Stem cell signalling in *Arabidopsis* requires CRN to localize CLV2 to the plasma membrane

Andrea Bleckmann¹, Stefanie Weidtkamp-Peters², Claus Seidel² and Rüdiger Simon¹*

¹ Institut für Genetik, Heinrich-Heine Universität Düsseldorf, Universitätsstr.1, 40225 Düsseldorf, Germany

² Institut für Molekulare Physikalische Chemie, Heinrich-Heine Universität Düsseldorf, Universitätsstr.1, 40225 Düsseldorf, Germany

* to whom correspondence should be addressed: 

ruediger.simon@uni-duesseldorf.de

Running Title:

CRN localizes CLV2 to the plasma membrane

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Abstract:

Stem cell number in shoot and floral meristems of Arabidopsis is regulated by the CLAVATA3 (CLV3) signalling pathway. Perception of the CLV3 peptide requires the receptor kinase CLAVATA1 (CLV1), the receptor-like protein CLAVATA2 (CLV2) and the kinase CORYNE (CRN). Genetic analysis suggested that CLV2 and CRN act together, and in parallel with CLV1. We studied the intracellular localization of receptor fusions with fluorescent protein tags, and their capacities for interaction via efficiency of fluorescence resonance energy transfer (E_{FRET}). We found that CLV2 and CRN require each other for export from the endoplasmatic reticulum (ER), and localization to the plasma membrane (PM). CRN readily forms homomers and interacts with CLV2 through the transmembrane domain and adjacent juxtamembrane sequences. CLV1 forms homomers independently of CLV2 and CRN at the PM. We propose that the CLV3 signal is perceived by a tetrameric CLV2/CRN complex and a CLV1 homodimer that localize to the PM and can interact via CRN.
Introduction:

In *Arabidopsis*, the stem cell number in the shoot apical meristem (SAM) is regulated by negative feedback regulation. Stem cell induction and maintenance is controlled by the homeodomain protein WUSCHEL (WUS), and WUS expression is in turn repressed by CLAVATA3 (CLV3) (Brand et al., 2000; Schoof et al., 2000), which encodes a 13 amino acid arabinosylated glycopeptide that is secreted from stem cells (Ohyama et al., 2009). Three genes have been identified that encode receptors for CLV3 signalling. Mutations in *CLAVATA1* (*CLV1*) (Clark et al., 1997), encoding a leucine rich repeat (LRR) receptor kinase, *CLAVATA2* (*CLV2*), encoding a LRR receptor-like protein (Jeong et al., 1999), or *CORYNE* (*CRN*), encoding a receptor-like kinase, disrupt CLV3 signalling and allow the stem cell domain to expand (Sablowski, 2007; Müller et al., 2008). Binding of CLV3 to the LRR domains of CLV1 was recently shown (Ogawa et al., 2008).

A simple readout for CLV3 signalling is carpel number. Stem cells of floral meristems are normally consumed with the production of two central carpels. Any reduction in CLV3 signalling, which results in increased WUS expression and production of more stem cells, causes an increase in carpel number. Mutations in *CLV1*, *CLV2* or *CRN* showed an intermediate carpel number phenotype and reduced CLV3 signalling (Müller et al., 2008). Double mutants of clv2 with crn were epistatic, but double mutants of clv1 with clv2 or crn were synergistic and abolished CLV3 signalling. This indicated that *CLV1* acts independently from, and in parallel with, *CLV2* and *CRN* to transmit the CLV3 signal. Furthermore, clv2 and crn mutants showed additional phenotypes, such as elongated pedicels and defects in stamen development, suggesting that *CLV2* and *CRN* act in a common pathway (Müller et al., 2008). Both CRN and CLV2 were proposed to be membrane localized, and may physically...
interact via their transmembrane domains, or immediately adjacent sequences (Fig. 1A). A loss-of-function mutation of CRN, crn-1, is caused by an amino acid exchange within the predicted transmembrane domain, suggesting that membrane localization, interaction with a partner protein, or both is essential for CRN function.

We have here investigated the intracellular localization of CLV1, CLV2 and CRN in plant cells, and their tendencies for protein-protein interactions. Using fluorescent protein tags, we show that CLV1 resides at the plasma membrane. We found that CLV2 and CRN require each other for transport from the ER to the plasma membrane. Via FRET, we show that CLV2 and CRN form complexes at the ER that then relocalize to the plasma membrane, and we identified the protein domains required for this interaction. Furthermore, we found that CLV1 homomerizes, but can also interact with CRN and CLV2, suggesting a mechanism for crosstalk between the two receptor complexes for CLV3 signalling.
Results:

Mutant complementation with fluorescent protein tagged receptors

For FRET based analyses of protein-protein interactions, the proteins CLV1, CLV2 and CRN were expressed as fusions with fluorescent proteins (FP). We found that constitutive expression of most receptor-GFP fusions from the CaMV35S promoter failed to rescue the corresponding mutants in transgenic Arabidopsis. We therefore decided to use an inducible gene expression system, which allows to study protein localization and interaction at variable protein concentrations.

