CLAVATA Components In Vitro and in Transient Expression

Lindsey A. Gish, Jennifer M. Gagne, Linqu Han, Brody J. DeYoung, Steven E. Clark

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, Michigan, United States of America

Abstract

The CLAVATA (CLV) signaling pathway is essential for shoot meristem homeostasis in Arabidopsis. CLV acts to limit the expression domain of the stem cell-promoting gene WUSCHEL (WUS). The closely related receptor-kinases CLV1 and BAM1 are key components in this pathway; however, the downstream factors that link the receptors to WUS regulation are poorly understood. The Arabidopsis gene At5g65480 was recently identified as a direct transcriptional target up-regulated by WUS. We have independently identified this gene which we term CCI1 as a CLV1 and BAM1 interacting protein in vitro and in transient expression. CCI1 has phosphatidylinositide-binding activity in vitro and localizes to the plasma membrane in transient expression. Furthermore, CLV signaling components and CCI1 both partition to detergent-resistant membrane microdomains characterized as lipid rafts.

Introduction

The aerial organs of the adult plant body are reiteratively initiated from a tightly maintained population of stem cells found at the shoot and flower meristems. Each meristem maintains a small number of stem cells in the center, surrounded by the more rapidly dividing and differentiating daughter cells [1]. The shoot meristems maintain a strict balance between proliferation and differentiation of stem cells throughout the life of the plant.

The shoot meristem (SM) in Arabidopsis is composed of three stem cell layers (L1, L2, and L3). Directly beneath L3 stem cells is the Organizing Center (OC) defined by the expression of the transcription factor WUSCHEL (WUS) [2]. Current evidence indicates that WUS protein moves from the OC to the overlying stem cell layers to maintain stem cell identity [3,4].

The components of the CLAVATA signaling transduction pathway act to spatially restrict WUS expression. The CLV pathway components include the CLV3 ligand, the leucine-rich repeat (LRR) receptor-kinase CLV1, the LRR receptor protein CLV2, and CRN, a transmembrane kinase-related protein. Mutations in the CLV components result in expanded WUS expression and enlarged meristem [5].

In addition, the CLV1-related BAM1, BAM2 and BAM3 proteins fulfill both redundant and unique roles. In the meristem center, the weakly expressed BAM proteins act redundantly with CLV1 to limit meristem size. However, BAM1 and BAM2 are predominantly expressed in the meristem periphery [6]. Loss of BAM receptors results in a reduction in stem cell accumulation [7]. In addition to their complex roles in meristem development, BAM receptors are expressed throughout the plant, and bam1 bam2 double mutants exhibit pleiotropic developmental defects ranging from seedling lethality, to reduced vascular branching to male sterility [6,8]. Critically, CLV1 and BAM receptors can cross-complement each other, indicating that the biochemical function of the individual receptors is largely interchangeable.

Several receptor complexes have been identified by various studies using both transient expression and in vivo analysis. The most commonly detected complexes are CLV1 and CLV1/BAM dimers and a complex of CLV2 and CRN [9–11]. Higher ordered interactions between CLV1 and CLV2 complexes have only been detected in transient expression.

The ligand, CLV3, is proteolytically processed to release the CLE peptide, which can then bind the extracellular domain of all of the detected receptor complexes [11,12]. CLV1, BAM1, BAM2 and CLV2 all have nearly identical binding affinities to the mature CLV3 ligand in vitro [11].

There is a conspicuous lack of understanding of signaling intermediates between the known CLV components and WUS. The only known verified signaling intermediates are the phosphatases POL and PLL1. Identified in a suppressor screen of the clv mutant phenotype, pol pll double mutants lack all stem cells and aerial tissues phenocopy wus mutants [13–16]. POL and PLL1 act downstream of CLV1 to maintain WUS expression. POL/PLL1 are plasma membrane localized in a fashion dependent on N-terminal myristoylation and palmitoylation [17]. This localization is required for protein function as the mutant phenotype can only
be complemented by expression constructs with both of these acylation sites intact. In addition, POL and PLL1 are phospholipid binding proteins whose phosphatase activity is stimulated by PI(4)P.

