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Analysis of interactions among the CLAVATA3 receptors reveals a direct interaction between CLAVATA2 and CORYNE in Arabidopsis

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SUMMARY

In Arabidopsis, CORYNE (CRN), a new member of the receptor kinase family, was recently isolated as a key player involved in the CLAVATA3 (CLV3) signaling pathway, thereby playing an important role in regulating the development of shoot and root apical meristems. However, the precise relationships among CLAVATA1 (CLV1), CLAVATA2 (CLV2), and CRN receptors remain unclear. Here, we demonstrate the subcellular localization of CRN and analyze the interactions among CLV1, CLV2, and CRN using firefly luciferase complementation imaging (LCI) assays in both Arabidopsis mesophyll protoplasts and *Nicotiana benthamiana* leaves. Fluorescence targeting showed that CRN was localized to the plasma membrane. The LCI assays coupled with co-immunoprecipitation assays demonstrated that CLV2 can directly interact with CRN in the absence of CLV3. Additional LCI assays showed that CLV1 did not interact with CLV2, but can interact weakly with CRN. We also found that CLV1 can interact with CLV2–CRN heterodimers, implying that these three proteins may form a complex. Moreover, CRN, rather than CLV1 and CLV2, was able to form homodimers without CLV3 stimulation. Taken together, our results add direct evidence to the newly proposed two-parallel receptor pathways model and therefore provide new insights into the CLV3 signaling pathway.

Keywords: CORYNE, CLAVATA1, CLAVATA2, Arabidopsis mesophyll protoplasts, *Nicotiana benthamiana*, firefly luciferase complementation imaging assay.

INTRODUCTION

In Arabidopsis, plasma membrane-located receptor-like kinases (RLKs) play critical roles in recognizing internal and external stimuli and triggering downstream signaling cascades, and are important for the regulation of plant growth and development. Among the RLKs, the leucine-rich-repeat (LRR) RLKs constitute the largest family in the Arabidopsis genome, which include some well-characterized members such as BRASSINOSTEROID INSENSITIVE 1 (BRI1), CLAVATA1 (CLV1), and FLAGELLIN SENSITIVE2 (FLS2) (Torii, 2000, 2004; Becraft, 2002). One of the best studied examples is CLV1, which directly binds CLAVATA3 (CLV3) and CLAVATA3/ESR (CLE) peptides to restrict stem cell proliferation in the shoot apical meristems (SAMs) (Ogawa *et al.*, 2008).

The stem cells residing at the SAMs of Arabidopsis are the source of all aerial plant organs (Stahl and Simon, 2005). The CLV3 signaling pathway negatively regulates stem cells through repression of the homeodomain transcription factor WUSCHEL (WUS), which promotes stem cell differentiation into various organs and tissues in adjacent cells (Mayer *et al.*, 1998; Brand *et al.*, 2000; Schoof *et al.*, 2000). Considerable progress has been made in recent decades in elucidating the molecular basis of the CLV3 signaling pathway. *CLV3* encodes a 96-amino-acid protein with a putative secretory signal peptide sequence in its N-terminal region that acts as an extracelluar peptide ligand (Fletcher *et al.*, 1999). *CLV1* encodes a predicted 105-kDa RLK that consists of 21 extracellular LRRs, a single transmembrane domain, and an intracellular Ser/Thr kinase domain, whereas *CLV2* encodes a receptor-like protein (RLP) similar to *CLV1* except that it only contains a very short cytoplasmic

tail. Therefore, the role of CLV2, which lacks a kinase domain, may be the stabilization of CLV1 (Clark *et al.*, 1997; Jeong *et al.*, 1999). It was believed that CLV3, as a secreted ligand, binds to the putative CLV1/CLV2 heterodimeric complex to regulate the fate of stem cells in the SAM (Fletcher *et al.*, 1999; Trotochaud *et al.*, 1999). In addition, the biochemical interactions between the LRR domain of CLV1 and the 12-amino-acid MCLV3 were recently identified by photoaffinity labeling using the membrane fraction of tobacco BY-2 cells as a system (Ogawa *et al.*, 2008). However, a recent genetic analysis isolated a novel membrane-associated receptor kinase, *CORYNE* (*CRN*), from a screen for suppressors of a weak meristem-arrest phenotype induced by *CLV3* overexpression (Müller *et al.*, 2008). It was found that *CRN* plays a key role in CLV3 signaling. The genetic evidence showed that the phenotype of the *crn–clv2* double mutant was similar to that of each single mutant. By contrast, the phenotype of the *crn–clv1* double mutant was more severe than that of either individual mutant. These genetic interaction experiments indicated that *CRN* and *CLV2* may function together, whereas *CRN* and *CLV1* might act in parallel (Müller *et al.*, 2008). Other molecular genetic work demonstrated that *SOL2* encodes the same RLK as *CRN*. Both *sol2* and *clv2* were similarly resistant to the 26 synthetic CLE peptides, indicating that *CRN/SOL2* possibly acts with *CLV2* in regulating the development of root meristems (Miwa *et al.*, 2008). Therefore, it was proposed that *CRN* probably acts with *CLV2* to mediate the development of shoot and root meristems, suggesting that the CLAVATA signaling pathway consists of two parallel receptor complexes involved in transmitting CLV3 signaling: one uses CLV1 homodimers and the other uses CLV2–CRN heterodimers (Müller *et al.*, 2008). However, such a presumption was controversial because no cell-based or biochemical evidence existed to verify these potential interactions.

Several powerful techniques, such as fluorescence resonance energy transfer (FRET) and bimolecular fluorescence complementation (BiFC), are well developed and have been applied successfully to the identification of protein–protein interactions in living plant cells (FRET: Shah *et al.*, 2001; Immink *et al.*, 2002; BiFC: Hu *et al.*, 2002; Walter *et al.*, 2004). The FRET technique is capable of detecting interaction intensity, but, because of autofluorescence, is of limited usefulness in a large-scale analysis with a low signal-to-noise ratio, especially for plant cells. In addition, FRET demands that the two fusion fluorescent proteins be expressed at nearly equivalent levels, which makes it hard to control (Bhat *et al.*, 2006; Dixit *et al.*, 2006). The BiFC technique is mainly suitable for examining stable interactions, but it is not able to detect dynamic interactions and quantify the intensity of interactions (Hu *et al.*, 2002). Recently, a new firefly luciferase complementation imaging (LCI) assay was developed for protein–protein interactions in living animals (Luker *et al.*, 2004). This method is based on the reconstruction of the firefly luciferase (LUC) enzyme upon the interaction of two proteins of interest fused to N- or C-terminal fragments of the whole LUC enzyme. Compared with previous techniques for protein interactions, such as FRET or BiFC, the LCI assay is the preferred method, especially for investigating highly dynamic protein–protein interactions, because of its high signal-to-noise ratio and quantum yield. It has been successfully applied to detect the interactions of nine protein pairs involved in the plant innate immunity pathway, and interactions between the nuclear histones 2A and 2B and between membrane proteins syntaxin (SYP) 51 in plants and SYP61 in Arabidopsis (Fujikawa and Kato, 2007; Chen *et al.*, 2008).

