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An Evolutionarily Conserved Pseudokinase Mediates Stem Cell Production in Plants

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Sequence comparisons, biochemical experiments, and studies with mutants in transgenic plants show that the *Arabidopsis* protein CORYNE, currently thought to be a kinase that acts as part of a receptor kinase complex, is likely to be a pseudokinase and not a kinase.

The apical stem cell niche in plants is restricted by a ligand and receptor-activated feedback loop that regulates accumulation of the transcription factor WUSCHEL (WUS) (Ceresa and Schmid, 2000). Binding of the secreted peptide ligand CLAVATA3 (CLV3) to the CLV1 leucine-rich repeat Ser-Thr kinase is central to this process. CLV2, a transmembrane leucine-rich repeat protein lacking an internal kinase domain, is also important for perception of CLV3 and other CLV3-like ligands throughout the plant (Wang and Fiers, 2010). In *Arabidopsis thaliana*, CORYNE (CRN), which encodes a predicted transmembrane Ser-Thr kinase with a short extracellular domain, has been shown to act with CLV2 in this process and may regulate CLV2 localization (Müller et al., 2008; Bleckmann et al., 2010). Because mutations in *CRN* and *CLV2* are additive with *CLV1* mutants, it has been proposed that CLV2 and CRN functionally assemble into a signaling complex that functions as a receptor kinase in parallel to CLV1 (Müller et al., 2008). The CLV/CRN-WUS circuit is conserved across diverse taxa, and determining how its signaling is activated is necessary for the understanding of plant stem cell regulation. Here, we demonstrate that CRN is unlikely to be a kinase and is likely a pseudokinase, indicating that the existing model needs revision.

CRN CONTAINS FEATURES OF A PSEUDOKINASE

CRN contains several features suggestive of nonfunctional kinases as described by Boudeau et al. (2006). CRN contains a His-Tyr-Asn (HYN) motif in the presumed catalytic loop instead of the His-Arg-Asp (HRD) motif typical of functional kinases and therefore lacks the critical active-site Asp (Figure 1A). The G-loop, GXGXXG, which binds and positions ATP, has diverged in CRN to GXDXXG. Similar mutations in VRK3 family members render the G-loop acidic, inhibiting ATP binding (Scheeff et al., 2009). The activation segment of CRN is shorter (16 amino acids between the DCG and APE motifs, compared with 20 to 35 in most active kinases in the National Center for Biotechnology Information conserved domain database), which should lead to a truncation of either the activation loop or the P+1 loop. Lastly, DCG replaces the DFG motif of the Mg^{2+} binding loop. Mutation of Phe is correlated with alterations in the positioning of the activation segment relative to the catalytic loop, affecting positioning of the upper and lower lobes (Nolen et al., 2004).

CRN LACKS KINASE ACTIVITY

To test experimentally the prediction that CRN is a kinase, we performed in vitro kinase assays with CRN and CLV1, a known Ser-Thr kinase, using purified wild-type CRN and CLV1 and mutants in the active site Lys of each (K146E and K720E, respectively). Of three CRN splice variants in the database, we chose the variant AT5G13290.2 as this represents the full kinase domain (see below). As expected, CLV1 autophosphorylated, while the $CLV1_{K720E}$ mutant did not (Figure 1B). No activity was seen for either CRN protein, suggesting that wild-type CRN lacks autokinase activity under standard conditions.

CRN DOES NOT REQUIRE KINASE ACTIVITY FOR WILD-TYPE FUNCTION IN VIVO

We next tested the ability of *CRN* and CRN_{K146E} to complement the *crn-1* mutant. There are no known *crn* null mutants. The *crn-1* allele is fully recessive, and over-expression of *crn-1* has no effect in wild-type plants, indicating the *crn-1* allele is a loss-of-function allele (Müller et al., 2008). We generated in-frame translational fusions of the CRN variants fused at the C terminus to mTFP1 by a 9-Ala linker. When expressed from the endogenous *pCRN* promoter (2.6 kb of upstream and 0.9 kb downstream), both *pCRN:CRN-mTFP1* and $pCRN:CRN1_{K146E}$-*mTFP1* complemented the *crn-1* mutant fully, indicating that CRN does not require kinase activity for wild-type function (Figures 1C and 1D). Similar results were obtained with fusions to TAG-red fluorescent protein (TAG-RFP), although no fluorescence signal was visible in any fusion class, consistent with previous results (Bleckmann et al., 2010). In total, more than 30 independent *pCRN*: $CRN1_{K146E}$ T1s displayed complete complementation of the *crn-1* mutant. Expression of endogenous *CRN* and the *CRN-TagRFP* transgene were confirmed by quantitative RT-PCR in reference lines (Figure 1E).

