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Membrane distributions of two ligand-binding receptor complexes in the CLAVATA pathway

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Genetic studies have suggested that transmembrane proteins CLAVATA1 (CLV1), CLV2, CORYNE (CRN), BAM1 and BAM2 all play a role in relaying the CLV3 signal and thus regulating stem cell homeostasis at the shoot meristem (SM). The extracellular domain of CLV1 was previously shown to bind the CLE peptide derived from CLV3, providing direct evidence that CLV3-CLV1 function as a ligand-receptor pair. How the other putative receptors function in the CLV pathway, however, remained unclear. We demonstrated in a recent *Plant Journal* article that the receptor-like protein CLV2 and the receptor-kinases BAM1 and BAM2 also bind to the CLV3 CLE peptide ligand with an affinity similar to that of CLV1. Critically, these ligand binding receptors form two distinct complexes in both transient expression in tobacco and in Arabidopsis meristem cells: a CLV2/CRN multimer and a CLV1/BAM multimer. Here we examine in detail the subcellular membrane partitioning for the receptor proteins in transient expression by two-phase partitioning and co-expression with known subcellular markers. All tested proteins measurably accumulate at the plasma membrane. While CLV1 primarily co-localizes with a plasma membrane marker, CLV2 shows greater co-localization with an endoplasmic reticulum (ER) marker.

Key words: CLAVATA2, CLAVATA1, CORYNE, subcellular localization, plasma membrane, endoplasmic reticulum, receptor complex, meristem

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The CLV signaling pathway regulates stem cell specification at the SM by regulating the asymmetric nature of L3 stem cell divisions, indirectly promoting differentiation of lateral stem cell daughters in the L1 and L2 layers.[1,2] Known components of this pathway include the CLV3 proprotein for the CLE peptide ligand,[3-5] the leucine-rich repeat receptor kinase (LRR-RK) CLV1,[6,7] and its close homologs BAM1 and BAM2,[8,9] the LRR receptor-like protein CLV2,[10,11] the transmembrane kinase-related protein CRN,[12,13] the type 2C protein phosphatases POLTERGEIST (POL) and PLL1,[14-16] and the homeodomain transcription factor WUSCHEL (WUS).[17,18] The CLV3 signal is believed to be perceived by the membrane-localized receptors and relayed through POL and PLL1, leading to the suppression in the L3 stem cells of *WUS*, which functions to promote stem cell identity in neighboring cells. Biochemical mechanisms of CLV signal perception and transduction are largely unknown. Our recent study in *Plant Journal*[19] presents evidence of ligand perception by different receptors and the formation of distinct receptor complexes, providing insights into the mechanisms of signal perception and receptor activation in the CLV pathway.

Using co-immunoprecipitation (co-IP) analysis in both transient expression in tobacco and in vivo in Arabidopsis meristem tissue, we demonstrated that CLV1 forms stable homomultimers and heteromultimers with BAM1 and BAM2, and that CLV2 forms a stable complex with CRN.[19] While the CLV2-CRN complex has been reported by two other studies using fluorescent-based association assays in transient expression systems, both of these studies also suggested the formation of a larger CLV1-CRN-CLV2 complex[20,21] and one study reported CLV1-CRN interaction,[20] raising the question of whether