In this study, we describe a novel protein CCI1 identified through interaction screens with both CLV1 and BAM1. We present evidence of CCI1 receptor interactions when transiently overexpressed in tobacco, plasma membrane localization, phospholipid binding, and membrane microdomain partitioning.

Results

Identification of a Novel CLV1-interacting Protein

We performed a protein interaction screen using the yeast Cytotrap system that involves interactions at the yeast plasma membrane (Supporting Figure 1) [18]. Yeast at the restrictive temperature require that hSos (a Ras GEF) localize to the plasma membrane to replace the temperature sensitive cdc25 isoform. hSos was fused to the CLV1 and BAM1 kinase domain and placed into yeast along with cDNA library from Arabidopsis meristem tissue placed behind a N-terminal myristoylation tag to drive plasma membrane localization. Only those yeast with a cDNA-encoded protein that bound to CLV1 or BAM1 would localize the hSos tag to the plasma membrane and survive at the restrictive temperature. The kinase domains used in this screen corresponded to residues 697–980 for CLV1 and 699–1003 for BAM1. Each bait was screened separately. Because CLV1 and BAM1 can replace each other’s function in Arabidopsis [6], we hypothesized that proteins interacting with both kinase domains were more likely to represent physiologically relevant partners. We sequenced 32 putative positive lines from yeast with the CLV1 bait protein and 52 lines from yeast with the BAM1 bait (Supporting Tables 1 and 2). Among these positives, two were identified from both CLV1 and BAM1 screens and only one, At5g65480, was identified multiple times in both screens. All positives for CLV1 and BAM1 screens and only one, At5g65480, were putative positive lines from yeast with the CLV1 bait (Supporting Tables 1 and 2). Among these positives, two were identified from both CLV1 and BAM1 screens and only one, At5g65480, was identified multiple times in both screens. All positives for CLV1 and BAM1 screens and only one, At5g65480, were putative positive lines from yeast with the CLV1 bait (Supporting Tables 1 and 2). Among these positives, two were identified from both CLV1 and BAM1 screens and only one, At5g65480, was identified multiple times in both screens.

At5g65480, which we have named CCI1 (inspired from the Clavata complex interactor) encodes a small protein of 153 amino acids. While having no known motifs, the genomes of all land plants we analyzed contained homologues of CCI1 named CCI2 (Figure 1). Expression profile mapping of the SM zone of the meristem, suggesting an overlap with CLV1 expression and function [19]. Very recently, a screen for direct transcriptional targets of WUS identified At5g65480 as one of the genes most highly induced by WUS activation. [20].

We first tested whether CCI1 directly interacts with the CLV1 kinase domain by expressing the corresponding proteins in E. coli as epitope-tagged fusion proteins. In pull-down experiments, GST-CCI1 showed direct interaction with the CLV1 kinase domain, but not in control reactions (Figure 2A). CCI2 also showed direct interaction with CLV1 (Figure 2A).

We next sought to determine whether the CLV1-CCI1 and BAM-CCI1 interactions could be replicated in a plant system. Because efforts to detect epitope-tagged CCI1 expressed in transgenic Arabidopsis was unsuccessful, we used transient expression in N. benthamiana to express the proteins [21]. We have successfully used this system to characterize CLV1 interactions both with CLV3 and with other signaling components [11,22]. To test the interactions between CCI1 and BAM1/CLV1, the full-length proteins were expressed as epitope tagged fusions under the cauliflower mosaic virus 35S cis elements. Two days after infiltration, leaf proteins were extracted and co-immunoprecipitation experiments were performed. When CCI1-FLAG and BAM1-GFP or CCI1-FLAG and CLV1-GFP were co-expressed in the same leaves we detected robust co-immunoprecipitation, suggesting a protein-protein interaction between CCI1-FLAG and the GFP-tagged full-length receptors (Figure 2B).