In this study, we performed fluorescence targeting assays to demonstrate the localization of CRN and utilized LCI assays to analyze the interactions among CLV1, CLV2, and CRN in both Arabidopsis protoplasts and the *Nicotiana benthamiana* leaves. We found that CLV2 can directly interact with CRN in the absence of the CLV3 peptide, and CLV1 can weakly interact with CRN, but it cannot interact with CLV2. Interestingly, CLV1, CLV2, and CRN may form a complex when co-expressed. In addition, CRN (rather than CLV1 and CLV2) was able to form homodimers, providing additional insight into the CLV3 signaling pathway.

RESULTS

Subcellular localization of CRN proteins in living Arabidopsis protoplasts

To determine the subcelluar localization of the new RLK, CRN, we performed fluorescence targeting experiments in Arabidopsis mesophyll protoplasts by fusing the enhanced yellow fluorescent protein (EYFP) to the C-terminus of CRN and transiently expressing the fusion proteins under the control of the constitutive 35S promoter. After overnight transfection, chlorophyll autofluorescence, YFP fluorescence, light field vision, and the image overlaying the chlorophyll autofluorescence, light field, and YFP fluorescence of the transfected protoplasts were examined simultaneously with a confocal microscope. We found that YFP fluorescence, representing the distribution of CRN proteins, was enriched primarily in the plasma membrane in Arabidopsis protoplasts. However, the distribution of YFP fluorescence at the membrane was not uniform, although the fusion protein was under the control of the constitutive 35S promoter (Figure 1). On the contrary, the control YFP construct, which did not carry CRN, was found in both the nucleus and the cytoplasm crossing the protoplasts, and the untransfected protoplasts did not show YFP fluorescence with only chlorophyll autofluorescence (data not shown).

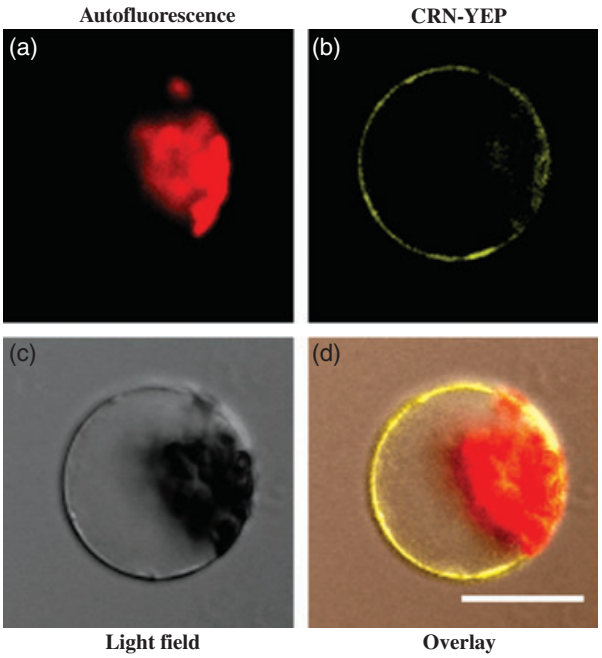

Figure 1. Confocal images of CORYNE (CRN)–YFP transfected Arabidopsis protoplasts.
(a) Chlorophyll autofluorescence.
(b) Yellow fluorescent protein fluorescence.
(c) Light field vision.
(d) Image overlay of (a–c).
Scale bar indicates 20 μm.

CLV2 can interact with CRN directly in Arabidopsis protoplasts

Recent studies have shown that *CRN* shares many functions with *CLV2*, and it has been suspected, based on their genetic analysis, that CRN and CLV2 may act together to transmit the CLV3 signal (Miwa *et al.*, 2008; Müller *et al.*, 2008). To test whether such an interaction can be detected with the LCI assay, CLV2–NLuc and CRN–CLuc were constructed and co-expressed in Arabidopsis protoplasts. Simultaneously, either CLuc or Pto–Cluc was selected as a negative control and co-expressed with CLV2–NLuc in protoplasts, in which Pto is a membrane-associated protein, similar to CRN, involved in the plant innate immunity signaling pathway (Tang *et al.*, 1996). After overnight co-transfection, the co-expression of CRN–CLuc and CLV2–NLuc resulted in strong LUC activity in protoplasts. In contrast, CLV2–NLuc co-expressed with Pto–CLuc, or CLV2–NLuc co-expressed with the CLuc vector, as two negative controls, showed only background levels of LUC activity. The average LUC activity of CLV2–CRN was from 7 to 10 times higher than the negative controls using CLV2–Pto or the CLV2 vector (Figure 2a). We further examined the expression of NLuc and CLuc fusion proteins by western blot and found that CLV2–NLuc proteins were expressed at a similar level with a size of approximately 125 kDa in all three samples, whereas the initial negative control CLuc protein was not observed on the Immun-Blot polyvinylidine fluoride (PVDF) membrane because it is so small (only 17 kDa in size). The second control, the Pto–CLuc (53 kDa) protein, was expressed at a higher level than CRN–CLuc protein (62 kDa) but had basal LUC activity (Figure 2a). Together, these results clearly show that the strong LUC activity detected from co-expressed CLV2–NLuc and CRN–CLuc was not caused by higher levels of CRN–CLuc and CLV2–NLuc proteins expressed in the protoplasts, but rather resulted from a specific interaction between CLV2 and CRN. The CLV2–CRN interaction was also verified by a reciprocal combination of CLV2–CLuc and CRN–NLuc in both Arabidopsis protoplasts and *N. benthamiana* leaves, which also exhibited strong LUC activity (see Figures 4a and 6–8).

Furthermore, the LCI assay was used to investigate whether exogenous application of the CLV3 peptide can enhance the intensity of CLV2–CRN interactions. First, we synthesized a 12-amino-acid CLV3 peptide according to Kondo *et al.* (2006) and tested its function using classic root assays. The application of 10 μM CLV3 peptide severely inhibited root growth in wild-type Col or L*er* seedlings, whereas the *clv2* mutant showed resistance to the peptide treatment and its root length was barely affected (Figure S1 in Supporting Information). These root assay results apparently show that the synthetic CLV3 peptide was functional. Then, two duplications of CLV2–NLuc/CRN–CLuc were co-expressed in protoplasts along with a negative control, CLV2–NLuc/CLuc. After overnight incubation, LUC activity was detected prior to CLV3 peptide stimulation, showing that both protoplasts co-expressed with CLV2–CRN, resulting in significant and nearly identical LUC activity. In contrast, the negative control CLV2–NLuc/CLuc showed only background LUC activity. Consequently, the middle duplicate containing co-expressed CLV2–NLuc/CRN–CLuc in protoplasts was treated with CLV3 peptide at a final functional concentration reaching 10 μM. After incubation in the dark for 15–60 min, the LUC activity was monitored under the same conditions. Interestingly, we observed little difference after peptide treatment compared with the untreated protoplasts co-expressed with CLV2–CRN, based on LUC activity. Western blots also showed that all fusion proteins were expressed at correct molecular sizes and similar levels (Figure 2b).