A set of transgenic Arabidopsis plants was generated that expressed fusions of CLV1, CLV2 or CRN with either GFP or mCherry at their C-terminus (CLV1-GFP, CLV1-mCherry etc) from an estradiol-inducible promoter (Zuo et al., 2002) (Fig. 1B,C). We studied the functionality of the different receptor-FPs by analyzing their capability to complement the corresponding loss-of-function mutant plants, clv1-11, clv2-1 or crn-1 (Fig1D-F). Transgenic plants were first cultivated for 28 days on soil, before transgene expression was induced with 20 µM β-estradiol on day 28, 30 and 32. At 8 weeks after germination, we assayed for restoration of CLV3 signalling by counting carpel number of 10 siliques on each plant. The non-transgenic control plants as well as mock-induced transgenic plants produced on average 3.9 carpels per silique. Importantly, siliques with less than three carpels were never observed under our growth conditions. Induced transgenic plants showing at least two consecutively formed siliques with wildtype carpel number were regarded as complemented, and those carrying a single rescued silique as partially complemented. We found that all our inducible transgenes were functional in vivo and complemented the respective mutations, albeit with different frequencies (Table
1). Although up to two flower primordia are normally initiated per day (Smyth et al., 1990), and transgene expression was induced during at least 5 days, we found that many transgenic plants carried only two siliques with 2 carpels. Transient restoration of CLV3 signalling could indicate either delayed floral primordium initiation, rapid loss of transgene activity due to cosuppression, or sensitivity of the system to receptor concentrations.

**Intracellular localization of CLV1, CLV2 and CRN**

In *Arabidopsis*, we detected induced CLV1-GFP fusion proteins in hypocotyl and root cells and very faint CRN-GFP signals when expressed from the CRN promoter, but failed to visualize CLV2-GFP. However, we were unable to reproducibly detect the fusion proteins in shoot or floral meristems (SupplFig. 1).

Because localization studies with stably transformed *Arabidopsis* plants proved not feasible, we used a transient expression system in *Nicotiana benthamiana* leaf epidermis cells for further experiments. Constitutively expressed CLV1-GFP localized to the plasma membrane, but we also noted formation of larger, fluorescing aggregates (SupplFig. 2). Similar aggregates were observed for CaMV35S::CLV2-GFP and CaMV35S::CRN-GFP, which could be caused by protein overexpression.

To control protein expression levels, we then used the estradiol inducible system. Vectors were transformed into intact leaves via *Agrobacterium* infiltration, and expression was induced by spraying leaves with β-estradiol. Signals from fluorescent proteins were first detectable 3 hours after induction. Upon extended induction (>12 hours), most cells carried large fluorescent protein aggregates, similar to those observed in our constitutive expression experiments (Fig. 2A, SupplFig. 1,2). Such an aggregation of receptor-FP fusions upon extended induction could be responsible for the only transient complementation of the *Arabidopsis* mutants. Therefore, all
measurements were performed with cells that did not show this overexpression phenotype (Fig. 2B). Integrity of the fusion proteins was confirmed by Western blotting using an αGFP antibody (Fig. 2C). The αGFP-antibody identified a single specific band for CLV1-GFP (138 kD), CLV2-GFP (110 kD) and CRN-GFP (75 kD), respectively. The sizes of the detected bands for CLV1-GFP and CLV2-GFP were slightly increased compared to the calculated fusion protein sizes. Similar discrepancies have been noted for other proteins carrying LRR domains, and are likely due to posttranslational modifications, such as glycosylation (van der Hoorn et al., 2005).

CLV1-GFP predominantly localizes to the plasma membrane (PM), which was confirmed by colocalization with the lipophilic fluorescent dye FM4-64 (Fig. 3A, Suppl Table 2). Staining was also observed in vesicles, but only faintly in the ER, which reflects transport to the PM and the site of synthesis (Fig. 3B). In contrast, cells expressing CLV2-GFP or CRN-GFP revealed a spotty GFP pattern near the PM, which did not colocalize with the PM marker FM4-64 (Fig. 3C,E). Transformation of CLV2-GFP or CRN-GFP together with an ER-tagged mCherry reporter (Nelson et al., 2007) revealed complete colocalization in the ER network (Fig. 3D,F). Because GFP positive transport vesicles were never observed, we conclude that CLV2 and CRN are predominantly ER localized. This contrasts our expectation of CLV3 perception and signalling at the PM.

Protein localization may depend on the establishment of functional complexes, and we therefore also tested our fusion proteins in coexpression experiments. Coexpression of CLV2-GFP with CLV1-mCherry (Fig. 3G), or CRN-GFP with CLV1-mCherry (Fig. 3H) did not affect their localization at the ER and PM, respectively. However, coexpression of CLV2-GFP and CRN-mCherry caused a relocation of both
proteins from the ER to the PM (Fig. 3I), and formation of transport vesicles. This relocation was confirmed by colocalization of CLV2-GFP with FM4-64 in the presence of CRN (Fig. 3J), and of CRN-GFP with FM4-64 in the presence of CLV2 (Fig. 3K). Co-transformed cells were easily detectable by their decreased ER fluorescence, the production of GFP-positive vesicles and PM staining. When CLV1, CLV2 and CRN were coexpressed, the tagged proteins were always located at the PM (Fig. 3L).