In addition to GFP-tagged BAM1 and CLV1, GFP-tagged full-length CRN, CLV2 and BR11 were also tested for interaction with CCI1-FLAG. Unexpectedly, we observed co-immunoprecipitation between CCI1-FLAG and all of the tested proteins (Figure 2C). Co-immunoprecipitations were also detected when the epitope tags were switched (i.e., CCI1-GFP with BAM1-FLAG and CLV1-FLAG) (Supporting Figure 2). Additional control reactions demonstrated that proteins interactions were not a result of non-specific antibody interactions (Figure 2C). Hypothesizing that these associations might be formed spuriously after membrane isolation, we next tested whether the associations of CCI1 with CLV signaling components required co-expression, or could occur by mixing membrane extracts expressing the corresponding proteins. These experiments revealed that co-expression is necessary for any interaction to occur, indicating that the CCI1-receptor interactions were not formed through spurious post-isolation interactions, but instead required that the proteins were expressed simultaneously in the same cells (Figure 2C).

Figure 1. Alignment of CCI1-related proteins from land plants. An alignment of Arabidopsis thaliana CCI1, CCI2 and related proteins from various plant species is shown. Conserved residues are shaded at 75%. Basic-rich regions are underlined. The top and bottom segments of CCI1 sequence correspond to the N and C-terminal half constructs used in the lipid-binding assays.
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CCI1 is Plasma-membrane Localized and Binds Phosphatidylinositols in vitro

Because CCI1 interacts with CLV1, which acts at the plasma membrane [23], we next tested whether CCI1 co-localized to the same subcellular compartment. CCI1 has no identifiable localization motif, nor any predicted transmembrane domain. Both CCI1-GFP and CCI1-FLAG were transiently expressed and localization was determined both by confocal microscopy and subcellular fractionation. The localization of CCI1-GFP was consistent with that of plasma membrane localization, with signal exclusively at the cell periphery (Figure 3A). However, the cytoplasm of these cells is largely appressed to the cell periphery, so that we could not exclude partitioning between the membrane and the cytoplasm. To resolve this issue, we fractionated extracts, separating membrane and soluble fractions. For CCI1-FLAG, we detected localization exclusively in the membrane fractions (Figure 4A). In addition, when these N. benthamiana leaf protein extracts were subjected to ultracentrifugation and fractionation by two-phase partitioning, CCI1-FLAG was detected in the plasma membrane-enriched PEG phase, and was absent from the plasma membrane-depleted dextran phase [24], (Figure 3B). This localization of CCI1 is consistent with the plasma membrane-localized H+ATPase PMA2, used as a control. These data collectively indicate CCI1 is plasma membrane bound.

These results raised the question of what motif(s) within CCI1 were driving exclusive plasma-membrane localization. As mentioned, neither CCI1 nor any analyzed homologue contains a known membrane-localization motif. Furthermore, CCI1 membrane localization was independent of CLV1 co-expression. One possibility emerged from attempts to use CCI1 as a bait protein in

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**Figure 2.** CCI1 interactions with CLV signaling components in vitro and in transient expression. A. Purified CLV1KD-GFP, GST, GST-CCI1 and GST-CCI2 proteins were mixed in various combinations, immunoprecipitated (IPd) with anti-GST antibodies, and the resulting immunoprecipitates were assayed on a protein gel blot probed with anti-GFP. The first lane shows CLV1KD-GFP input. B. Total membrane extracts from N. benthamiana leaves expressing CCI1-FLAG and full-length CLV1-GFP and BAM1-GFP IPd with anti-GFP antibodies and co-IP detected with anti-FLAG antibodies. Lanes 1, 2 and lanes 4, 5 are replicates. Note, CLV1-GFP did not express detectably in the lane 4 replicate, nor was there co-IP detected. Experiments represented by lanes 1–3 used an aliquot from the lane 7 expression of CCI1-FLAG alone (*). Co-IP was detected when both CCI1 and BAM1 were co-expressed in the same leaf (CCI1 co), but not when mixed post expression (CCI1 post). C. Total membrane extracts from N. benthamiana leaves expressing CCI1-FLAG and full-length BR11-GFP, BAM2-GFP, CLV2-GFP and CRN-GFP IPd with anti-GFP antibodies and co-IP detected with anti-FLAG antibodies. Experiments represented by lanes 1–4 used an aliquot from the lane 9 expression of CCI1-FLAG alone (*). CoIP was detected when CCI1 and the receptors were co-expressed in the same leaf (CCI1 co), but not when mixed post expression (CCI1 post). doi:10.1371/journal.pone.0066345.g002

