Long-Distance Signaling in Nodulation Directed by a CLAVATA1-Like Receptor Kinase

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Proliferation of legume nodule primordia is controlled by shoot-root signaling known as autoregulation of nodulation (AON). Mutants defective in AON show supernodulation and increased numbers of lateral roots. Here, we demonstrate that AON in soybean is controlled by the receptor-like protein kinase GmNARK (Glycine max nodule autoregulation receptor kinase), similar to Arabidopsis CLAVATA1 (CLV1). Whereas CLV1 functions in a protein complex controlling stem cell proliferation by short-distance signaling in shoot apices, GmNARK expression in the leaf has a major role in long-distance communication with nodule and lateral root primordia.

Multicellular organisms need to control the proliferation of pluripotent stem cells, also referred to as meristematic cells in the apices, cambium, and pericycle of flowering plants. Because organ differentiation of plants is predominantly postembryonic and does not involve cell migration, plant stem cells need to be controlled by short- and long-distance signals to achieve equilibrium between cell proliferation and differentiation. The role of short-distance signaling in plant development has been more extensively researched, and some of the key genes involved have been identified (1–8).

Legume nodulation is important in supplying nitrogen to ecological and agricultural systems. Nodule meristems form in response to mitogenic signals from symbiotic bacteria called rhizobia (6), but nodule proliferation is restricted by autoregulation of nodulation (AON) (9–12). Mutations affecting nodule meristems have been readily identified, including ones that confer supernodulation as a result of a defect in AON (Fig. 1A).

Allelic supernodulating (nts) mutants of soybean were first isolated by EMS (ethylmethane sulfonate) mutagenesis (11–13). These mutants altered at the NTS-1 locus also develop more lateral roots, leading to a bushy root system in the absence of nodulation, and reduced root growth in the presence of proliferative nodulation. Subsequently, additional mutations in the NTS-1 locus were induced chemically or by fast neutrons (14–16). Isolation of mutants in other legumes confirmed the generality of AON (17–19), and reciprocal grafting of supernodulating and wild-type genotypes showed that long-distance signaling was involved and that the leaf genotype controlled proliferation of nodule primordia (20–22).

To elucidate the mechanisms of this long-distance signal exchange, we used map-based cloning to isolate the NTS-1 locus. Mutant alleles were mapped to soybean linkage group H close to restriction fragment length polymorphism (RFLP) marker pA132. A subclone of pA132, pUTG132a, was placed 0.7 cM from NTS-1 in a F2 population of nts382 (G. max Bragg) × G. soja (PI468.397) (23, 24) and 1.3 cM from NTS-1 in a G. max nts246 × G. soja CPI 100070 population (25, nts382 and nts246 were identified in our original mutant screen (11). Amplified fragment length polymorphism (AFLP) marker UQC-IS1 also flanked NTS-1 1.9 cM away (Fig. 1B) (25). UQC-IS1 was the closest of 11 AFLP markers shown by bulk segregant analysis and genetic mapping to be linked to NTS-1 (25).

Bacterial artificial chromosome (BAC) clones derived from a soybean PI437.654 library (26) were isolated by filter-hybridization to pUTG132a and UQC-IS1, and were verified to contain either pUTG132a or UQC-IS1 by sequencing each marker from the respective BAC clone. Both the pUTG132a and UQC-IS1 BAC contigs were oriented relative to NTS-1 by mapping polymorphic BAC ends on F2 recombinants (Fig. 1B).

Confirmation of mapping was aided by the fast neutron mutant F3N7 (16). Physical mapping of markers and complete BAC sequencing of BAC17107 (135 kb) (Fig. 1B) showed that this mutant contains a chromosomal deletion in the NTS-1 region. The southern and northern deletion breakpoints were localized within the BAC17107 sequence and close to marker UQC-IS4, respectively (Fig. 1B). Arrangement of putative open reading frames from BAC17107, sequenced BAC ends, and markers in the NTS-1 region showed contiguous microsynteny to Arabidopsis chromosomes 2 and 4 (Fig. 1B). Seven genes (three from the northern contig and four from the southern contig) were syntenic between the NTS-1 region and Arabidopsis. BAC92D22 contained three expressed sequence tags, highly syntenic...
with *Arabidopsis* chromosome 2, but was not demonstrated to overlap with either the northern or southern contig.