**PM localization requires transmembrane and extracellular domains of CRN**

*In silico* studies indicated that CRN consists of a signal peptide (SP) (aa 1 to 33), a short extracellular domain (EC) (aa 34 to 61), the transmembrane domain (TM) (aa 62 to 84) and the protein kinase domain (aa 118 – 393) (Müller et al., 2008). To identify the protein domains required for interaction with CLV2 and PM localization, we designed CRN derivatives and coexpressed them with CLV2. Deleting the SP, EC and TM of CRN (CR(ΔSP-TM)) resulted in a fusion protein of 56 kD that was detected in the cytoplasm and the nucleus, and failed to relocate CLV2 to the PM upon coexpression (Fig. 4A,B). *crn-1* encodes a mutant protein that carries an amino acid exchange in the TM, which shortens the predicted TM from 23 aa length to only 19 aa (Müller et al., 2008). The mutant phenotype was explained by CRN protein mislocalization, or instability. We found that crn-1-GFP was stably expressed and ER localized (Fig. 4C). Coexpression of crn-1-GFP with CLV2-mCherry induced only a partial relocation of both proteins from the ER to the PM, and very few transport vesicles were detectable. We then swapped the TM of CRN with the TM of BRI1-ASSOCIATED RECEPTOR KINASE (BAK1) (Russinova et al., 2004; Chinchilla et al., 2007), a PM localized LRR receptor protein kinase acting in the brassinosteroid and
flagellin signalling pathways (CR(<>B1TM)). Even when coexpressed with CLV2, CR(<>B1TM) was mostly retained in the ER, indicating that the specific TM of CRN is essential for CLV2-dependent PM localization (Fig. 4D). Similarly, deletion of the EC of CRN (CR△EC) caused a complete retention in the ER, even when CLV2 was coexpressed (Fig. 4E). The extracellular juxtamembrane domains (28 aa) of CRN and CLV2 are oppositely charged, with pl 11.7 for the EC of CRN, and pl 3.7 for the EC of CLV2. Close apposition and neutralization of these charges may be required to permit the relocalization from the ER to the PM. Next, we addressed the role of the kinase domain of CRN. An exchange against the kinase domain of CLV1 (CR(<>C1Ki) caused increased retention of CLV2 and CR(<>C1Ki) at the ER when coexpressed, suggesting that the CRN kinase domain contributes to PM localization (Fig. 4F). However, deletion of the entire kinase domain (CR△Ki) still allowed PM expression together with CLV2 (Fig. 4G), which shows that the localization of CRN is independent of its kinase domain or function, and depends only on sequences that allow the interaction with CLV2. Presence of the CLV1 kinase domain in CR(<>C1Ki) may destabilize the fusion protein, or interfere with CLV2 interaction. A fusion of CLV2 with the intracellular domain of CRN (C2-CRKi) was retained in the ER, even in the presence of coexpressed CLV2 (Fig. 4H). We conclude that interaction of the TM and EC of CRN with CLV2 is essential to direct both proteins to the PM. Integrity of the CRN derivates was confirmed by Western blotting using an αGFP antibody (SupplFig. 3).

**CLV1 and CRN homomerisation**

We measured FRET efficiency, \( E_{FRET} \), between FP labeled receptor proteins for more detailed interaction studies. \( E_{FRET} \) has to be considered as “apparent”, because the
signal was not corrected for background contributions, spectral dependent crosstalk and sensitivity (Raicu, 2007). As FRET pair we used the combination of GFP with mCherry, which were previously described to be sufficiently stable to enable $E_{\text{FRET}}$ measurements (Albertazzi et al., 2009). Emission spectra of these proteins were fully separable from auto-fluorescence of plant cells. We then quantified donor (GFP) fluorescence after photobleaching of the acceptor (mCherry), and calculated $E_{\text{FRET}}$ as the resulting % GFP expression change (Fig. 5A). Unless noted otherwise, all measurements were performed at the PM, which required coexpression of untagged CRN for CLV2-FPs, and of untagged CLV2 for CRN-FPs. $E_{\text{FRET}}$ depends on the orientation of chromophores to each other, and on their distance. As a control for minimum distance, we fused GFP with mCherry to the C-termini of CLV1 (C1-G-C), CLV2 (C2-G-C) and CRN (CR-G-C) (Fig. 5B). Intramolecular $E_{\text{FRET}}$ ranged from 13 to 19 %. Even in the absence of mCherry, GFP fluorescence fluctuated by 3 to 4 % during the course of an acceptor photobleaching experiment. This fluctuation could result from GFP chromophore reconstitution during the bleaching period (Fig. 5B). A similar level of $E_{\text{FRET}}$ “background” was previously observed by others for the CFP/YFP pair (Karpova et al., 2003). In all further experiments, only $E_{\text{FRET}}$ significantly higher than 4 % was regarded to indicate close proximity or physical interaction between proteins (SupplTable1). These significance levels were confirmed by independent fluorescence measurement of donor lifetimes (Weidtkamp-Peters et al., 2009).

