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ADDENDUM

The *Medicago truncatula* CLAVATA3-LIKE CLE12/13 signaling peptides regulate nodule number depending on the CORYNE but not the COMPACT ROOT ARCHITECTURE2 receptor

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ABSTRACT

We previously showed that the *rdn1* and *sunn* supernodulation mutants of *Medicago truncatula* respond differentially to overexpression of the rhizobial CLAVAT3/EMBRYO SURROUNDING REGION (CLE) signaling peptides MtCLE12p and MtCLE13p, allowing the order of action of the genes to be determined in the autoregulation of nodulation (AON) signal transduction pathway. We tested the same gene constructs that lead to the production of proteolytically processed peptides (indicated by a p after the name) in plants mutant for two other proteins that control nodule number (CRN and CRA2) and were able to determine that CRN is involved in the same signaling pathway as MtCLE12p and MtCLE13p, while regulation in CRA2 mutants responds normally to the peptides, suggesting CRA2 likely signals separately from SUNN, RDN1, and CRN. Based on the analysis of the double mutant of *cra2-2* and *sunn-4*, we also confirm recent findings that CRA2 acts independently of SUNN in nodule number regulation.

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Introduction

CLE peptides are involved in many signal transduction pathways affecting plant root growth and development (for review see[1,2]). A subset of CLE peptides in legumes have been shown to be involved in the autoregulation of nodulation (AON). In soybean, *Lotus japonicus* and *Medicago truncatula*, some CLE peptides negatively regulate nodule development[3-7] and evidence supports a model in which the peptides genes are upregulated in the nodule meristem and peptides transported to receptors in the shoot of the plant.[6,7] A subsequent signal back to the root results in a halt to further nodule development, and recent findings implicate cytokinins[8] and a microRNA[9] in this signal.

We showed that overexpression of the *M. truncatula* nodulation regulatory peptides MtCLE12p and MtCLE13p in *rdn1-2* hypernodulation mutants yielded genetic evidence that the hydroxyproline O-arabinosyltransferase enzyme encoded by *RDN1* modifies MtCLE12p. Further, that modification was necessary for regulatory signaling by MtCLE12p but not signaling by MtCLE13p.[10] The receptor kinase SUNN, mutation of which also cause hypernodulation,[11] was shown genetically to be the receptor for both MtCLE12p and MtCLE13p and *sunn-4* plants were used as a negative control unresponsive to the peptides.[10] In *M. truncatula, SUNN* has the highest homology to *CLV1* in Arabidopsis.[12]

Two other molecules are predicted to associate with SUNN. The pseudokinase CRN has been shown by bimolecular fluorescence complementation to associate with SUNN, and mutations in *CRN* co-segregate with an increased nodule phenotype.[13] Likewise, CLV2 associates with both SUNN and CRN[13] and mutations in *CLV2* cause an increased nodule phenotype.[14] Evidence from Arabidopsis also indicates that CLV1, CLV2 and CRN associate with each other and signal together in some pathways.[15–18]

In contrast, mutation of the *CRA2* receptor kinase gene in *M. truncatula* has the opposite effect of *CRN* gene mutations, reducing nodule number in a systemic manner.[19] Genetic evidence suggests the CRA2 is the receptor for the CEP1 peptide.[20] The CEP peptides are part of a signaling system for nitrogen demand.[21] Since high nitrate reduces or eliminates nodulation, the authors of the work above postulated that CRA2 might be involved in SUNN nodule regulatory signaling as well.[20]

Based on this data, we hypothesized that constitutive expression of *MtCLE12* and *MtCLE13* could require *CRN* to regulate nodule number. If *CRA2* is involved in *SUNN* regulatory signaling, we postulated constitutive expression of *MtCLE12* and *MtCLE13* would affect systemic nodule number signaling in *CRA2* mutants as well. We performed the same experiments in[10] in a *M. truncatula crn* mutant and the *cra2-2* mutant identified in.[19] Before beginning, we confirmed that we could rescue the hypernodulation phenotype of the *crn* mutant used in[13] with the *CRN* message, proof that the *Tnt1* insertion in the *crn* mutant is the cause of the phenotype. Testing both mutants allowed us to determine that cross talk between the nodule regulatory pathway and the nitrogen demand signaling pathway does not involve the *M. truncatula* rhizobia-induced CLEs, despite the fact that both *sunn* and *rdn1* mutants have altered phenotypic plasticity in response to nitrate in the absence of rhizobia.[22] Phenotypic analysis of the *sunn-5; cra2-2* double mutant further confirmation this finding of independence.