For the UQC-IS1 contig, UQC-IS2 ( southern end of BAC 95P14) mapped 1.2 cm away from the locus (Fig. 1B). UQC-IS2 was used to identify additional BAC clones, of which BAC3K21 was demonstrated to extend toward *NTS-1* by AFLP fingerprinting. BAC-end UQC-IS3 was cloned and used to identify additional BAC clones, of which BAC129E8 was shown by AFLP fingerprinting to extend further toward *NTS-1*. BAC-end UQC-IS4 from BAC129E8 was cloned and used to select BAC75M10, which was AFLP fingerprinted in comparison to BAC129E8. One AFLP marker called UQC-IS5, 650 base pairs (bp) in length and located within BAC 75M10, was cloned and sequenced, revealing complete identity to a known gene called *GmCLV1B* (27). This gene encodes a predicted protein showing 75% amino acid similarity to the shoot meristem—expressed *CLAVATA1* of *Arabidopsis* (4). *clv1* mutants of *Arabidopsis* have increased numbers of undifferentiated stem cells in shoot and floral meristems, and extra organs within flowers (4).

*GmCLV1B* was a good candidate for *NTS-1* because it mapped to the correct region, was absent in deletion mutant *FN37* (Fig. 2), and the homologous *CLV1* is involved in control of cell proliferation in shoot meristems. The full-length genomic sequence of the candidate *NTS-1* gene, including about 600 bp upstream of the translation start, was obtained (GenBank accession number AF166655). Given the difficulty of transforming soybean, confirmation of the gene was obtained by sequencing an allelic series rather than by complementing the mutant phenotype. Sequencing of *GmCLV1B* from several wild-type and mutant lines (Table 1) identified changes in the coding sequence that strongly indicated it was the gene responsible for control of nodule meristem proliferation (Fig. 1). This gene was renamed *GmNARK* (*Glycine max* nodule autoregulation receptor kinase) to reflect its putative biochemical and functional role in root nodulation.

*GmNARK* encodes a predicted receptor-like protein kinase (RLK) composed of a 24-amino acid NH$_2$-terminal signal peptide (MRSCV-CYTLIFFFIWLRVATCS), an extracellular domain composed of 19 tandem copies of a 24-amino acid leucine-rich-repeat (LRR), a transmembrane domain (TRVIVIVIALGTA-ALLVATVYVYM), and a COOH-terminal cytoplasmic kinase domain (28). A 49-amino acid island interrupts the LRR domain between repeats 11 and 12. Overall, the protein contains 15 potential N-glycosylation sites. We were able to correlate the type of mutational change with the severity of the nodulation phenotype (Table 1 and Fig. 1C). Allele *nts1007* (causing a greater than 10-fold increase in nodule number) is a nonsense mutation that truncates the protein at glutamine residue 106 (Q106*), eliminating most of the LRRs and the entire protein kinase domain. The Q106* mutation was confirmed in the Australian variety PS55, carrying the *nts1007* allele. Alleles *nts246*, *en5600*, and *nts382*, conferring extreme phenotypes almost identical to *nts1007*, also truncate the protein by nonsense mutations; *nts246* (K115*) is located immediately downstream of *nts1007*, also in the LRR domain; *en5600* (K606*) is immediately upstream of the transmembrane domain; and
nts382 is in the kinase domain (Q920*). The deletion mutant FN37 also has an extreme supernodulation phenotype. Because all five mutations (FN37 deletion, three receptor nonsense mutations, and a kinase nonsense mutation) showed indistinguishable extreme supernodulation, the loss of the kinase activity is sufficient to confer the extreme phenotype. In contrast, the weak allele nts1116 conferred two- to three-fold increased nodulation. It is caused by a transition of valine to alanine at position 837, also in the kinase domain (Fig. 1C). Thus, gene identification was confirmed by the characterization of six independent mutant alleles, in which the predicted impact of the molecular alteration of the protein is precisely reflected in the severity of the phenotype. We confirmed that gene structure and amino acid sequence are conserved in wild-type soybean cultivars Clark, Williams, Bragg, G. soja PI468.397, and G. soja CPI 100070. Some silent mutations exist in the wild-type G. soja lines.

