogawa *et al.* (2008): 500-μg membrane protein preparations or receptor proteins immunoprecipitated with 50 μl protein A agarose beads were resuspended in 200 μl binding buffer [50 mM 2-(*N*-morpholine)-ethanesulphonic acid (MES)-KOH,

100 mM sucrose, pH 5.5) that contained 100 000 cpm of [<sup>125</sup>I]CLV3, with or without 20 μM cold CLV3 peptide for competition. After being incubated on ice for 30 min, the reactions were loaded on top of 1 ml of washing buffer (50 mM MES-KOH, 500 mM sucrose, pH 5.5) and were subjected to a 10-min 28 000 g centrifugation to remove unbound [<sup>125</sup>I]CLV3. If necessary, after removing most of the supernatant, another 10 min 28 000 g spin was carried out to ensure a tight pellet. For binding kinetics, 500 μg CLV1-GFP, 1 mg CLV2-MYC, 100 μg BAM1-GFP or 100 μg BAM2-GFP membrane protein preps were used in binding assays with a series of tracer concentrations from 0.82 nM to 200 nM. Specific binding was calculated by subtracting the background binding when 20 μM cold peptide is present from the total binding. For receptor specificity studies, 1 mg CLV1-GFP, 1 mg CLV2-MYC, 500 μg BAM1-GFP or 500 μg BAM2-GFP membrane protein preparations were used in binding assays, with or without cold competition from different CLE peptides at the concentration of 10 μM. All the peptides used in this assay are listed in Table S2. Radioactivity in the pellets were determined with an automatic gamma counter (Micromedic 4/600 Plus; ICN Biomedicals Inc., now part of MP Biomedicals, <http://www.mpbio.com>). Saturation curves and Scatchard plots were generated using GRAPH PAD PRISM (GraphPad Software Inc., <http://www.graphpad.com>). All the binding assays were performed with at least three replicates.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Tagged receptor constructs complement meristem mutants.

**Figure S2.** Control co-immunoprecipitations with GFP.

**Figure S3.** Receptor interactions require co-expression.

**Figure S4.** Additional receptor interactions.

**Figure S5.** Scatchard plots for CLV3 CLE receptor binding.

**Table S1.** Antibodies used in this study.

**Table S2.** CLE peptides used in this study.

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