To analyze formation of receptor homomers (Fig. 5C), we coexpressed CLV1, CLV2 and CRN fused to GFP and mCherry, respectively. Importantly, only homomers formed between receptors labelled with different fluorophores will be quantified by these measurements, and interactions such as CLV1-GFP / CLV1-GFP and CLV1-
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mCherry / CLV1-mCherry are undetectable. CLV1 clearly formed homomers at the PM (9.5 % ± 0.8). Importantly, homomers were formed in the absence of exogenous CLV3, suggesting that CLV1 homointeraction is ligand independent. $E_{\text{FRET}}$ values for CLV2 were close to background (4.3 % ± 0.8), indicating no dimerisation. Furthermore, CLV2 homointeraction was not stimulated by coexpression of CRN (4.8 % ±1.0). However, CRN showed significant self-interaction already in the ER (8.3 % ± 1.0). Deleting the kinase domain (CRΔKi) increased $E_{\text{FRET}}$ (15.4 % ± 1.1), possibly due to decreased distance between the chromophores. Alternatively, the kinase domain could promote the disassembly of the protein complex. CRN homomerisation must be mediated by the TM and immediately flanking sequences. Coexpression of CLV2 relocalized CRΔKi to the PM, and resulted in even higher $E_{\text{FRET}}$ values (19.4 % ± 1.1), suggesting formation of a CRN/CLV2 complex.

We conclude that both CLV1 and CRN self-interact, and that the kinase domain is not required for CRN homodimers. CRN homomeric complexes are already formed in the ER, and are translocated in the presence of CLV2 to the PM. For CLV2, no homomerisation was detected.

**CRN interacts with CLV2 in the ER and at the PM**

The interdependence between CRN and CLV2 for PM localization suggested that both proteins may bind to each other (Fig. 5D). We found the CRN/CLV2 interaction to be the strongest we observed in all our $E_{\text{FRET}}$ measurements, giving 13.9 % ± 0.6 for CRN/CLV2 in both GFP/mCherry combinations, and 21.8 % ± 1.7 for the CRΔKi/CLV2 interaction. This suggested that the TM and juxtamembrane domains are not only essential for PM localization, but also for interaction of CRN with CLV2.
Consistent with this, CR(ΔSP-TM)-GFP and CLV2-mCherry did not interact (4.8 % ± 0.9). Further, the mutant crn-1 protein which showed reduced PM localization interacted only weakly with CLV2 (7.7 % ± 0.7). Replacing the CRN TM with that of BAK1 abolished CLV2 interaction (3.6 % ± 0.8). Interestingly, deleting the EC of CRN, which caused complete ER retention, did not affect the interaction with CLV2 (12.4 % ± 0.7). We conclude that CRN and CLV2 form complexes via their TM domains, and that the EC of CRN is required for ER exit and localization of the complex to the PM.

**CRN mediates binding of CLV2 to CLV1**

We did not detect interaction between CLV1 and CLV2 (3.2 % ± 0.5) (Fig. 5E). This is not unexpected, because CLV2 is localized to the ER (in the absence of CRN), and only a subpopulation of CLV1 is found in the ER before transfer to the PM. Deleting the CLV1 kinase should shorten the distance between the FP tags that are located at the C-termini of CLV1 and CLV2; however, no significant E\text{FRET} was observed (6.2 % ± 0.7). Importantly, when we coexpressed untagged CRN, significant E\text{FRET} between CLV1 and CLV2 was recorded (7.4 % ± 0.5). We then tested whether CLV1 can directly interact with CRN. In the absence of CLV2, we observed no significant interaction of CRN with CLV1 (5.5 % ± 0.7). Deleting the kinase domains (CRΔKi and C1ΔKi) should increase the sensitivity of the FRET assay. For these deletion constructs, we found significant interaction between CRΔKi and C1ΔKi in the ER (7.4 % ± 0.6). Upon coexpression with untagged CLV2, CLV1/CRN heteromers were now found at the PM, and E\text{FRET} values increased further (9.6 % ± 0.6 for CRN and CLV1; 12.6 % ± 0.9 for CRΔKi and C1ΔKi).
To test for the specificity of the observed interactions, we analyzed the behaviour of BAK1, a LRR-receptor kinase that was previously shown to heterodimerize with BRI1 (Russinova et al., 2004), FLS2 (Chinchilla et al., 2007) and possibly other LRR-RLKs as coreceptor (Kemmerling et al., 2007). BAK1-GFP localized to the PM as expected, but we did not observe significant \( E_{\text{FRET}} \) with mCherry tagged CLV1, CLV2, CRN, or combinations of them (Fig. 5E, SupplTable1).