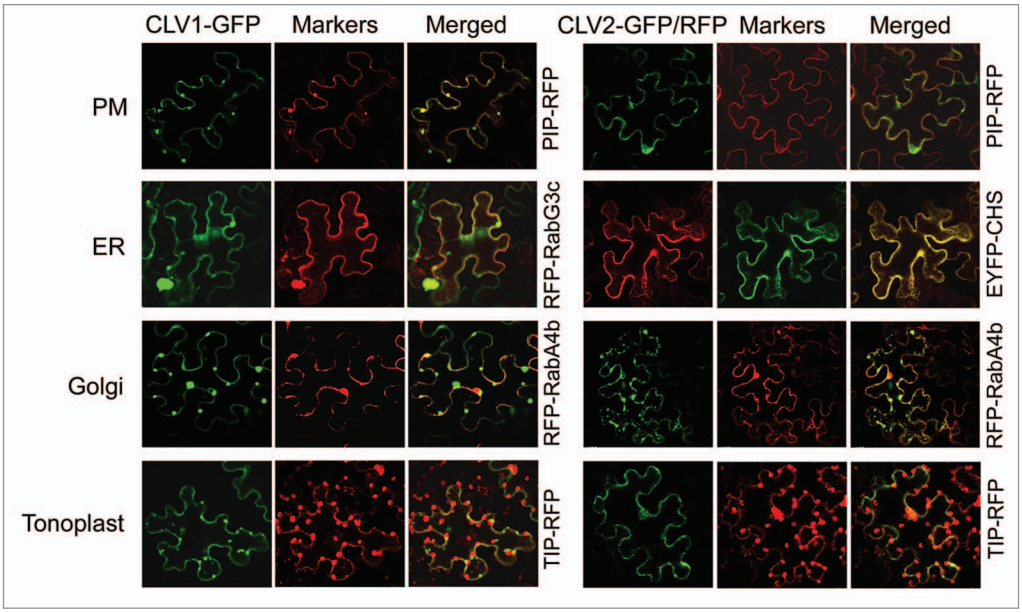

Figure 1. Co-localization of CLV1 and CLV2 with fluorescent markers in *N. benthamiana* leaves. Shown are confocal images of fluorescent-tagged receptors and co-expressed fluorescent markers two days post-inoculation. Tobacco leaf infiltration and confocal analysis were performed as described[19] except that leaves were viewed at a 40x magnification.

CLV2-CRN and CLV1 function in two distinct complexes or in one large supercomplex. By using a quantitative approach to measure the efficiency of co-IPs, we showed that the interactions between components of the two complexes such as CLV1-CLV2, CLV1-CRN, BAM1-CLV2 and BAM2-CLV2 are significantly weaker in transient expression and not detectable in vivo.[19] Consistent with a model that the two ligand-binding receptor complexes function independently from each other, we showed that ectopic expression of BAM1, BAM2 and to a less extent, CLV1, rescued the *clv2* mutant phenotype, suggesting that BAM and CLV1 can replace CLV2 function in vivo.[19]

If CLV2 forms a distinct complex with CRN, what then is the role of the large CLV2 extracellular domain? We found that CLV2 binds to the CLV3 CLE ligand with the same affinity as CLV1. Because CLV2 lacks a cytosolic signaling domain, CLV2 perception of CLE is likely relayed through its stable association with the transmembrane kinase-related protein CRN in vivo. CLV1/BAM on the other hand, contains both extracellular CLE-binding domains as well as intracellular protein kinase domains to relay signal. Thus, the CLV pathway features two distinct ligand-binding complexes with similar ligand affinity, a novel mode of signal perception.

Consistent with the predicted plasma membrane (PM) localization of the receptors, in our recent study we showed that when transiently expressed in tobacco leaves, all tested CLV receptor proteins accumulated in the membrane fraction and GFP/RFP-fused proteins showed fluorescence primarily at the cell periphery.[19] To further examine the subcellular localizations of the receptors, we co-expressed CLV1 and CLV2 with fluorescent markers for various membrane localizations, including a trans-Golgi marker RFP-RabA4b,[22] a tonoplast marker TIP-RFP,[23] a PM marker PIP-RFP,[23] and endoplasmic reticulum (ER) markers EYFP-CHS[24] and RFP-RabG3c[22] (**Fig. 1**).