**Figure 3.** CCI1 is plasma membrane localized. A. Confocal image of CCI1-GFP transiently expressed in N. benthamiana leaves 48 hours after infiltration. Signal is detected at cell periphery. B. Two-phase membrane partitioning of CCI1-FLAG transiently expressed in N. benthamiana leaves. Endoplasmic reticulum marker BiP2 and plasma membrane marker PMA2 are used to mark the lower and upper phases, respectively. doi:10.1371/journal.pone.0066345.g003
the Cytotrap yeast system. Here we observed that CCI1 alone localized to the yeast plasma membrane (as evidenced by auto-activation, data not shown). As shown previously for the animal protein Tubby, Cytotrap auto-activation can result from lipid binding activity of the bait protein [25]. Furthermore, the CLV1 downstream signaling phosphatases POL and PLL1 autoactivate in the Cytotrap system, localize to the plasma membrane, and bind to phospholipids [17]. To test whether CCI1 has lipid-binding activity, E.coli expressed GST-CCI1 was incubated with lipid strips blotted with phosphatidylinositides and other lipids. The human FAPP protein, which has been shown to specifically bind phosphatidylinositol-4 phosphate (PI(4)P), was used as a positive control [26]. Full-length CCI1 bound PI-monophosphates and cardiolipin, with weak association observed to some PI-di- and tri-phosphates (Figure 5).

Examination of the protein revealed the N-terminal half contains several polybasic stretches of amino acids, conserved across most plant species, while the C-terminal domain has more extensive conservation across land plants (Figure 1). Several phosphatidylinositol-binding domains utilize polybasic patches to interact with negatively charged phosphates on the inositol head group of PI-mono and di-phosphates [27]. When the N- and C-terminal regions were expressed separately as fusion proteins, the N-terminal 83 amino acids of CCI1 were sufficient to bind a similar profile of lipids, while the C-terminal 70 amino acids showed no detectable binding (Figure 5). Deletion constructs targeting individual polybasic regions in the N-terminal portion appeared to attenuate but not abolish lipid binding activity (Supporting Figure 3).

Plasma membranes are not homogeneous with respect to protein and lipid-type distribution [28,29]. Isolation and visualization of membrane raft microdomains have suggested that specific protein and lipid enrichments in microdomains in the plasma membrane act as hubs to recruit signal transduction pathway components. Some microdomains are sufficiently enriched in sterols, phosphatidylinositols and saturated lipids that they become insoluble to specific detergent treatments [30,31]. Relative to the total plasma membrane, detergent-resistant membranes (DRMs) are enriched in phosphatidylinositides, such as PI(4)P and PI(4,5)P2, over structural phospholipids such as phosphatidylcholine and phosphatidylethanolamine [32]. Furthermore, we have previously observed that CLV3 binding to the CLV1, BAM and CLV2 receptors could only be detected for receptors in DRM fractions, potentially reflecting lipid raft...
localization [11]. Taken together, we hypothesized CCI1 lipid binding might be associated with membrane microdomain partitioning as part of signaling complexes.

CCI1 was found in both the soluble membrane and detergent-resistant membrane fractions from N. benthamiana transient expression (Figure 4A). CLV2 partitions in a similar pattern in Arabidopsis, while BAM1 and BAM2 are found predominantly in the soluble membrane fraction with detectable partitioning to the DRM fraction (Figure 4B). To test if these receptors were truly localized to lipid rafts, we assayed their sedimentation in sucrose gradients. While the control clathrin was found exclusively in denser soluble membrane fractions, a portion of CLV2 and all detectable CRN from Arabidopsis meristems were found in lighter fractions consistent with lipid raft partitioning (Figure 4C).