In contrast to CLV1, which is present as a single copy in Arabidopsis, in soybean GmNARK (GmCLV1B) is strongly homologous to a duplicated gene called GmCLV1A (27). GmNARK and GmCLV1A were previously investigated in a study of stem fasciation (27). Both genes were reported to be wild type in fasciated soybean mutants but linked to pA381-1 (27), a RFLP marker in the vicinity of NTS-1 (24). The GmCLV1A gene differs from GmNARK by only ~10% at the nucleotide level, but a greater sequence divergence in the 3’ untranslated regions (UTRs) allowed genespecific amplification (27). Interpretation of polymerase chain reaction (PCR) and Southern analyses was aided by the availability of deletion mutant FN37, which lacks GmNARK and several NTS-1-linked markers (Figs. 1B and 2, A and B). GmNARK, but not GmCLV1A, is located on overlapping BAC clones BAC75M10 and BAC112J23 (Fig. 2B). GmCLV1A is only weakly detected in Southern analysis of FN37, presumably through the presence of a duplicated copy of GmCLV1A elsewhere in the genome.

Reverse transcription (RT–PCR) analysis with GmNARK 3’-UTR-specific oligonucleotide primers was also used to determine tissue-specific transcript levels. Whereas Arabidopsis CLV1 expression is restricted to the shoot apical meristem (SAM) (4), GmNARK is expressed in nodulated roots and shoots of the wild type (Fig. 2, C and D). No GmNARK transcript was detected in FN37 (Fig. 2C). Quantitative RT-PCR also revealed that leaf GmNARK transcript levels are substantially higher than those in the SAM tissue (Fig. 2D) for wild type, nts1007, and nts382. Such expression of GmNARK is consistent with a primary role of the leaf in AON (20–22).

GmNARK contains a single intron (465 bp) in the kinase domain, in precisely the same position as the intron (79 bp) in CLV1 (4). The similarity of gene structure and protein sequence suggests that GmNARK shares functional and evolutionary similarities with CLV1 (Fig. 1D). CLV1 controls stem cell proliferation in shoot meristems, and mutations of CLV1 lead to apical and floral meristem changes, whereas GmNARK functions in the leaf and exerts long-distance control of nodulation with no detectable SAM (Fig. 2E), floral, or leaf phenotypes. Also, GmNARK displays differential tissue-specific expression to CLV1. It is therefore likely that GmCLV1A is the immediate functional CLV1 ortholog and that GmNARK is a duplicated version of CLV1, but with a different expression pattern and divergent function involved in long-distance control of nodulation.

The discovery of a receptor-like protein kinase as part of the signaling circuit for AON opens the possibility of characterizing associated long-distance signals in plant development. Because GmNARK is closely related to CLV1, one can presume that both upstream and downstream signal transduction are broadly similar in their mode of action. It is therefore likely that the predicted extracellular LRR domain of GmNARK interacts with another protein

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**Table 1. Mutant alleles of GmNARK.** The asterisk denotes nonsense termination mutation.

<table>
<thead>
<tr>
<th>Genetic background</th>
<th>Allele†</th>
<th>Supernodulation phenotype</th>
<th>Mutation</th>
<th>Reference‡</th>
</tr>
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<tbody>
<tr>
<td>G. max Bragg</td>
<td>nts382</td>
<td>Extreme</td>
<td>Q920*</td>
<td>(11, 12)</td>
</tr>
<tr>
<td>G. max Bragg</td>
<td>nts1007</td>
<td>Intermediate</td>
<td>Q105*</td>
<td>(11)</td>
</tr>
<tr>
<td>G. max Bragg</td>
<td>nts1116</td>
<td>Intermediate</td>
<td>Q105*</td>
<td>(11)</td>
</tr>
<tr>
<td>G. max PSS5</td>
<td>nts1007</td>
<td>Extreme</td>
<td>K115*</td>
<td>(11)</td>
</tr>
<tr>
<td>G. max nts246 × G. soja CPI 100070</td>
<td>nts382</td>
<td>Extreme</td>
<td>K606*</td>
<td>(11)</td>
</tr>
<tr>
<td>G. max Enrei</td>
<td>en6500</td>
<td>Extreme</td>
<td>K606*</td>
<td>(11)</td>
</tr>
<tr>
<td>G. soja PI468.397</td>
<td>FN37</td>
<td>Extreme</td>
<td>K606*</td>
<td>(11)</td>
</tr>
</tbody>
</table>

†First description of allele. ‡Supernodulating F2 plants derived from G. max nts246 × G. soja CPI 100070.
similar to *Arabidopsis* CLV2 and an extracellular peptide similar to *Arabidopsis* CLV3 (1, 5). Other proteins involved in signal transduction have also been shown to interact with CLV1 in *Arabidopsis* (5).