We observed that CLV1-GFP co-localized largely with the PM marker PIP-RFP and to a lesser extent with the ER maker RFP-RabG3c. CLV2-RFP co-localized primarily with the ER marker EYFP-CHS. Neither CLV1-GFP nor CLV2-GFP co-localized well with the tonoplast or the Golgi marker (**Fig. 1**). Fluorescence at ER could be an effect of overexpression from accumulation of newly synthesized receptor proteins as they transport to the PM. ER localization of CLV receptors, on the other hand, could also be a regulatory step during receptor activation and signal transduction. A recent study using the similar tobacco transient system indicated that CLV1, CLV2 and CRN all showed ER localization, CLV2 and CRN were predominantly ER-localized and co-expression of CRN and CLV2 brought both proteins to the PM.[21] Interestingly, CLV signaling function requires the ER-resident molecular chaperone SHEPHERD proteins.[25] Furthermore, the ER quality control machinery is emerging as an important facet of brassinosteroid receptor function.[26,27]

To further test membrane localization of the CLV receptors, we performed two-phase partitioning on membrane fractions (**Fig. 2**). Protein gel blot analysis showed that CLV1-GFP, CLV2-GFP, CRN-GFP, BAM1-GFP and BAM2-GFP all partially partition in the upper phase which is highly enriched for plasma membranes, as demonstrated by the PM-localized PIP2 control.[23] However, we also observed significant accumulation for all receptors in the lower fraction, which is enriched for chloroplast, mitochondrial and ER membranes, as exemplified by the ER-localized BIP control.[28]

Consistent with data from marker co-localization, CLV1 and the related BAM receptors were detected more strongly

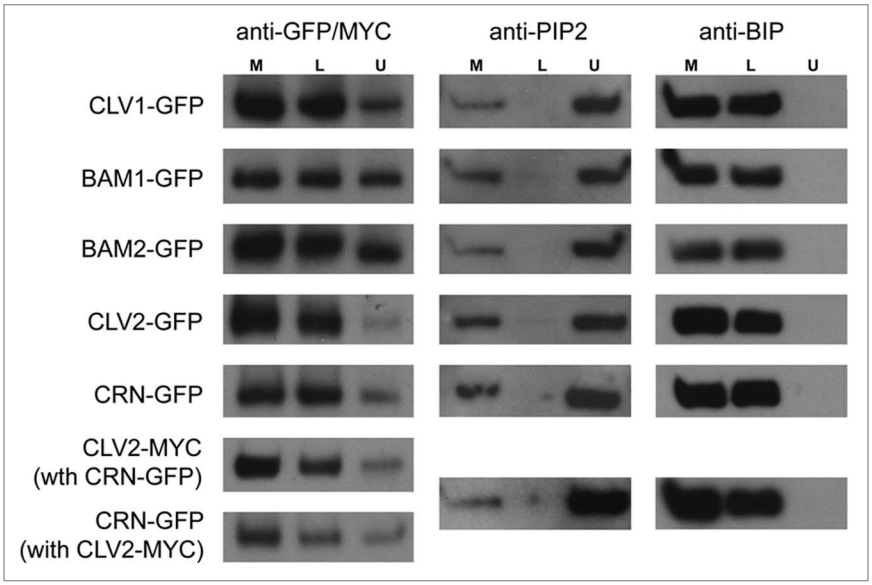

Figure 2. Two-phase partitioning of membranes isolated from tobacco leaves expressing different receptor proteins. Data from CLV2-GFP alone, CRN-GFP alone and CLV2-MYC/CRN-GFP co-expression are shown. Two-phase partitioning[14] and protein gel blot analysis[19] were performed as described. The rabbit anti-PIP2 [29] and mouse anti-BiP (SPA-818; Stressgen) antibodies were used to detect the PM and ER marker proteins. M, total microsomal fraction; U, upper phase enriched in PM proteins; L, lower phase depleted of PM.

in the plasma membrane compared to CLV2 and CRN. In addition, we did not observe significant increase in PM partitioning for CLV2 or CRN when they were co-expressed, which is in contradiction to a previous report[21] (**Fig. 2**). The discrepancy between our results and the Bleckmann et al.[21] study could be the result of different experimental approaches and different expression strategies in the tobacco transient system (inducible promoter in their study versus 35S promoter in this study).

In summary, as an interesting addition to our recent study, the confocal and two-phase partitioning data presented here support the idea of partial PM localization of the CLV pathway receptors. However, significant accumulation in interior membranes was also observed, especially for CLV2 and CRN. To what extent these localizations in transient expression reflect in vivo membrane partitioning of these proteins in meristem cells and how this might be affected by signaling activation are critical questions for future studies.

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