The potato sucrose transporter StSUT1 partitions to a DRM fraction of the membrane. Immunoprecipitation of StSUT1 from potato tissue co-immunoprecipitated over 40 associated proteins [33]. This broad array of interactions is thought to result from co-localization to the DRM fraction. In other words, immunoprecipitating a raft-localized protein can pull down the membrane microdomain and all of their associated proteins. Similarly, the co-immunoprecipitation of CCI1 with CLV signaling components could result from their co-localization to DRMs and not necessarily from direct protein-protein interactions. To test this hypothesis, co-IP experiments were performed on both total membrane and DRM-depleted soluble membrane fractions from N. benthamiana co-expressing CCI1-FLAG and CRN-GFP, as well as CCI1-FLAG and BAM2-GFP. When the DRM fraction was removed from the membrane fraction, the CCI1/BAM2 interaction was still detectable while the CCI1/CRN interaction was not (Figure 4D). This suggests the interaction between CCI1 and CRN depends on co-localization to the DRM and does not necessarily reflect a direct protein-protein interaction.

Genetics Analysis of CCI1 Function

We have characterized all three available alleles for At5g65480. cai1-1 is a JIC SM line (GT_5_40258), which contains an enhancer/suppressor-mutator mobile element inserted into At5g65480 [34]. The insertion in cai1-1 is located 33bp after the start ATG; however, RT-PCR analysis readily detected transcripts from the downstream portion of the gene (Supporting Figure 4). Sequencing the insertion junction revealed that the insertion created an in-frame methionine, leading to a potentially functional protein that was transcribed (Supporting Figure 5). Thus, we conclude that cai1-1 is not a null allele and may not be hypomorphic. cai1-1 plants lacked any identifiable mutant phenotype.

cai1-2 (GABI_541D11) is a GABI-KAT line inserted near the end of the first exon, interrupting the 124th codon, leaving intact the phospholipid binding domain and the conserved domain in the C-terminal portion [35]. Thus, it is not possible to conclude that cai1-2 is a null allele because the bulk of the coding sequence is left intact. cai1-2 homozygous mutant plants had no identifiable phenotype.

cai1-3 (GABI_102G06) is also a GABI-KAT line inserted into the intron between the first and second exons. Homozygous cai1-3 plants could not be identified in segregating populations from heterozygous parent plants. Sequencing the right border of the T-DNA insertion indicated centromeric satellite sequences, suggesting a possible chromosomal aberration. Analysis of progeny of cai1-3 heterozygotes indicated a 1:1 ratio of wild-type to heterozygous plants (32:30), consistent with lethality due to chromosomal abnormalities. To test this hypothesis, reciprocal crosses were performed between wild-type and cai1-3 heterozygous plants. Among the F1 progeny, we observed transmission of the cai1-3 allele through both the male and female gametes. Thus, the failure to observe cai1-3 homozygous progeny is readily explained by the chromosomal rearrangement associated with the T-DNA insertion.
Cells use these raft microdomains in pathogen response, protein trafficking and they are the site of signaling hubs in the plasma membrane. Lipid rafts can both concentrate signaling components while at the same time insulating the raft members from negative signaling regulators such as phosphatases [29]. Signal transduction pathways utilizing membrane rafts have been well-characterized in animal immune response and G-protein signaling [28,40]. Membrane rafts in plants are enriched in proteins associated with signaling including LRR receptor kinases [41]. Auxin signaling and redox systems in membrane rafts in plants have also been characterized [42,43].