To further verify the physical interaction between CLV2 and CRN, we performed co-immunoprecipitation assays in Arabidopsis protoplasts. 35S:CLV2–HA and 35S:CRN–FLAG were constructed and co-transfected into Arabidopsis protoplasts. We chose BAK1–FLAG as a negative control, because its membrane localization was similar to that of CRN (Li *et al.*, 2002; Nam and Li, 2002). Western blot results

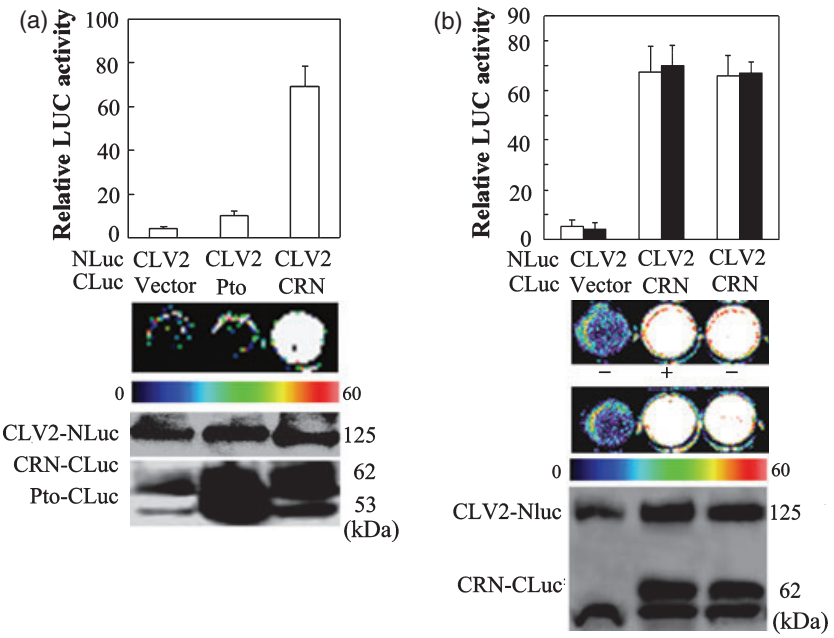

Figure 2. CLAVATA2 (CLV2) can interact with CORYNE (CRN) in Arabidopsis protoplasts.
Quantification of the average firefly luciferase (LUC) activity (top panels). Microtiter plates containing protoplasts expressing the indicated constructs (middle panels). The pseudocolor bars indicate the range of luminescence intensity in each image. Western blots of co-transfected proteins isolated from protoplasts (bottom panels).
(a) CLV2–NLuc co-expressed with CRN–CLuc resulted in strong LUC activity in the protoplasts. In contrast, CLV2–NLuc co-expressed with Pto–CLuc, or CLV2–NLuc co-expressed with CLuc vector both showed only background or negligible levels of LUC activity. The LUC activity of CLV2–CRN was about seven to eight times higher than the average intensities of the negative control CLV2–Pto or CLV2 vector.
(b) CLV3 peptide treatment did not apparently affect the intensity of CLV2–CRN interaction.
In the first panel, white and black histograms showed the quantification of LUC activity before and after peptide treatment, respectively. The '+' indicates applied peptide treatment to the indicated co-transfected protoplasts, in contrast, the '–' represents no peptide treatment. Western blots showed that all fusion proteins were expressed at their correct weights. All of the above data represent three independent replicates and repeated three times with similar results, the pooled data are shown as means ± standard errors. *$P < 0.01$

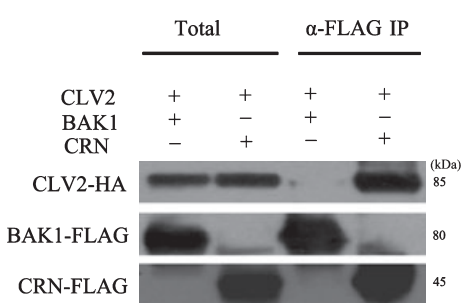

Figure 3. The interaction between CLAVATA2 (CLV2) and CORYNE (CRN) confirmed by co-immunoprecipitation assays.
The CLV2–HA construct was co-expressed with CRN–FLAG or BAK1–FLAG in Arabidopsis protoplasts, and total protein was extracted after transfection for 12–16 h. Total protein was then incubated with an agarose-conjugated anti-FLAG monoclonal antibody. The presence of CLV2–HA, BAK1–FLAG, and CRN–FLAG was detected by western blot with anti-HA or anti-FLAG antibodies. The CLV2–HA was present in the CRN–FLAG immune complex. In contrast, CLV2–HA did not appear in the BAK1-FLAG immune complex. See Figure S2 for a full version of this figure.

demonstrated that all fusion proteins were expressed at their expected molecular sizes (Figure 3). CLV2–HA (85 kDa) was present in the CRN–FLAG (45 kDa) immune complex. In contrast, CLV2–HA did not appear in the BAK1–FLAG immune complex.

CLV1 does not interact with CLV2 in Arabidopsis protoplasts

It has long been suspected that the CLV1–CLV2 receptor complex perceives CLV3 signaling (Fletcher *et al.*, 1999; Trotochaud *et al.*, 1999; Rojo *et al.*, 2002; Fiers *et al.*, 2005). However, it remains unclear whether this receptor complex exists. Here, we constructed CLV1–NLuc and CLV1–CLuc vectors for LCI assays. CLV1–CLuc and CLV2–NLuc were co-expressed in Arabidopsis protoplasts to determine whether CLV1 could interact with CLV2. The CLV1–CLuc/NLuc vector was simultaneously used as a negative control and CLV2–CLuc plus CRN–NLuc was used as a positive control. As shown in Figure 4(a), we found that co-expression of CLV2–CLuc and CRN–NLuc resulted in strong LUC complementation, similar to the CLV2–NLuc/CRN–CLuc, whereas co-expression of CLV1–CLuc and CLV2–NLuc only led to negligible LUC activity, similar to the negative control CLV1–CLuc/NLuc co-expression. After incubation with 10 μM CLV3 peptide, the co-expression of