Our experiments revealed that CRN binds to CLV2 via its TM. The CRN/CLV2 heteromer can interact with CLV1 at the PM. Translocation to the PM requires the EC domain of CRN. CLV2 interacts with CLV1 only indirectly, probably as part of the CRN/CLV2 heteromer. Thus, CRN mediates both the localization of the CRN/CLV2 heteromer to the PM, and also its interaction with CLV1 monomers or homomers.
Discussion

CLV3 controls stem cell proliferation in shoot and floral meristems, and requires CLV1, CLV2 and CRN for its activity. Genetic studies suggested that the CLV3 signal is transmitted by CLV1, and CLV2 with CRN in two separate receptor pathways that can crosstalk (Müller et al., 2008). It is always desirable to analyze interaction and signalling capacity of receptor proteins in their native context, i.e. in those cells where they are normally expressed. Attempts to express components of the CLV3 signalling pathways in Arabidopsis suffered from low expression signals, when the endogenous promoters were used, or from protein aggregation and degradation when strong constitutive promoters were employed. Inducible expression of receptor-FP fusions allowed complementation of the respective mutants, but in most cases only in a limited number of floral meristems, indicating integrity of the receptor fusions, and that their dosage can be critical for in vivo function. To analyze receptor localization and interaction, we therefore switched to the Nicotiana benthamiana leaf system, which allows to precisely control and monitor expression levels and to avoid any overexpression artefacts. Our transient expression studies of translational fusions with GFP and mCherry now showed that all three receptor proteins can localize to the plasma membrane, and have the capacity to undergo multiple interactions.

First, we found that CLV1 forms homomers at the PM of Nicotiana benthamiana cells. Because this interaction was revealed by FRET between differentially labelled CLV1 fusion proteins, we have no information on the exact composition of this complex. The native ligand for CLV1, CLV3, is normally expressed only from stem cells and not in epidermal cells, indicating that receptor oligomerization is not induced by ligand binding, and that CLV1 can preassemble into
binding-competent homotypic complexes. We also noted that addition of a 12 amino acid CLV3 peptide, which suffices to activate CLV signalling (Kondo et al., 2006), did not further stimulate heterotypic interactions (Suppl. Fig. 4). This indicates that the signalling mode for CLV1 differs from that of other peptide-binding LRR-RLKs, such as the flagellin receptor FLS2. Here, interaction with BAK1 together with mobility reduction was induced by flagellin, indicating that ligand binding is required to trigger heteromerization (Ali et al., 2007; Chinchilla et al., 2007). A capacity for homotypic interaction was also found for CRN, but not for CLV2. Notably, both CRN and CLV1 homomeric complexes may assemble already in the ER.

Second, CRN and CLV2 interact in the ER, and require each other for localization to the plasma membrane. Deletion mapping and domain swaps showed that the TM of CRN specifically mediates binding to CLV2. The mutation in crn-1 introduces a charged amino acid into the TM, which decreases interaction with CLV2 and also PM localization. A similar mutation in the GABAA receptor caused ER retention, but was also associated with protein degradation via the ERAD pathway (Gallagher et al., 2005), whereas crn-1-GFP fusions remained expressed at levels comparable to wildtype protein.

The CLV2 / CRN interaction is not sufficient for PM localization, but requires also the EC domain of CRN, which may serve to neutralize charged residues in the juxtamembrane domain of CLV2. Because CLV2 also remains in the ER if CRN is absent, we propose that the formation of a CLV2/CRN complex has to shield ER retention signals that are found in both proteins. Such quality control via a trafficking checkpoint was previously described for the GABA receptor. Here, a possible ER retention signal RXR at the C-terminal domain of the GB1 receptor must be shielded by GB2 for plasma membrane expression of only functionally assembled receptor.
complexes (Margeta-Mitrovic et al., 2000). Although a related sequence motif is found at the cytoplasmic tail of CLV2, it cannot be solely responsible for ER retention, because complexes between CLV2 and CRN lacking the EC domain still fail to localize to the PM. The cytoplasmic juxtamembrane domain of CRN further contributes to complex assembly in the absence of CLV3, and together with the TM and EC sequences forms the pre-ligand-binding assembly domains (PLAD) of CRN. Such receptor preassembly in the ER has been proposed to assist in signalling specificity and rapidity (Chan et al., 2000). Furthermore, it represents a mechanism to avoid surface expression of unassembled receptors. This could be especially important to safeguard against interactions at the PM between CRN and CLV2-related RLPs that respond to danger signals and trigger plant immune responses (Boller and Felix, 2009).

It is possible that both CLV2/CRN heteromers and CLV1 homomers assemble already in the ER, which could be investigated using fluorescence-based techniques such as FLIM with high local resolution (Weidtkamp-Peters et al., 2009). Given that the expression patterns of CRN, CLV2 and CLV3 overlap in meristem cells of the L3 layer, CLV3 could act there as an intracrine signal, which may serve to sharpen the boundary between stem cells and the organizing centre.