Intriguingly, *GmNARK* is most similar to CLV1, whereas two receptor-like kinase genes in the regions on chromosomes 2 and 4 of *Arabidopsis* syntenic with the soybean NTS-I region are much more distantly related (Fig. 1D). There is no synteny between the NTS-I region of soybean and the vicinity of CLV1 (29). One possible explanation for this finding is that a localized gene recombination or conversion-like event may have occurred in evolution involving the CLV1 ortholog and another receptor-like kinase gene, such as to change the chromosomal location of CLV1 in either *Arabidopsis* or soybean.

Other receptor-like kinases have been shown to participate in environmental sensing—for example, in the perception of hormones, pathogens, symbionts, or cellular interactions (1–3, 7–8). The discovery of a divergent *Arabidopsis* CLV1 ortholog in soybean affecting long-distance nodulation control extends this spectrum of activities to cell division events in a distal organ that are first sensed, then homeostatically controlled.

Our findings suggest evolutionary mechanisms for the development of the root nodule symbiosis. Duplication of genes followed by divergence in function is a common theme in evolution (30). Ancestral duplication of a gene controlling stem cell proliferation in the SAM may have led to a variant mechanism in which shoot control of cell proliferation is extended to root tissue. Research in legumes into CLAVATA-related signaling will undoubtedly facilitate the understanding of key developmental processes such as nodulation that are absent in the model plant *Arabidopsis*.

**References and Notes**

28. Abbreviations for amino acid residues: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Arg; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
29. I. R. Searle et al., unpublished data.

**Modulation of ATP-Dependent Chromatin-Remodeling Complexes by Inositol Polyphosphates**

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Eukaryotes use adenosine triphosphate (ATP)–dependent chromatin-remodeling complexes to regulate gene expression. Here, we show that inositol polyphosphates can modulate the activities of several chromatin-remodeling complexes in vitro. Inositol hexakisphosphate (IP₆) inhibits nucleosome mobilization by NURF, ISW2, and INO80 complexes. In contrast, nucleosome mobilization by the yeast SWI/SNF complex is stimulated by inositol tetrakisphosphate (IP₄) and inositol pentakisphosphate (IP₅). We demonstrate that mutations in genes encoding inositol polyphosphate kinases that produce IP₄, IP₅, and IP₆ impair transcription in vivo. These results provide a link between inositol polyphosphates, chromatin remodeling, and gene expression.

In eukaryotes, the SWI2/SNF2 family of ATP-dependent chromatin-remodeling complexes is widely used to regulate DNA accessibility for transcription. Four related classes of protein complexes (SWI2/SNF2, ISWI, Mi2, and INO80) use the energy of ATP hydrolysis to alter nucleosome architecture (1–3). Although there have been significant advances in understanding the mechanism and function of chromatin-remodeling complexes, the interaction of these complexes with cell signaling pathways has not been widely explored. One major mechanism for communicating environmental signals is the inositol signaling pathway. Activation of phosphorylatedinositol-specific phospholipase C at the cell membrane leads to cleavage of phosphatidylinositol 4,5-bisphosphate (PIP₂), generating secondary messengers inositol 1,4,5-trisphosphate (IP₃), a regulator of calcium release and diacylglycerol (DAG), an activator of protein kinase C (4, 5). IP₃ can undergo additional phosphorylation to IP₄, IP₅, or IP₆, and di-phosphorylated derivatives (6). Recent advances have revealed multiple and varied functions for IP₄, IP₅, and IP₆ in nuclear acid and viral metabolism (7–11).

The regulation of INO1, encoding inositol-1-phosphate synthase (12), by SNF2, ISW2, and INO80 (13–16), encoding the core ATPases of three chromatin-remodeling complexes, prompted us to consider whether soluble inositol metabolites could influence ATP-dependent chromatin remodeling. We investigated this question by an in vitro nucleosome mobilization assay, which uses native gel electrophoresis to distinguish between nucleosomes at different locations on a DNA fragment. The *Drosophila* ISWI-containing complex NURF mobilizes reconstituted nucleosomes to favor one dominant position (N3) on hsp70 promoter DNA (17–19). We found that IP₆ inhibits nucleosome mobili-