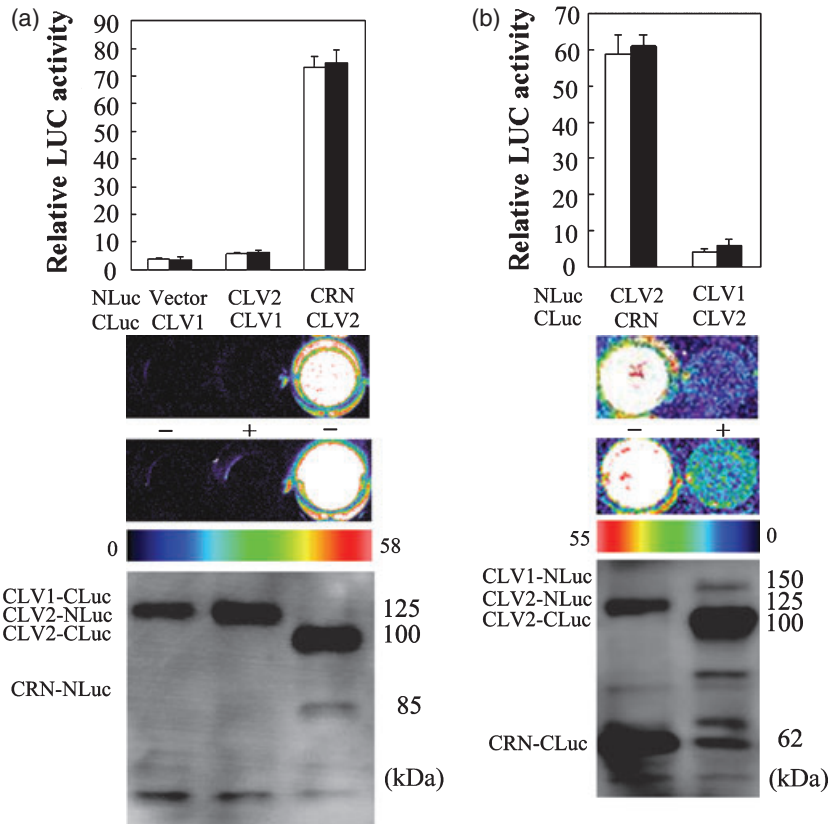

Figure 4. CLAVATA1 (CLV1) does not interact with CLV2 in Arabidopsis protoplasts.
(a) Co-expression of CLV1–CLuc and CLV2–NLuc or negative control, CLV1–CLuc and NLuc vector in protoplasts both showed background levels of firefly luciferase (LUC) activity. In contrast, the co-expression of CLV2–CLuc and CRN–NLuc resulted in strong LUC complementation.
(b) CLV1–NLuc co-expressed with CLV2–CLuc also failed to show LUC complementation in protoplasts. In contrast, the positive control CLV2–NLuc/CRN–CLuc, showed significant LUC activity.
In the top panels, white and black histograms represent the quantification of LUC activity before and after peptide treatment, respectively. The '+' indicates peptide treatment, whereas the '–' indicates no peptide treatment. Western blots showed that all fusion proteins were expressed at their indicated weights. These data are representative of three independent experiments.

CLV1–CLuc/CLV2–NLuc still did not show detectable LUC activity. The co-expressed proteins were extracted from microfilters on a 1:1 basis, and western blot was performed. Coincidently, the molecular weights of the CLV1–CLuc and CLV2–NLuc proteins were nearly identical (125 kDa), making it difficult to distinguish whether both proteins were normally expressed together, although the protein band for CLV1–CLV2 was slightly thicker than the negative control, CLV1–CLuc/NLuc.

To overcome this problem, we constructed a new pair of vectors, CLV1–NLuc (150 kDa) and CLV2–CLuc (105 kDa), and co-expressed them in Arabidopsis protoplasts. In Figure 4(b), we show that little LUC activity was observed from the co-expression of CLV1–NLuc and CLV2–CLuc, which was consistent with the co-expression of CLV1–CLuc/CLV2–NLuc. In contrast, the positive control CLV2–NLuc/CRN–CLuc showed significant LUC activity. To further investigate whether exogenous peptide treatment could affect CLV1–CLV2 interaction, the co-expressed CLV1–CLV2 protoplasts were incubated with 10 μM CLV3 peptide. Then, LUC activity was examined at 15, 30, 45, and 60 min time points. The protoplasts co-expressed with CLV1–CLV2 still did not show any detectable LUC activity. Western blots further showed that all fusion proteins were expressed at their correct sizes (Figure 4b).

CLV1 can interact weakly with CRN in Arabidopsis protoplasts

We were also curious as to whether there was an interaction between CLV1 and CRN, because we cannot exclude a possible interaction between these two RLKs. Therefore, LCI assays were again used. CLV1–NLuc (150 kDa) and CRN–CLuc (62 kDa) were co-expressed in Arabidopsis protoplasts, using CLV1–NLuc/CLuc as a negative control (data not shown) and CLV2–NLuc plus CRN–CLuc as a positive control. As indicated in Figure 5(a), it was obvious that LUC reconstitution activity resulted from co-expression of the positive control, CLV2–NLuc and CRN–CLuc. In contrast, in the co-expressing CLV1–NLuc/CRN–CLuc or CLV1–NLuc/CLuc protoplasts, almost no discernible LUC activity could be seen. After applying peptide treatment to the co-expressing CLV1–CRN protoplasts (10 μM), there was still no detectable LUC activity. Western blots further showed that all co-transfected fusion proteins were expressed at their correct molecular weights.

Moreover, we also constructed CRN–NLuc and co-expressed it with CLV1–CLuc to confirm whether luciferase fragment interchange would affect CLV1–CRN interaction. Surprisingly, co-expression of CLV1–CLuc/CRN–NLuc