We have presented evidence supporting DRM partitioning and possible lipid raft association for several CLV pathway components, including CCI1. In addition, CLE binding to CLV2, BAM and CLV1 can only be detected in DRM fractions, suggesting CLV signaling depends on receptor localization to membrane microdomains [11]. The expression profiling data compiled by Yadav et al. and the direct interaction of CCI1 with the partially raft-associated kinases CLV1 and BAM1 combined with the co-immunoprecipitation of CCI1 with DRM-associated proteins when transiently overexpressed in tobacco suggest a role for CCI1 in lipid-raft based signal transduction in the shoot meristem. While we provide evidence of the biochemical properties of CCI1 in transient expression, the physiological role of CCI1 in the Arabidopsis meristem remains an open question. This is in part due to the lack of a clear cci1 null allele as well as the lack of phenotypes in the homozygous alleles that can be identified. Independent evidence, however, suggest a role for CCI1 in meristem function. First, CCI1 is preferentially expressed within the central zone of the meristem, overlapping CLV1 expression. Second, CCI1 was recently identified as one of the most highly induced gene by WUS activation and identified as a direct WUS target [20]. Interestingly, WUS also represses CLV1 transcription [44] suggesting that there may be several layers to the feedback regulation in the CLV/WUS pathway, including a potential role for CCI1.

Methods

Lipid Binding

Sequences encoding CCI1, CCI2 and the last 70 amino acids of CCI1 (C-term CCI1) were amplified from cDNA and inserted into pGEX-5X-1 with BamHI and NotI sites. The sequence encoding the first 83 amino acids of CCI1 (N-term CCI1) was inserted using BamHI and SalI sites. The N-terminus GST fusion proteins were expressed in E. coli protein expression strain BL21 CodonPlus (Stratagene). The GST-hFAPP expression construct was kindly provided by Erik Nielsen. The expressed proteins were purified using glutathione sepharose (GE Healthcare).

PPIP strips and membrane lipid strips were obtained from Echelon Biosciences. The strips were blocked with 3% fatty acid free BSA in PBS-T for 1 hour at room temperature. The CCI1 and CCI2 proteins were incubated with the blots at a concentration of 1 nM for 1 hour at room temperature. Lipid-protein interactions were detected using a 1:10,000 dilution of an anti-GST HRP-conjugated antibody (GenScript).

For deletion construct blots, nitrocellulose membrane was blotted with PIP strips and PE from Echelon Biosciences.

Co-immunoprecipitation and Fractionation of Transiently Expressed and Arabidopsis Proteins

Binary vectors containing 35S::BR1, CLV1, BAM1, BAM2, CLV2, CRN C-terminal GFP and CLV1 and BAM1 C-terminal fusion constructs, as well as BAM1-FLAG, BAM2-FLAG and
CLV2-MYC have been previously described [6,11,45]. CCI1-GFP was generated by replacement of the CLV1 coding sequence in the 35S:CLV1-GFP construct. To generate the 35S:FLAG-CCII cassette, the CCI1 coding sequence was amplified and cloned into pENTR/D-TOPO to create entry vectors for subcloning into pEarleyGate 202 via LR clonase reaction.

For transient expression, binary vector constructs were transformed into Agrobacterium tumefaciens strain GV3101 and infiltrated along with P19, a viral silencing suppressor [21], into N. benthamiana leaves. After 48 hours, proteins were extracted in buffer (50 mM Tris-HCl, 150 mM NaCl, 10 mM EDTA, 10% glycerol, 10 mM NaF, 10 mM NaVO3, 2% plant specific protease inhibitor cocktail (Sigma), 10 ug/ml chymostatin and 2 ug/ml aprotinin). For stable expression lines, 8–10 Arabidopsis meristems were added to the 100,000 g supernatant and boiled for 5 minutes. The pellet from each fraction was resuspended in extraction buffer with 1% Triton X-100 and centrifuged at 2400 g for 10 minutes at 4°C to remove flocculate. Supernatants were centrifuged at 100,000 g for 1 hour at 4°C to separate soluble microsomal fractions. The microsomal fractions were then solubilized using 1% triton X-100 with gentle agitation at 4°C.

When immunoprecipitation was performed with anti-GFP antibodies, the antibody was incubated with the solubilized membrane fraction at 4°C for 2 hours, then protein A agarose was added and incubated for an additional two hours. When immunoprecipitration was performed with anti-FLAG antibodies, anti-FLAG M2-Agarose was incubated with the solubilized membrane fractions for 4 hours at 4°C. Agarose was pelleted at 100 g, washed three times, and boiled in SDS buffer containing β-ME.