CRN HOMOLOGS ENCODE APPARENT PSEUDOKINASES

CRN homologs have been identified in taxonomically diverse species, although functional data exist for *Arabidopsis* CRN only (Miwa et al., 2009). All CRN homologs examined encoded apparent
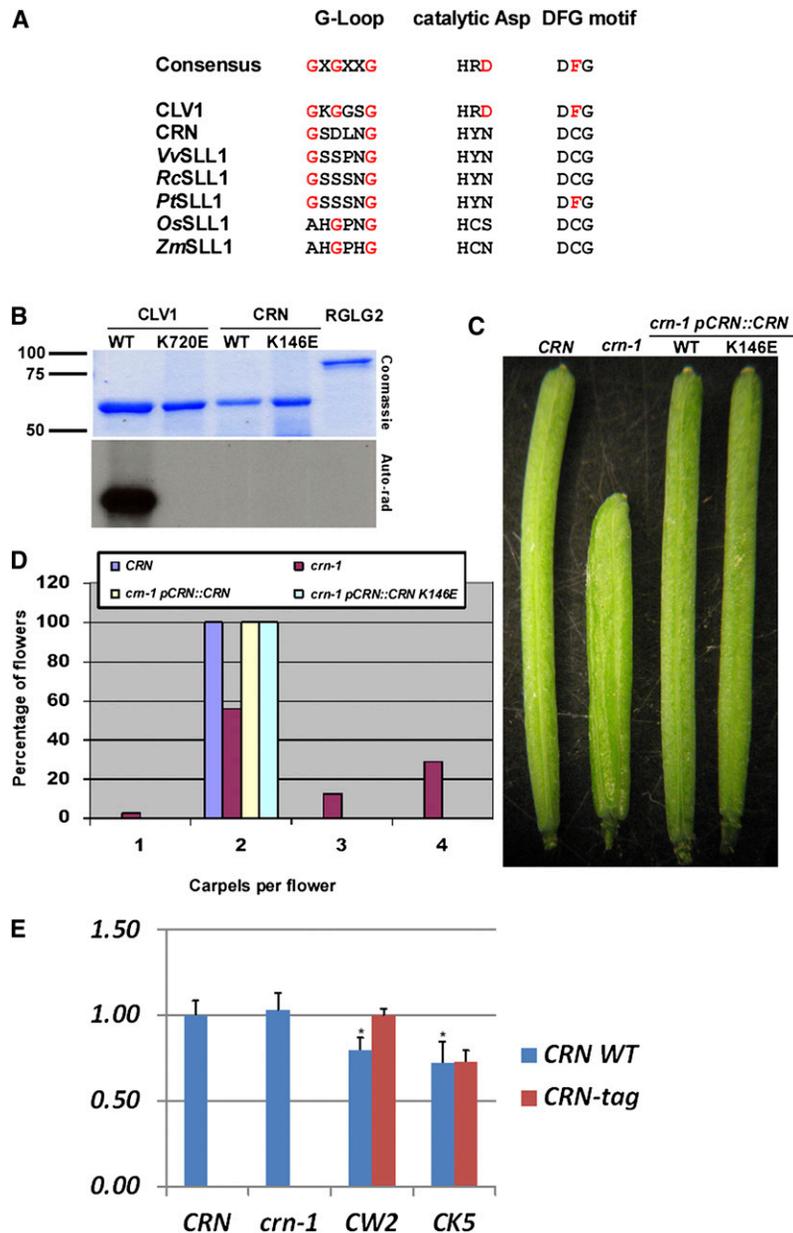
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Figure 1. CRN Encodes an Apparent Pseudokinase.

(A) CRN lacks consensus residues necessary for kinase activity in known kinases. Alignment of critical CRN residues deviating from the consensus kinase sequences. CLV1 is included as an example of an active kinase. Other sequences refer to CRN homologs (referred to as SOL2-like1 [SLL1]) as identified by Miwa et al. (2009). *Zm*SLL1 represents *Zea mays* sequence NP_001142196.

(B) CRN lacks autophosphorylation activity. Coomassie blue–stained gel of purified CLV1 and CRN kinase domains. GST-tagged CLV1 or CRN were purified using standard purification techniques, eluted, and allowed to autophosphorylate on beads in the kinase assay buffer (see Methods). Purified protein was then eluted and subjected to SDS-PAGE, followed by staining with Coomassie blue (top panel), and processed and exposed to film for autoradiography (bottom panel). GST-RGLG2, a ubiquitin ligase (Yin et al., 2007), was used as a negative activity control. WT, wild type.