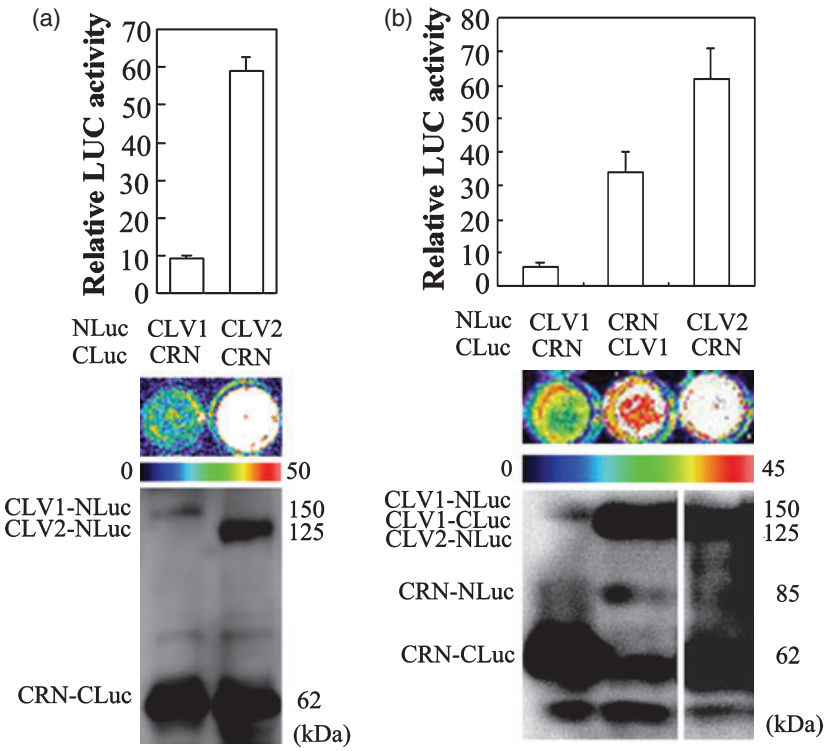

Figure 5. CLAVATA1 (CLV1) can interact weakly with CORYNE (CRN) in Arabidopsis protoplasts.
(a) Co-expression of CLV1–NLuc and CRN–CLuc resulted in little firefly luciferase (LUC) activity. In contrast, CLV2–CRN co-expression resulted in significant LUC complementation.
(b) Co-expression of CLV1–CLuc and CRN–NLuc led to partial LUC activity, almost equivalent to one-third of the LUC activity of the positive control, CLV2–CRN. In contrast, CLV1–NLuc/CRN–CLuc co-expression still showed barely detectable LUC activity. Western blots further showed that all fusion proteins were expressed at their correct weights.
These data are representative of three independent experiments with three replicates.

resulted in weak but detectable LUC activity, representing about one-third the activity of the positive control, CLV2–CRN. In contrast, CLV1–NLuc/CRN–CLuc still showed only background levels of LUC activity (Figure 5b). Further Western blots showed that all fusion proteins were expressed at their correct molecular weights.

CRN rather than CLV1 or CLV2 was able to form homodimers in Arabidopsis protoplasts

To investigate whether CLV1, CLV2, and CRN can form homodimers, three pairs of vectors, CLV1–NLuc/CLV1–CLuc, CLV2–NLuc/CLV2–CLuc, and CRN–NLuc/CRN–CLuc were co-expressed separately in Arabidopsis protoplasts. Moreover, the negative control, CLuc, was also co-expressed with CLV2–NLuc (data not shown), whereas the CLV2–NLuc/CRN–CLuc and CLV2–CLuc/CRN–NLuc pairs were used as positive controls. After overnight co-transfection, it was obvious that co-expression of both the positive controls, CLV2–NLuc/CRN–CLuc and CLV2–CLuc/CRN–NLuc, resulted in strong LUC activity. In contrast, both protoplasts co-expressing CLV1–NLuc/CLV1–CLuc and CLV2–NLuc/CLV2–CLuc had negligible LUC activity (Figure 6a). Interestingly, CRN–NLuc/CRN–CLuc co-expression also exhibited obvious LUC activity similar to positive controls (Figure 6b). To verify whether peptide treatment could affect these interactions, the co-expressing protoplasts, CLV1–CLV1 and CLV2–CLV2, were incubated with 10 μM CLV3 peptide for 15–60 min in the dark. The results demonstrated that the LUC activity of both co-expressed protoplasts was still not detectable (data not shown). Western blots further confirmed that all proteins were expressed at their correct molecular weights.

CLV1, CLV2, and CRN can form complexes in Arabidopsis protoplasts

Although we demonstrated that CLV1 did not interact with CLV2 but can weakly interact with CRN, it remains to be validated whether CLV1, CLV2, and CRN can bind together to form complexes when these three receptors are co-expressed. Therefore, CLV1–CLuc, CLV2–NLuc, and CRN–NLuc were co-transfected into Arabidopsis protoplasts. Presumably, CLV2–CRN interaction would not result in LUC activity because both proteins were both fused with the same N-terminal luciferase fragment. In addition, we used CLV2–CLuc/CRN–NLuc as a positive control, and CLV2–NLuc/CLV1–CLuc as a negative control. As shown in Figure 7, the co-expression of CLV2–NLuc/CLV1–CLuc only resulted in background levels of LUC activity. In contrast, CLV1–CLuc/CLV2–NLuc/CRN-NLuc co-expression resulted in strong LUC activity, similar to that of the positive control CLV2–CRN. Furthermore, the co-expression of both CLV1–CLuc/CLV2–NLuc/CRN–FLAG and CLV1–CLuc/CLV2–HA/CRN–NLuc showed similar and detectable LUC activity (Figure S3). Western blot results further confirmed that all the fusion proteins were expressed at their correct molecular weights.

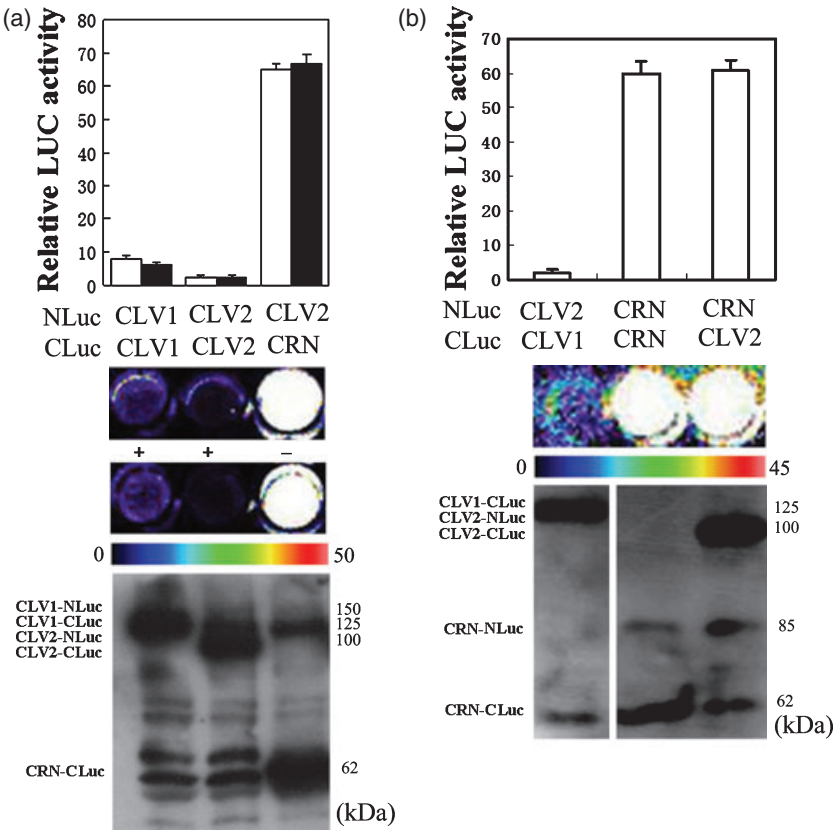

Figure 6. CORYNE (CRN) rather than CLAVATA1 (CLV1) or CLV2 was able to form homodimers in Arabidopsis protoplasts.
(a) Co-expression of CLV1–NLuc and CLV1–CLuc resulted in little firefly luciferase (LUC) activity. Similarly, co-expression of CLV2–NLuc with CLV2–CLuc in protoplasts resulted in only background levels of LUC activity. In contrast, in the positive control, CLV2–CRN co-expression showed significant LUC complementation. The reconstituted average LUC activity of CLV2–CRN was approximately six to eight times greater than the CLV1–CLV1 or CLV2–CLV2 combination. After application of peptide treatment to the CLV1–CLV1 and CLV2–CLV2 co-expressed protoplasts and incubation for 15–60 min in the dark, no obvious differences in LUC activity were found. The '+' indicates peptide treatment, whereas the '–' indicates no peptide treatment. Western blots showed that all fusion proteins were expressed and the amount of protein loaded in each lane was roughly equivalent.
(b) Co-expression of CLV1–CLuc/CLV2–NLuc showed only background levels of LUC activity. In contrast, both CRN–NLuc/CRN–CLuc and the positive control, CRN–NLuc/CLV2–CLuc, led to strong LUC activity with similar intensity. Western blots also showed that all fusion proteins were expressed at their correct molecular sizes. These data represent three independent experiments with three replicates every time.