Sucrose Gradients
Tissue from 10 apices each of BAM1-FLAG, BAM2-FLAG, CLV1-GFP, and CLV2-myc were collected, placed in tubes containing 200 µL of detergent- and glycerol-free extraction buffer (50 mM Tris pH 8.0, 10 mM EDTA, 100 mM NaCl, 5% protease inhibitor cocktail [Sigma, P9599]) on ice, and homogenized as previously described [6]. Homogenized tissue was centrifuged at 2400 g for 10 minutes at 4°C. The supernatants were pooled and centrifuged again at 2400 g for 10 minutes at 4°C. SDS sample buffer was added to a portion of the supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes. The supernatant was centrifuged at 100,000 g for 1 hour at 4°C. SDS sample buffer was added to the 100,000 g supernatant and boiled for 5 minutes.

Two-phase Partitioning
The membrane fraction from tobacco leaves transiently expressing FLAG-CCII was isolated as described above and resuspended in microsome resuspension buffer containing 330 mM sucrose, 2 mM DTT, 5 mM KH2PO4, 10 mM EDTA, 10 mM NaF, 10 mM NaVO3, 2% plant specific protease inhibitor cocktail (Sigma), 10 ug/ml chymostatin and 2 ug/ml aprotinin, pH 7.8. The plasma membrane fraction was extracted using PEG-dextran phases containing 6.4% (w/w) PEG 3350 and 6.4% dextran (w/w) [24]. Antibodies against plasma membrane marker PMA2 [46] and endoplasmic reticulum marker Bip2 (SPA-818; Stressgen) were used as controls.

E. coli Expressed Protein Co-immunoprecipitation
The coding sequence for the CLV1 kinase domain and C-terminal GFP tag was cloned into pDEST12 and expressed in BL21 codon plus cells. Soluble sonicate from GST-CCI1 or GST-CCI2 and CLV1 KD-GFP were combined, then incubated with glutathione sepharose 30 minutes at room temperature. The sepharose was washed 3 times and eluted. The co-immunoprecipitation was detected with ab65556 (Abcam).

Supporting Information
Figure S1 The Cytotrap system uses Ras recruitment to identify protein-protein interactions. The cd25 temperature sensitive mutant is complemented when the human homologue hSo, fused to the bait protein, is recruited to the plasma membrane upon a protein-protein interaction with a myristoylated cDNA library target. (TIF)

Figure S2 Solubilized membrane extracts from N. benthamiana leaves expressing CCI1-GFP and full-length BAM1-FLAG and CLV1-FLAG (three replicates in lanes 2–4) were IPd with anti-GFP antibody and the co-IP was detected with anti-FLAG antibody. (TIF)

Figure S3 Lipid binding of CCI1 deletion isofroms. Deletion of several regions of N-terminal CCI1 did not abolish lipid binding activity. (TIF)

Figure S4 Reverse transcriptase PCR detected CCI1 transcript in the cci1-1 allele. Tubulin was used as a control. (TIF)

Figure S5 The cci1-1 insertion allele contains an upstream in-frame methionine. The junction of insertion sequence and CCI1 coding sequence leads to a possible ORF. CCI1 coding sequence is in blue. Ds insertion sequence is in red. (TIF)

Figure S6 The mean number of carpels per flower in wild-type, cci1-1, and cci1-1 combined with mutants of CLV pathway. Error bars represent standard error of the mean. (TIF)

Table S1 Positives from Cytotrap protein-protein interaction screen with the BAM1 kinase domain. (DOCX)

Table S2 Positives from Cytotrap protein-protein interaction screen with the CLV1 kinase domain. (DOCX)

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Author Contributions
Conceived and designed the experiments: LAG JMG LH BJD SEC. Performed the experiments: LAG JMG LH BJ D. Analyzed the data: LAG JMG LH BJ D SEC. Wrote the paper: LAG SEC.

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