Based on our results, we propose here that CLV1 homodimers and CLV2/CRN heterotetramers coexist at the PM of shoot meristem cells (Fig. 6). Both receptor complexes should independently bind the processed CLV3 peptide, and activate intracellular signal transduction. Formation of a larger complex, comprising CLV1, CLV2 and CRN, could allow for crosstalk between the receptors, and may be necessary to fine-tune the response to CLV3 signalling. In addition to its role in CLV3 signalling, the CLV2 / CRN complex would contribute to the regulation of other
developmental pathways, including the regulation of pedicel length, anther development and root meristem growth (Müller et al., 2008).

The secreted bone morphogenetic proteins (BMPs) control many aspects of vertebrate development, and are perceived by homomeric and heteromeric receptor complexes, consisting of different receptor types. Importantly, separate signalling pathways are activated when BMP-2 binds to preassembled complexes, or when receptors are first recruited by BMP-2 (Gilboa et al., 2000; Nohe et al., 2002). We now need detailed studies on downstream events to elucidate the consequences of CLV3 signalling via different receptor complexes that comprise CLV1, CLV2 and CRN.
Materials and Methods

Plant Materials and growth conditions

*Nicotiana benthamiana* plants were grown for 4 weeks in a greenhouse under controlled conditions. *Arabidopsis* mutant lines were obtained from the Nottingham Arabidopsis Stock Centre. *clv1-11* (Dievart et al., 2003), *clv2-1* (Jeong et al., 1999) and *crn-1* (Müller et al., 2008) mutations are in a Landsberg erecta background and were described previously.

Construction of inducible receptor-fusions

The destination plasmids pABindGFP, pABindmCherry and pABindFRET were designed by inserting coding regions of GFP(S65T) (pABindGFP), mCherry (pABindmCherry) or GFP(S65T) and mCherry (pABindFRET) 3’ to the Gateway cassette of pMDC7. To create receptor fusions, attB sites were added via PCR mediated ligation to coding regions of *CLV1* (AT1G75820.1), *CLV2* (AT1G65380.1), *BAK1* (AT4G33430.1) or *CRN* (AT5G13290.2), and recombined into pDONR™201 according to the manufacturer’s instructions (Gateway manual, Invitrogen™). The following derivatives were created via PCR mediated mutagenesis: (1) CRΔEC: deletion of aa 36 – 61; (2) CR(<B1TM): exchange of aa 62 to 84 from CRN against aa 225 – 247 of BAK1; (3) CRΔKi: deletion of aa 88 – 401; (4) CR(<C1Ki) exchange of the aa 91 – 401 against the aa 665 - 980 of CLV1; (5) C2-CRKi: fusion of the aa 1 – 705 of CLV2 with aa 86 – 401 of CRN; (6) CR(ΔSP-TM): deletion of aa 1-92.

Construction of transgenic *Arabidopsis thaliana*
The transgenes were transformed into *Arabidopsis* plants via the floral dip method and selected as described previously (Stahl et al., 2009). Transgene expression was induced 4 weeks after germination by spraying with 20 µM ß-estradiol / 0.1 % Tween (three times every 48 h).

**Phenotypical Analysis**

Carpel number analysis was used to determine CLV signalling pathway activity. Mature siliques were individually observed with a dissection microscope. The siliques were examined to determine the carpel number, and a partially formed carpel was counted as one. Photographs were taken with a Canon Powershot G2 digital camera or with a AxioCam ICc3 mounted onto a Zeiss dissecting microscope. Digital photographs were collated with Adobe Photoshop™.

**Transient gene expression in *Nicotiana benthamiana* leaves**

The *Agrobacterium tumefaciens* strain GV3101 pMP90 was transformed with expression clones and cultured on dYT media supplemented with rifampicin (50 µg/ml) gentamycin (50 µg/ml) and spectinomycin (100 µg/ml). Bacterial cultures were grown, precipitated and dissolved in 5 % (w/v) sucrose, 150 µM acetosyringone, 0.01 % (v/v) silvet. To reduce gene silencing *in planta*, cultures were mixed with an *Agrobacterium* culture that allows expression of the silencing suppressor p19 (Voinnet et al., 2003), giving an OD$_{600}$ of 0.3 for each strain. Leaves of 4 weeks old *N. benthamiana* plants were infiltrated with the culture. Transgene expression was induced 48 to 96 hours after infiltration with 20 µM ß-estradiol + 0.1 % Tween20, and analyzed within 4 to 24 hours.
Confocal Microscopy