(C) Kinase activity apparently is not required for CRN function in planta. *crn-1* plants (L*er* background) were transformed with either wild-type *CRN* or the *K146E* active-site mutant of CRN driven by *pCRN* (see Methods for details).

(D) Kinase activity apparently is not required for CRN function in planta. Quantification of carpel number in flowers from the wild type (L*er*), *crn-1*, *crn-1 pCRN: CRN-WT*, or *pCRN:CRN-K146E*. Flowers 3 to 21 were counted for individual plants or T1s, and the total number of flowers was combined. Graph represents

pseudokinases, arguing that CRN function has been conserved independent of kinase activity for at least 150 million years, perhaps predating the monocot/dicot divergence (Figure 1A). It is therefore implausible that the CRN/CLV2 complex transmits CLV3 signals using a mechanism analogous to that of CLV1. *CRN* gives rise to two additional splice variants (AT5G13290.1 and AT5G13290.3) that contain large deletions of the kinase catalytic domain, including the entire ATP binding G-loop and a significant portion of the VAVK motif of subdomain II. Thus, none of the three *CRN* splice variants would appear to give rise to active enzymes with kinase activity.

CLUES TO CRN FUNCTION

Evolutionarily conserved pseudokinases play a role in several Tyr ligand-receptor signaling systems in animals. Pseudokinase roles include steric inhibition of active kinases and scaffolding functions (Boudeau et al., 2006). The additive nature of *crn* and *clv1* alleles suggests that CRN may play a scaffolding role, perhaps to aid export of CLV2 to the plasma membrane and/or to assemble higher-order CLV1 or CLV1 substrate complexes. Consistent with our findings, the CRN kinase-related domain is not necessary for plasma membrane accumulation of CLV2 in transient expression assays (Bleckmann et al., 2010). Previous genomic analysis has suggested that up to 20% of all *Arabidopsis* receptor kinases, including CRN and members of the SUB family, encode kinase-defective variants (Chevalier et al., 2005; Castells and Casacuberta, 2007). Elucidating the role of CRN in CLV1/CLV2 perception of CLV3 perception should provide a model for understanding the function of this large family of kinase homologs in plants.

METHODS

Vector Construction

The *CRN* cDNA open reading frame (AT5G13290.2) was amplified from Columbia-0 RNA using CRN forward (Fwd) primer (5′-GGCCATGGGGATCC-ATGAAGCAAAGAAGAAGAAGAAATGG-3′) and CRN reverse (Rvs) primer (5′-GCCATGGATCG-GGCTGCCGCAGCGGCAGCAGCCGCAGCAG-GAAAGCTGTGCAGTTGTGTAAGCATG-3′) that generates a NcoI site at either end of the PCR product, deletes the endogenous stop codon, and adds a linker encoding a Pro and nine Ala residues. PFU1 II Ultra was used to minimize mutations (Stratagene). The K146E mutation was generated by recombinant PCR using the CRN Fwd and CRN K-E Rvs primer (5′-CAAGT-GAGCCTAGTCTTTCGACTGCAACCACTAG-3′) and the CRN Rvs primer and CRN K-E Fwd primer (5′-CTAGTGGTTGCAGTCGAAAGACTA-GGCTCACTTG-3′) in the first round of amplification, followed by PCR amplification of the first round products with the CRN Fwd and CRN Rvs primers using the first round products to generate the *CRN K146E* full-length mutant. The resulting PCR product was subcloned into pCR2.1 (Invitrogen) and sequenced. These products were cloned into a pBJ based shuttle vector containing either mTFP1 or TAG-RFP vector modified to contain a unique NcoI site to allow in-frame fusions at the N terminus to generate a C-terminal tagged *CRN Bam*HI fragment. The CRN promoter (Pro) was generated by recombinant PCR using CRN Pro Fwd (5′-GGGCGGCC-GCGGAGATAAATGAAGCTATTTTTCTTCTCG-3′) and CRN *Bam*HI Rvs (5′-AGTACGTTGGGGGA-TCCTGCTGCTTCTACGAATAAAAG-3′) and CRN Pro Rvs (5′-GGGCGGCCGCGTAAGTTCTTGTA-GAATCCCCAATACGTG-3′) and CRN *Bam*HI Fwd (5′-CTTTTATTCGTAGAAGCAGCAGGATC-CCCCAACGTACT-3′) in the first round, followed by amplification using CRN Pro Fwd and CRN Pro Rvs using the first-round products. This fragment was chosen as it has been shown to provide complementation of the *crn-1* mutant (Müller et al., 2008). This was cloned into pCR2.1, sequenced, and cloned as a *Not*I fragment into a modified pMOA33 (Barrell and Conner, 2006)

in which the *Bam*HI site was destroyed, thus generating *pCRN*. The *CRN* fusions were then cloned into *pCRN* as *Bam*HI products.