Interactions among CLV1, CLV2, and CRN by *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves

To confirm the interaction results in Arabidopsis protoplast system, we constructed serial vectors, including pCAMBIA 1300–CLV1–NLuc, pCAMBIA1300–CLV1–CLuc, pCAMBIA1300–CLV2–NLuc, pCAMBIA1300–CLV2–CLuc, pCAMBIA1300–CRN–NLuc, and pCAMBIA1300–CRN–CLuc for LCI assays in *N. benthamiana* leaves. After co-infiltration for 40–48 h, we found that co-infiltration of *Agrobacterium tumefaciens* containing both CLV2–CLuc/CRN–NLuc and CRN–NLuc/CRN–CLuc resulted in strong LUC complementation, confirming that the CLV2–CRN interactions by split-LUC assay can be reproduced in the intact tobacco epidermal cells that retain the cell wall. Similarly, the co-infiltration of CLV1–NLuc/CRN–CLuc or CLV1–CLuc/CRN–NLuc resulted in weaker but detectable LUC activity, compared with that of CLV2–CRN or CRN–CRN co-infiltration. However, the co-infiltration of CLV1–NLuc/CLV2–CLuc or CLV1–CLuc/CLV2–NLuc had only background levels of LUC activity. In addition, the co-infiltration of both CLV1–NLuc/CLV2–CLuc/CRN–CLuc and CLV1–CLuc/CLV2–NLuc/CRN–NLuc showed apparent LUC activity (Figure 8). In contrast, the co-infiltration of the negative control CLV2–NLuc/CLuc had little LUC activity (data not shown).

DISCUSSION

The CLV3 signaling pathway is considered to play a central role in regulating the fate of stem cells in the SAM of Arabidopsis (Mayer *et al.*, 1998; Brand *et al.*, 2000; Schoof *et al.*, 2000). Previous studies proposed that CLV1–CLV2 heterodimers perceive and transmit a secreted extracellular CLV3 peptide signal (Fletcher *et al.*, 1999; Trotochaud *et al.*, 1999; Rojo *et al.*, 2002; Fiers *et al.*, 2005). Recently, a new receptor kinase, *CRN*, was isolated by genetic analysis and hypothesized to act with *CLV2* in regulating the development of SAMs independent of *CLV1* (Müller *et al.*, 2008). However, this new hypothesis lacks direct cell biological or biochemical evidence. The analysis of interactions among these three important membrane proteins will help us to acquire a more detailed understanding of the CLV3 signaling pathway.

In our study, fluorescence localization assays clearly demonstrated that CRN was localized to the plasma membrane, in agreement with its structural predictions, which indicates that it may interact with CLV2 at the plasma membrane through their single overlapping transmembrane domain. Moreover, using the newly developed LCI assay, a direct physical interaction between CLV2 and CRN was observed in Arabidopsis protoplasts. To avoid an artifact due to the over-expression of CLV2 and CRN proteins, we used serial concentrations of DNA to transfect

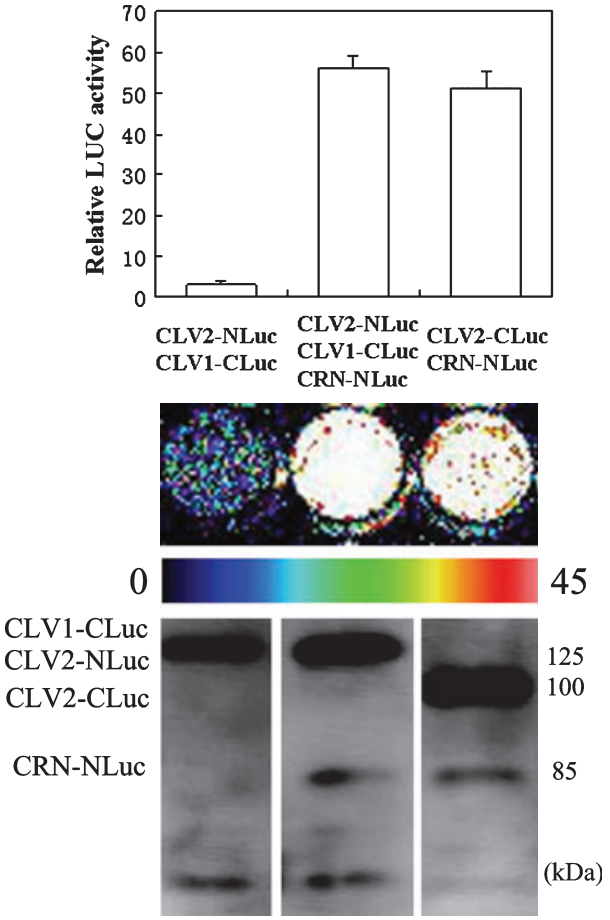

Figure 7. CLAVATA1 (CLV1), CLV2 and CORYNE (CRN) can form complexes in Arabidopsis protoplasts.
The co-expression of CLV2–NLuc and CLV1–CLuc, as a negative control, resulted in background levels of firefly luciferase (LUC) activity. In contrast, CLV1–CLuc/CLV2–NLuc/CRN–NLuc co-expression led to strong LUC activity that was somewhat greater than that of the positive control, CLV2–CRN. Western blots further showed that all fusion proteins were expressed at the indicated molecular sizes.