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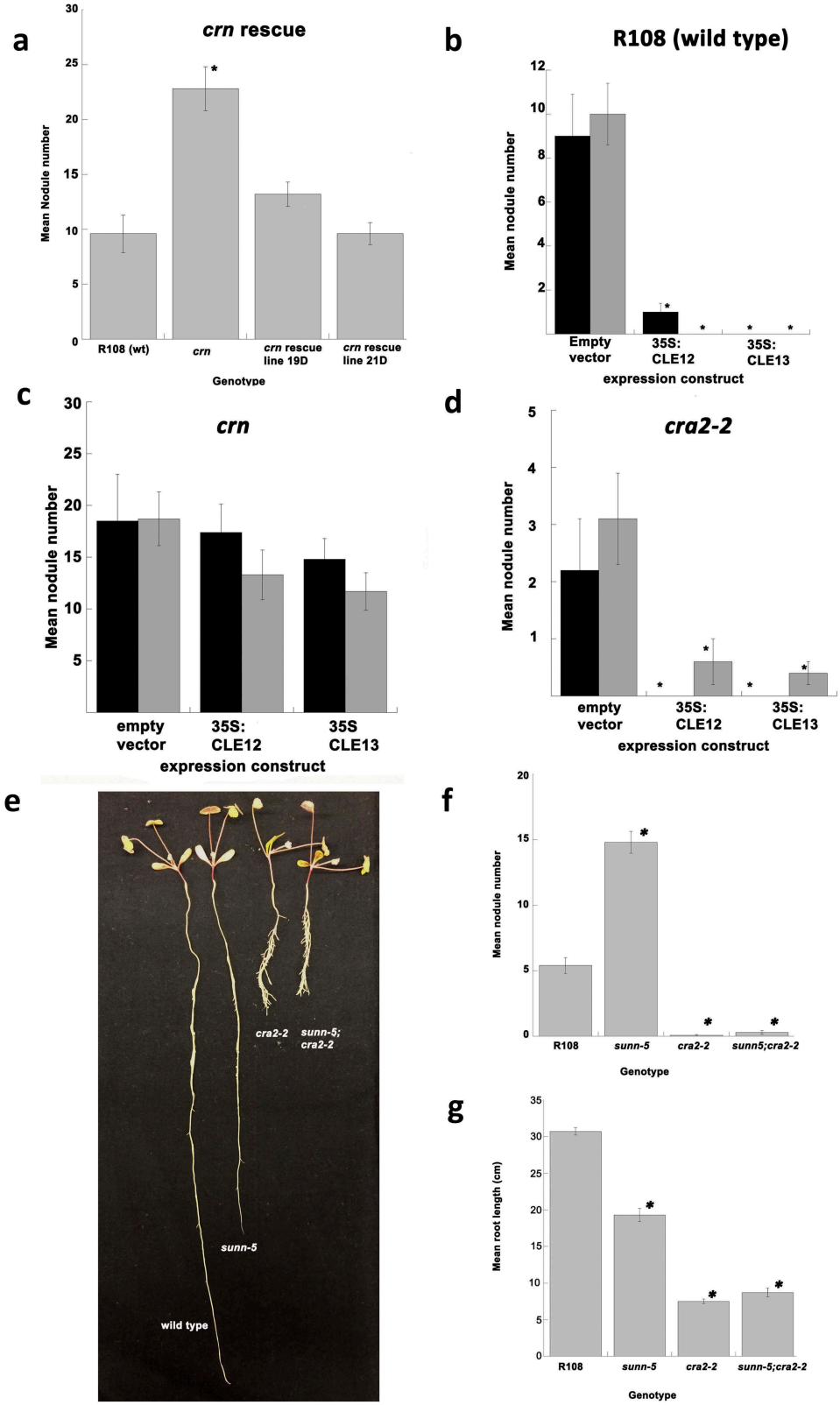

Figure 1. MtCLE12 and MtCLE13 are dependent on CRN but not CRA2 for AON signaling. (a) Expression of wild type *CRN* in *crn* plants rescues the mutant phenotype. Nodule number in wild type R108 and the mutant *crn* line compared to progeny of each of two lines from independent transformations of *crn* mutants expressing wild type *CRN* under the CaMV 35S promoter (21D and 19D). (n = 10–12 per genotype). * indicates significant difference from wild type as determined by Student's t-test, $p < 0.001$. (b and c) *MtCLE12* and *MtCLE13* overexpression effects depend on *CRN*. Data are mean number of nodules 14 days post inoculation with *S. medicae* in transgenic hairy roots constitutively expressing *MtCLE12* or *MtCLE13* under the CaMV 35S promoter. Grey and white bars indicate the results of two independent experiments. (b) Wildtype R108, n = 8–10 plants per construct per experiment (c) *crn* n = 4–8 plants per construct per experiment (d) *MtCLE12* and *MtCLE13* overexpression effects do not depend on *CRA2*. *cra2-2* n = 5–9 plants per construct per experiment. Error bars indicate standard error of mean, * indicates significance of group from empty vector control, Student's t-test, $p < 0.01$. (e) The *cra2-2;sunn-5* double mutant displays the *cra2-2* phenotype. Photo left to right is of wild type, *sunn-5*, *cra2-2* and *sunn-5;cra2-2* double mutant plants. (f) Nodule phenotypes of F2 progeny of *cra2-2* crossed to *sunn-5*. Error bars indicate standard error of mean, * indicates significance of group from wild type as determined by Student's t-test, $p < 0.001$. (n = 10 (wt) 15 *(sunn-5)* 14 (*cra2-2*) and 16 (*sunn-5;cra2-2*). (g) Root length phenotypes of the same genotypes, uninoculated. (n = 10–16). Error bars indicate standard error of mean, * indicates significance of group from wild type as determined by Student's t-test, $p < 0.001$.