Generation of Transgenic Plants and Plant Growth

pCRN constructs were transformed into Landsberg *erecta* (L*er*) or the *crn-1* (Müller et al., 2008) mutant using the GV3101 *Agrobacterium tumefaciens* strain and the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on B5 media plates containing 30 μg/mL kanamycin and transferred to soil and grown as described before (Clark et al., 1993).

In Vitro Kinase Assays

The regions corresponding to amino acids Met-662 to Phe-980 for CLV1 and Val-85 to Phe-401 for CRN were PCR amplified. For the kinase dead version of these proteins, the catalytic Lys of CLV1 (Lys-720) was mutated to Glu (K720E) and for CRN Lys (Lys-146) was mutated to Glu (K146E) using a recombinant PCR strategy. These products were TOPO cloned into pENTRD/TOPO and moved into pDEST15 by LR recombination to generate N-terminal glutathione *S*-transferase (GST) fusions. Proteins were expressed in BL21-AI at room temperature for 4 h following the manufacturer's recommendations (Invitrogen). GST-CLV1, GST-CRN, and GST-RGLG proteins were purified with the Glutathione Sepharose 4B affinity matrix.

For the kinase assay, proteins still bound to the GST beads (GE Life Sciences) were quantified, and the appropriate bead volume was used to give a total of 1 μg of bound protein for each kinase reaction. For each reaction, 0.5 mCi of [γ-^{32}P]ATP (MP Biomedicals) was added to the kinase reaction buffer (20 mM Tris-HCl, 10 mM MgCl$_2$, 10 mM MnCl$_2$, 1 mM DTT, and 10 mM cold ATP) and incubated at room temperature or 1 h. The beads were washed three times in cold kinase reaction buffer (lacking ATP) and resolved by SDS-PAGE electrophoresis. After electrophoresis, the gel was stained with Coomassie Brilliant

Figure 1. (continued).

percentage of flowers displaying different carpel numbers. Six plants were counted for L*er* and *crn-1*, totaling 120 flowers each. Nine T1s were counted for *pCRN:CRN-WT*, totaling 180 flowers. All *pCRN:CRN-WT* plants displayed full complementation. Fifteen T1s were counted for *pCRN:CRN-K146E*, totaling 300 flowers. All *pCRN:CRN-K146E* lines displayed full complementation. Similar results were obtained with *pCRN:CRN-WT* and *-K146E* lines tagged with TAG-RFP (data not shown).

(E) Endogenous *crn-1* and CRN transgenes are coexpressed in transgenic lines. Quantitative real-time PCR of endogenous *CRN* transcripts (*CRN WT*) and transgenic CRN (*CRN-tag*). CW2, complementing *pCRN:CRN WT-TagRFP* line; CK5, complementing *pCRN:CRN K146E-TagRFP* line. See Methods for RNA extraction and transcript quantitation. No CRN-tag transcripts were detected after 40 cycles in *CRN* or *crn-1* plants. The asterisk denotes a P value < 0.05.

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Blue R 250 (Bio-Rad) and dried onto Whatman 3-mm chromatography paper. The dried gel was exposed to film for 18 h.

Gene Expression Analysis

RNA was harvested from inflorescence tissue using the RNeasy Mini Kit (Qiagen). cDNA was synthesized from 1 mg DNase1-treated (Invitrogen) total RNA using SuperScript II (Invitrogen). Quantitative real-time PCR was done using SYBR green (Quantance; SensiMix). Data were analyzed using the $\Delta\Delta$Ct method and normalized with the expression of the reference genes tubulin and NM_128399. Wild-type *CRN* levels were calculated in reference to wild-type *CRN* (L*er*), and *CRN-TAG* levels were calculated in reference to transgenic line CW2. For the quantification of wild-type *CRN* transcript, the following primers were used: CRN-WT Fwd, 5′-AGACCGGC-CTTCAAGTGATGA-3′; and CRN-WT 3′UTR, 5′-GAATATATTGATGCAACTGCAGATG-3′. For the quantification of *CRN-TAG* transcript, the CRN-WT Fwd and CRN-TAG Rvs (5′-GTTGTTCACG-GTGCCCTCCA-3′) primer pair was used. Reference genes were quantified using the primers Tubulin F primer, 5′-AAACTCACTACCCCCAG-CTTTG-3′; Tubulin R primer, 5′-CACCAGACAT-AGTAGCAGAAATCAAGT-3′; NM_128399 Fwd, 5′-GGATTTTCAGCTACTCTTCAAGCTA-3′; and NM_128399 Rvs, 5′-TGCCTTGACTAAGTTGACACG-3′.

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