Epidermis cells were examined with a 40x 1.3 NA Zeiss oil immersion objective using a Zeiss LSM 510 Meta confocal microscopy system. GFP was excited with a 488 nm Argon laser, with emission detection through the meta-channel at 497 to 550nm. mCherry was excited at 561 nm using a Diode, and emission was detected at 572 to 636 nm via the meta-channel. $E_{\text{FRET}}$ was measured via GFP fluorescence intensity increase after photobleaching of the acceptor mCherry. Frame size was kept constant at 256 x 256 pixel, with a pixel time of 2.55 µs/pixel. A region of interest (r.o.i.) around the plasma membrane was bleached after 5 detection frames with 100 % laser intensity of the 561 nm Diode and 120 iterations. 15 frames were recorded after photobleaching. The GFP fluorescence intensity change was analyzed around the plasma membrane in the r.o.i.. Only measurements with less than 10 % GFP intensity fluctuations before acceptor bleaching were further analyzed. The percentage change of the GFP intensity directly before and after bleaching was analyzed as $E_{\text{FRET}} = \frac{(\text{GFP}_{\text{after}} - \text{GFP}_{\text{before}})}{\text{GFP}_{\text{after}}} \times 100$. FM4-64 (Invitrogen™) staining was performed at a final concentration of 20 µM for 5-20 min. A minimum of 15 measurements were performed for each experiment. Significance was analyzed using Student’s t-test.

Western Blot analyses

Approximately 0.1 g of Nicotiana benthamiana leaf tissue was homogenized with a Precellys Homogenisator (PeqLab™) for 20 seconds in 740 µl extraction puffer (0.1 M Tris-HCl pH 8.3; 5 mM DTT; 5 mM EDTA; 5 µl protease inhibitor cocktail (Sigma P9599)). After 1 h incubation at 4°C, 90 µl of 10 X SDS loading buffer (0.25 M Tris; 1.92 M glycine; 1 % (w/v) SDS) were added, heated for 10
min at 95°C and separated on an 8 % (v/v) SDS PAGE. The separated proteins were transferred to a PVDF membrane and probed with the primary α-GFP antibody (Roche™) and a secondary α-mouse alkaline phosphatase conjugated antibody (Dianova™). NBT/BCIP was used as detection substrate.

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<td>4</td>
<td>16 (33%)</td>
<td>4 (8%)</td>
<td>28 (59%)</td>
</tr>
<tr>
<td>iCLV2-FP clv2-1</td>
<td>8</td>
<td></td>
<td>2 (9%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>iCRN-FP crn-1</td>
<td>3</td>
<td>11 (55%)</td>
<td>4 (20%)</td>
<td>5 (25%)</td>
</tr>
<tr>
<td>crn-1</td>
<td>1</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>10 (100%)</td>
</tr>
</tbody>
</table>

**Table 1: Rescue of clv-signalling mutants by inducible expression of receptor-FP fusions**

Transgenic T1 plants of the indicated genotypes were estradiol induced at 4 weeks after germination. *clv1-11, clv2-1* and *crn-1* mutants carry siliques with on average 3.9 ± 0.1 carpels (Müller et al., 2008). Carpel number of ten siliques were analyzed per plant. N = number of T1 lines; C = number of complementing T1 plants, and % of total, carrying more than 1 silique with only 2 carpels (wild-typic); PC = partially complemented, carrying 1 silique with 2 carpels; NC = non complemented, producing only mutant carpels.
References


Figure 1: Inducible transgene expression rescues the corresponding mutants

(A) Speculative model for interactions of CLV3 with receptor complexes. CLV3 peptide is proposed to bind to two separate receptor systems, consisting of CLV1, and CLV2 together with CRN. CLV2 homodimers could interact with CRN via their transmembrane domains. Receptor activity restricts stem cell fate, and the separate receptor complexes may interact through their kinase domains. (B) T-DNA for inducible expression of translational fusions with the fluorescent proteins (FPs) GFP, mCherry or both. G10-90: constitutive promoter; XVE: chimeric transcription factor that activates transcription from the lexA-46 35S promoter upon estradiol induction. FPs: GFP in pABindGFP; mCherry in pABindmCherry and GFP-mCherry in pABindFRET. (C) Schematic representation of CLV1-FP, CLV2-FP and CRN-FP. (D to F) Examples of partial phenotypic restoration in clv1-11, clv2-1 and crn-1 mutants upon induced expression of the corresponding FP fusion protein. Left panel: whole plant; panel right top: higher magnification of the primary shoot, panel right below: mutant silique with 4 carpels, and rescued silique (asterisk) with 2 carpels. (D) clv1-11 carrying the iCLV1-GFP transgene. Inducing iCLV1-GFP expression lead to the formation of a single silique with 2 carpels, whereas older and younger siliques form four carpels. (E) clv2-1 carrying iCLV2-GFP. iCLV2-GFP induction restored carpel number to 2 in 4 siliques. (F) crn-1 carrying iCRN-GFP. iCRN-GFP induction led to the formation of 3 siliques with only 2 carpels, all older and younger siliques consisted of four carpels.