Results

The CRN coding sequence rescues the CRN mutation

The *CRN* coding sequence used in[13] was cloned and transformed by tissue culture into the *crn* mutant as described in[23] and multiple T0 whole plant transgenics carrying the construct were obtained. Two independent transgenic lines carrying the construct (T1) were selected for analysis because they segregated only plants carrying the construct. T2 plants from these lines were grown in an aeroponic chamber in nodulation medium and inoculated as in.[10] The *crn* mutant plants carrying the *CRN* construct displayed wild type nodule numbers when compared to the R108 wild type (no statistical difference, Student's t-test) and different from the parental *crn* mutant used for transformation (Student's t-test, $p > 0.001$, Figure 1(a)). The results confirm the suggestion in[13] that the lesion in the *CRN* gene is responsible for the hypernodulation phenotype of *crn* mutants.

CRN is part of the SUNN/CLE signaling pathway, but CRA2 is not

The *MtCLE12* and *MtCLE13* peptide genes as well as an empty vector control were constitutively expressed in composite hairy roots of R108 ecotype wild type plants as in.[10] The construct carries a DS-Red marker which allows identification of transformed roots by microscopy and only these roots were used in the analysis. Inoculation with rhizobia resulted in a normal number of nodules in the empty vector control. Significantly reduced nodulation was observed in plants constitutively expressing either the *MtCLE12* or the *MtCLE13* gene (Figure 1(b) $p < 0.001$, Student's t-test), in agreement with previous findings for this experiment performed in wild type plants of the A17 ecotype.[4,10] However, expression of both the empty vector control and either the *MtCLE12* or the *MtCLE13* gene in composite hairy roots had no effect on the increased nodule number observed in *crn* mutant plants (Figure 1(c)). This is the same result we and other observed for the experiment done in a *sunn* mutant background[4,10] and indicates that both CLE peptides signal through a pathway that involves the CRN pseudokinase. While it is possible CRN is a receptor for the CLEs, it is more likely based on experiments on the interactions of CRN with other molecules in Arabidopsis and *M. truncatula* that this is evidence of a downstream effect. Since CRN and SUNN have been shown to physically interact[13] it may be that CRN responds to the binding of the CLEs to the SUNN kinase with which it associates, and this response does not occur in a *crn* mutant.

In contrast, when the *MtCLE12* and *MtCLE13* peptide genes were constitutively expressed in *cra2-2* mutant plants in the R108 background, the low nodule phenotype of these plants was significantly lower than mutants expressing the empty vector (Figure 1(d), $p < 0.01$, Student's t-test), similar to the effect of expression of these genes in wild type plants. From this, we conclude that MtCLE12p and MtCLE13p are not involved in the CRA2 nodule regulatory pathway. Further evidence of independence is the phenotype of plants carrying mutations in both *sunn-5*[13] and *cra2-2* .[20] The *sunn-5* allele in the R108 background contains a *Tnt1* transposon insertion in an exon near the end of the extracellular domain of the receptor, resulting in a three-fold increase in nodule number over the corresponding wild type (Figure 1(f)). The *sunn-5;cra2-2* double mutant, identified by PCR among F2 plants from a *cra2-2* cross to *sunn-5*, was analyzed as in Figure 1(a) for nodule number and the compact root architecture phenotype of *cra2-2*. Plants homozygous for both mutations displayed the *cra2-2* compact root with many laterals and low nodule number phenotypes (Figure 1(e–g)). The presence of the *cra2-2* allele drastically reduced the nodule number in plants carrying the *sunn-5* allele (Student's t-test, $p < .001$), however, there was no statistical difference in nodule number between the double mutant and plants containing only the *cra2-2* allele. Recent work with an allelic series of *cra2* mutants in the A17 background revealed an intermediate nodule phenotype close to wild type nodulation in their *sunn;cra2* double mutants, [24] but the less severe reduction in nodulation is likely due to the alleles and ecotype used to create the double mutant. The *cra2-2* allele in the R108 genotype has a severe reduction in nodule number (many plants don't make a nodule at 14 days post inoculation) in our aeroponic system compared the *cra2* alleles in the A17 genotype in their pouch system (3–5 nodules per plant at 14 days post inoculation) and the *sunn-5* allele in R108 is not as strong (3x increase) as the *sunn-4* allele in A17 (10x increase) in our system. However, our results support their conclusion that the CEP/CRA2 and CLE/SUNN systemic pathways act independently from the shoots to regulate nodule number, [24] and implicate CRN in the CLE/SUNN/CRN systemic pathway.

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