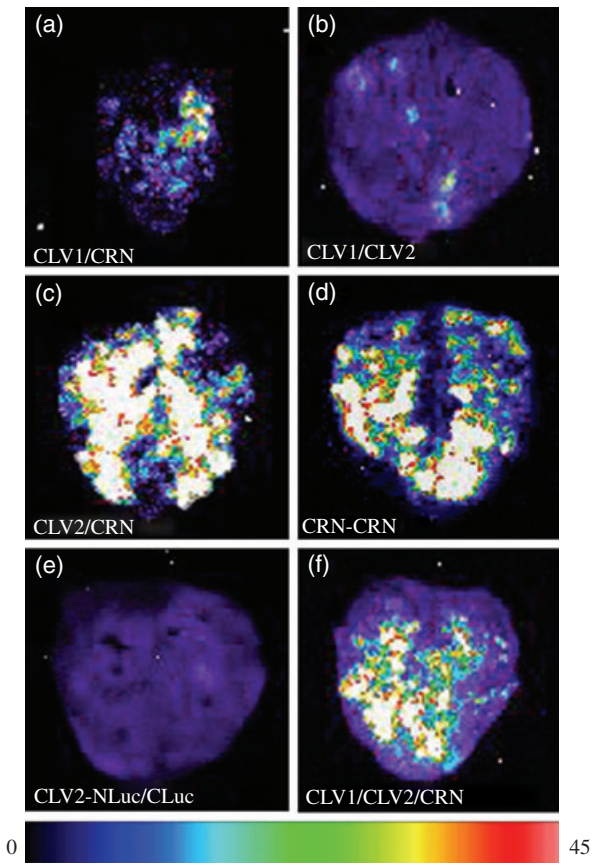

Figure 8. The interactions among CLAVATA1 (CLV1), CLV2, and CORYNE (CRN) analyzed by *Agrobacterium tumefaciens*-mediated LCI assays in *Nicotiana benthamiana* leaves.
(a–f) The firefly luciferase (LUC) images of *N. benthamiana* leaves co-infiltrated with the agrobacterial strains containing the indicated fusion proteins, respectively. (a) The co-infiltration of both CLV1–NLuc/CRN–CLuc and CLV1–CLuc/CRN-NLuc resulted in weak but detectable LUC activity. (b) The co-infiltration of CLV1–NLuc/CLV2–CLuc or CLV1–CLuc/CLV2–NLuc only showed background levels of LUC activity. (c) The co-infiltration of CLV2–CLuc/CRN–NLuc resulted in strong LUC activity. (d) The co-infiltration of CRN–NLuc/CRN–CLuc also resulted in apparent LUC activity. (e) The co-infiltration of CLV2–NLuc/CLuc, as a negative control, showed no detectable LUC activity. (f) The co-infiltration of CLV1–NLuc/CLV2–CLuc/CRN–CLuc or CLV1–CLuc/CLV2–NLuc/CRN–NLuc resulted in apparent LUC activity.
Data were collected 40–48 h after co-infiltration. The data are representative of three independent experiments with two replicates every time.

the protoplasts. Even at very low expression levels, CLV2–CRN co-expression exhibited obvious LUC activity. Further co-immunoprecipitation assays also confirmed the constitutive interaction between CLV2 and CRN. Interestingly, LCI assay results showed that exogenous CLV3 peptide treatment apparently did not affect the intensity of the interaction between CLV2 and CRN. Although different peptide concentrations from 10 to 50 μM were applied, there was still little difference. We cannot yet explain the precise mechanism for this phenomenon. The most probable explanation is that only stem cells localized in the SAM and root apical meristem (RAM) can respond to synthetic CLV3 peptide. The protoplast system would not respond to the exogenous ClV3 peptide as sensitively as that of SAM or RAM. Alternatively, our LCI assay is designed to verify protein–protein physical interaction based on transient overexpression, and it is not sensitive enough to detect the effects of ligand stimulation. Another possibility is that the CLV2–CRN association may be ligand independent.

Further LCI results revealed that CLV1 did not interact with CLV2 under both unstimulated and CLV3 peptide-treated conditions. Surprisingly, we found that co-expression of CLV1–NLuc and CRN–CLuc resulted in only background levels of LUC activity, whereas the co-expression of CLV1–CLuc and CRN–NLuc led to detectable LUC activity. These results were probably due to the low expression level of CLV1–NLuc, which was not high enough to generate a detectable LUC signal in our Arabidopsis protoplast system.

Consistent with our results, it was reported that CLV1 was difficult to express at an efficient level for biochemical assays in plants, because CLV1 is a negative regulator of cell division (Ogawa *et al.*, 2008). Compared to CLV1–NLuc, CLV1–CLuc had a higher expression level and it can interact with CRN–NLuc in LCI assays in Arabidopsis protoplasts. It is worth noting that the intensity of the CLV1–CRN interaction is much weaker than that of the CLV2–CRN interaction. Moreover, when CLV1–CLuc, CLV2–NLuc, and CRN–NLuc were co-expressed in protoplasts, strong LUC activity was observed, indicating that CLV1 may interact with the CLV2–CRN heterodimers. Further experiments which had one construct free of NLuc or CLuc fusion showed that the co-expression of both CLV1–CLuc/CLV2–HA/CRN–NLuc and CLV1–CLuc/CLV2–NLuc/CRN–FLAG resulted in similar and detectable LUC activity, confirming that CLV1 and the CLV2–CRN heterodimer may form a complex when they are co-expressed. In addition, our LCI results revealed that CRN was inclined to form homodimers in the absence of the CLV3 peptide, but CLV1 and CLV2 were not able to form homodimers. CLV2 did not homodimerize, probably because it preferentially formed a heterodimer with CRN. Although the co-expression of CLV1–NLuc and CLV1–CLuc did not result in detectable LUC activity, we cannot conclude that CLV1 did not form homodimers due to the low expression level of CLV1–NLuc in Arabidopsis protoplasts.

Agrobacterium tumefaciens-mediated transient expression in intact *N. benthamiana* leaves provides a rapid and useful method for studying proteins of interest (Fischer *et al.*, 1999; Goodin *et al.*, 2008). Recently, it was also reported to be adopted in LCI assays (Chen *et al.*, 2008). In the present investigation, the interactions among CLV1, CLV2, and CRN were analyzed by *A. tumefaciens*-mediated LCI assays in *N. benthamiana* leaves. The co-infiltration of both CLV2–CRN and CRN–CRN resulted in apparent LUC activity, confirming the physical binding between CLV2–CRN and CRN homodimers. Co-infiltration of CLV1–CRN showed weak but detectable LUC activity, while the co-infiltration of CLV1–CLV2 showed background levels of LUC activity in *N. benthamiana* leaves, in accordance with our results from Arabidopsis protoplasts. Moreover, the CLV1–CLV2–CRN co-infiltration resulted in strong LUC activity, similar to that of co-expression of these three proteins in protoplasts. Taken together, these interaction results were consistent with our previous results from the Arabidopsis protoplast system. Nevertheless, these results were still based on transient high-level expression in a heterogeneous plant system. The relationships among CLV1, CLV2, and CRN may be more complex than that indicated in the two-parallel-receptor pathway model (Müller *et al.*, 2008). Future study should investigate the relationships among CLV1, CLV2, and CRN in the SAM which would precisely reflect their *in vivo* physiological conditions.