Scale bar = 1 cm
Figure 2: Analysis of receptor – GFP fusion protein expression

(A-B) Transient expression of iCLV1-GFP in leaf epidermis cells of *N. benthamiana*. Scale bar = 20 µm. (A) Long induction (>12 hours) of iCLV1-GFP causes formation of fluorescent aggregates (inset: close-up). (B) At 4 hours after induction, CLV1-GFP localizes predominantly to the plasma membrane. (C) Western Blot analysis of protein extracts from *N. benthamiana* leaf cells transiently expressing CLV1-GFP, CLV2-GFP or CRN-GFP. An α-GFP antibody was used for detection, sizes of protein markers are given in kDa. The PonceauS stained protein bands of Ribulosebisphosphatecarboxylase (Rubisco) is shown as loading control.
Figure 3: Intracellular localization of CLV1, CLV2 and CRN

Transient expression of fluorescent-protein tagged receptor chimeras in epidermis cells of *N. benthamiana*. Confocal section of epidermis cells either through the middle of a cell (A-A’’) (C-C’’) (E-E’’) (G-L’’)) or beneath the outer cell wall (B-B’’;D-D’’; F-F’’): (A-A’’; C-C’’; E-E’’; J-J’’;K-K’’): Receptor-GFP colocalization with FM4-64. (B-B’’;D-D’’; F-F’’): Receptor-GFP colocalization with an mCherry ER-reporter. (A-A’’): CLV1-GFP colocalizes with the FM4-64 dye at the plasma membrane and in a few transport vesicles (arrows). (B-B’’): Weak CLV1-GFP expression in the ER. (C-C’’): CLV2-GFP is found next to the PM in the ER, and does not colocalize with FM4-64. (D-D’’): Colocalization of CLV2-GFP with ER-reporter mCherry. (E-E’’): Like CLV2, CRN-GFP is not found at the PM. (F-F’’): CRN-GFP co-localization with the ER-mCherry protein. (G-G’’): Coexpression of CLV1-mCherry with CLV2-GFP or, (H-H’’): Coexpression of CLV1-mCherry with CRN-GFP does not effect their localization in the cell. (I-I’’): Coexpression of CLV2-GFP with CRN-mCherry leads to their relocation to the plasma membrane and the formation of transport vesicles (arrows). (J-J’’): CLV2-GFP colocalizes with FM4-64 at the PM if CRN is coexpressed. (K-K’’): CRN-GFP colocalizes at the plasma membrane with FM4-64 in the presence of CLV2. (L-L’’): CRN-GFP colocalized with CLV1-mCherry at the plasma membrane in the presence of CLV2.

Scale bar = 20 µM
Figure 4: CRN localization at the PM requires the TM domain and adjacent sequences

Transient expression of receptor-FP fusions in Nicotiana benthamiana. Confocal section through the middle of an epidermis cell. First to third column: Coexpression of CRN-GFP or derivatives with CLV2-mCherry. Fourth column: Localization of CRN-GFP or derivatives in absence of CLV2, PM labeling with FM4-64 (red). (A-A''): CRN with CLV2 localize to the PM. (B-B''): CR(ΔSP-TM)-GFP is found in the cytoplasm and nucleus, CLV2 is now found in the ER. (C-C''): The point mutation in crn-1 reduces PM localization. (D-D''): The TM of BAK1 is insufficient to replace the TM of CRN. (E-E''): Deleting the EC of CRN abolishes PM localization of CRN and CLV2. (F-F''): Exchanging the CRN kinase domain against the CLV1 kinase domain weakly interferes with PM localization. (G-G''): Deleting the CRN kinase domain does not affect PM localization. (H-H''): Fusion of CLV2 to the CRN kinase domain abolishes PM localization. Insets: close ups
**Figure 5: Receptor interaction revealed by E\textsubscript{FRET}**

(A): Principle of E\textsubscript{FRET} measurement by acceptor photobleaching. Fluorescence intensities of GFP and mCherry are recorded. E\textsubscript{FRET} is calculated as relative increase of GFP fluorescence intensity (%) after photobleaching of the mCherry FRET acceptor. (B): E\textsubscript{FRET} control measurements: GFP background fluctuations of C1-G, C2-G and CR-G at the plasma membrane, and measurement of intramolecular FRET. (C): Receptor homomers. (D): Capacity of CRN deletion derivatives and domain swaps to interact with CLV2. (E): Formation of receptor heteromers.

Grey bars show mean values, with standard error indicated. Asterisks mark E\textsubscript{FRET} levels significantly different from GFP fluorescence fluctuation.

Red line at 4 % indicates background fluctuation level of GFP. C1 = CLV1; C2 = CLV2; CR = CRN; B1 = BAK1; C = mCherry; G = GFP; squared brackets: coexpression of unlabelled protein; TM = transmembrane domain; Ki = kinase domain; <-> domain exchange
**Figure 6: Model of CLV receptor complexes**

A CRN dimer interacts with two CLV2 receptors via the TM domain. Juxtamembrane sequences are required to secure interaction, and for localization of the complex at the plasma membrane (left). CLV1 forms homodimers (right), which can also bind the tetrameric CLV2/CRN complex (middle). This interaction is mediated by CRN. Binding of CLV3 to the three complexes may trigger different signal transduction cascades.