In summary, our fluorescence localization and interaction results have substantially enriched our knowledge of the CLV3 signaling pathway and revealed previously unclear aspects of the relationships among CLV1, CLV2, and CRN. We showed that CLV2 can physically interact with CRN in the absence of CLV3, and CLV1 can weakly interact with CRN but cannot interact with CLV2. Interestingly, CLV1 and the CLV2–CRN heterodimer may form a complex when they are co-expressed. In addition, CRN, rather than CLV1 and CLV2, was able to form a homodimer, providing additional insight into CLV3 signaling.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana ecotype Columbia plants were grown in a controlled growth room at 24°C/20°C day/night with 12 h daylight and 70% humidity. Five- or 6-week-old Arabidopsis plants were used for protoplast isolation. Seven- to 10-week-old *N. benthamiana* plants were used for *A. tumefaciens*-mediated transient expression.

Vector construction

The two sets of plant gene expression plasmids, 35S:NLuc and 35S:CLuc, for LCI assays have been previously described, and include a pUC19-based plasmid designed for transfection of protoplasts and pCAMBIA1300-based plasmid for *A. tumefaciens*-mediated transient expression (Chen *et al.*, 2008). Derivative NLuc and CLuc fusion constructs were made by polymerase chain reaction (PCR) amplification of the full-length CLV1, CLV2, and CRN genes, digestion with *Kpn*I and *Sal*I, and insertion into the 35S:NLuc or 35S:CLuc plasmids. To construct the transient expression vectors for the co-immunoprecipitation assays, full-length CLV2 cDNA was inserted into the 35S–HA–pUC vector at *Kpn*I and *Sal*I sites, and full-length CRN cDNA was inserted into the pUC19–35S–FLAG–RBS vector at *Kpn*I and *Csp*451 (Promega, http://www.promega.com/) sites. For the YFP constructs, the open-reading frame of CRN was amplified by PCR, and the PCR product was digested with *Bam*HI and *Sac*I and cloned into 35S:YFP–pCAMBIA1205 (Quan *et al.*, 2007). All constructs were verified by sequencing. Primers are listed in Table S1.

Protoplast preparation and transfection

Protoplasts were isolated from 5- or 6-week-old ecotype Columbia plants according to Sheen (http://genetics.mgh.harvard.edu/sheenweb/faq.html). About 2×10^5 protoplasts were co-transfected with the indicated constructs and incubated in a 24-well microtiter plate under weak light for 10–16 h before LUC activity was measured in the LCI assays. In co-immunoprecipitation assays, about 4×10^6 protoplasts were co-transfected with the indicated pairs of constructs before protein extraction. About 2×10^5 protoplasts were transfected with the fusion fluorescence proteins under the same conditions.

Agrobacterium-mediated transient expression in *N. benthamiana* leaves

Agrobacterium tumefaciens (strain GV3101) bacteria containing the indicated pCAMBIA1300-based constructs for LCI assays were grown in LB medium supplemented with kanamycin and rifampicin at 28°C overnight, harvested by centrifugation at 800 ***g*** for 20 min,

resuspended in infiltration medium containing 10 mM $MgCl_2$, 10 mM 2-(*N*-morpholine)-ethanesulfonic acid (MES; pH 5.7), and 200 μM acetosyringone (3,5-dimethoxy-4′-hydroxy-acetophenone), and incubated at room temperature for at least 3 h. The cultures were then resuspended in fresh infiltration medium and adjusted to an OD_{600} of 0.6. The agrobacterial cultures for co-infiltration were mixed with the post-transcriptional gene silencing suppressor p19 protein in a 1:1 ratio, which was able to enhance the transient expression in tobacco (Voinnet *et al.*, 2003; Lindbo, 2007). The mixed bacterial suspensions were then infiltrated into young, but fully expanded, leaves of *N. benthamiana* plants using a needleless syringe. After infiltration, plants were immediately covered with plastic bags and placed at 23°C for 40–48 h before detection of LUC activity.

Firefly luciferase complementation imaging assays

The LCI assays were performed as previously described (Chen *et al.*, 2008). Before LUC activity detection, excess luciferin was added to transfected protoplasts or sprayed on the leaves of *N. benthamiana*, and then a 24-well microtiter plate or *N. benthamiana* leaves were kept in the dark for 10 min to quench background fluorescence. A low-light cooled CCD imaging apparatus (CHEMIPROHT 1300B/LND, 16 bits; Roper Scientific, http://www.roperscientific.com/) was used to capture the LUC image. The camera was cooled to −110°C by liquid nitrogen and then relative LUC activity was measured with the parameters of an exposure time of 2 min with 3 × 3 binning. Each data point consisted of two or three replicates, and three independent experiments were performed for each assay. Student's *t*-test was performed to determine the significance of differences at $P < 0.01$. After LUC detection, total protein was extracted from equal amounts of protoplasts or tobacco leaves using the extraction buffer containing 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM DDT, 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)–HCl pH 8.0, and a 50 × protease inhibitor cocktail, and western blotting was performed using the rabbit anti-full-length firefly LUC antibodies (Sigma, http://www.sigmaaldrich.com/), which react with both the N-terminal and C-terminal firefly LUC fragments. Western blot results were detected with the ECL plus kit (Amersham Biosciences, http://www.amershambiosciences.com).

Peptide treatment

The exogenous CLV3 peptide was synthesized by SBS Genetech (http://www.sbsbio.com.) with the sequence Arg-Thr-Val-Hyp-Ser-Gly-Hyp-Asp-Pro-Leu-His-His (Kondo *et al.*, 2006) and dissolved in a filter-sterilized sodium phosphate buffer (50 mM, pH 6). The peptide was confirmed to be functional by root assays before use in LCI assays (Figure S1).

Co-immunoprecipitation assays

Total protein was extracted from equal amounts of 4 ml of transfected protoplasts with 1 ml of extraction buffer (150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM DDT, 50 mM TRIS–HCl pH 8.0, and a 50 × protease inhibitor cocktail). After being vortexed vigorously for 30 sec, the samples were centrifuged at 17 945 ***g*** for 10 min at 4°C. The supernatant was incubated with pre-washed anti-FLAG-agarose beads for more than 4 h at 4°C with 360° shaking. The beads were collected and washed six times with washing buffer (150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 1 mM DDT, and 50 mM TRIS–HCl pH 8.0). Co-immunoprecipitated proteins were analyzed by western blot with anti-HA and anti-FLAG antibodies (Xiang *et al.*, 2008). The protein bands with appropriate molecular weights were shown.

Confocal microscopy

After 10–12 h transformation under weak light, a 510 Meta confocal laser scanning microscope (Carl Zeiss, http://www.zeiss.com/) excited at 514 nm with an argon laser was used to examine the localization of the fusion proteins CRN–YFP. Chlorophyll autofluorescence, YFP fluorescence, and light field vision were recorded in separate channels, after which these three were merged into an overlay image. All samples were imaged with the 40 × objective.

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SUPPORTING INFORMATION

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

Figure S1. Root growth assays for peptide treatment.

Figure S2. The full version of Figure 3.

Figure S3. The co-expression of CLV1–CLuc/CLV2–NLuc/CRN–FLAG and CLV1–CLuc/CLV2–HA/CRN–NLuc in Arabidopsis protoplasts.

Table S1. Primers used for plasmid construction.

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