R gene-controlled host specificity in the legume–rhizobia symbiosis

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Leguminous plants can enter into root nodule symbioses with nitrogen-fixing soil bacteria known as rhizobia. An intriguing but still poorly understood property of the symbiosis is its host specificity, which is controlled at multiple levels involving both rhizobial and host genes. It is widely believed that the host specificity is determined by specific recognition of bacterially derived Nod factors by the cognate host receptor(s). Here we describe the positional cloning of two soybean genes *Rj2* and *Rfg1* that restrict nodulation with specific strains of *Bradyrhizobium japonicum* and *Sinorhizobium fredii*, respectively. We show that *Rj2* and *Rfg1* are allelic genes encoding a member of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance (R) proteins. The involvement of host *R* genes in the control of genotype-specific infection and nodulation reveals a common recognition mechanism underlying symbiotic and pathogenic host–bacteria interactions and suggests the existence of their cognate avirulence genes derived from rhizobia. This study suggests that establishment of a root nodule symbiosis requires the evasion of plant immune responses triggered by rhizobial effectors.

soybean | nodulation | nitrogen fixation | defense

Plants of the legume family, such as alfalfa, clovers, peas, and beans, can make their own nitrogen fertilizer by forming symbioses with a diverse group of nitrogen-fixing soil bacteria known as rhizobia. This cross-kingdom collaboration is characterized by the formation of the root nodule, a specialized plant organ that provides an optimized environment for the bacteria to convert atmospheric nitrogen into ammonia. The legume–rhizobia association is highly specific, such that each rhizobial strain establishes a symbiosis with only a limited set of host plants and vice versa. Despite recent advances in our understanding of the Nod-factor signaling pathway (1, 2), the host control of nodulation specificity remains poorly understood.

The symbiotic specificity is determined by a fine-tuned exchange of molecular signals between a host plant and its bacterial symbiont (3). The best-known bacterially derived signal is the Nod factor, a family of lipo-chitooligosaccharides with various strain-specific chemical decorations (4–6). The ability to induce the production of bacterial Nod factors in response to host-secreted flavonoids and to subsequently perceive the signal by the cognate host receptor(s) is widely thought to play a key role in defining the host range (3, 7–10). For example, allelic variation in the pea *sym2*, a putative Nod-factor receptor, leads to natural polymorphisms in Nod-factor perception and thus symbiotic specificity (7, 8). Moreover, introduction of the putative Nod-factor receptors *NFR1* and *NFR5* of *Lotus japonicus* into *Medicago truncatula* enables nodulation of the transgenic roots by the *L. japonicus* symbiont *Mesorhizobium loti* (9, 10).

Besides Nod factors, rhizobia also use surface polysaccharides or secreted proteins to modulate host range (11–13). Rhizobial surface polysaccharides (e.g., exopolysaccharides, lipopolysaccharides, and cyclic glucans) have been implicated in facilitating infection thread formation and nodule development (14). It has been proposed that surface polysaccharides play a role in the evasion or suppression of host defense responses, a feature that is shared by pathogenic and symbiotic bacteria (12–14). Another striking similarity between pathogenic and symbiotic bacteria is that many, but not all, rhizobial strains also possess a type III secretion system (T3SS) that delivers effectors [so-called nodulation out proteins (Nops)] into the host cells (11). Rhizobial T3SSs modulate the host range in a genotype-specific manner, but are dispensable for rhizobial infection and nodulation. This latter observation reveals an important difference between T3SSs of mutualistic rhizobia and pathogenic bacteria, because T3SSs of pathogens are required for causing disease in susceptible hosts and for eliciting the hypersensitive response in resistant hosts (15). For these rhizobial signals, the host presumably possesses the yet unknown corresponding recognition mechanisms that control the compatibility of the legume–rhizobia interaction. To unravel such mechanisms, it is critical to characterize natural variation in symbiotic specificity within a host species, but such variation remains largely unexplored in the two model legumes *M. truncatula* and *L. japonicus*.

Natural variation in symbiotic specificity has been well documented in soybeans since the 1950s (16). Genetic analysis of these naturally occurring variations identified several dominant genes (e.g., *Rj2*, *Rj4*, and *Rfg1*) that restrict nodulation with specific rhizobial strains (17–19). The dominant nature of these genes is in contrast to those "loss-of-function" recessive alleles in Nod-factor receptors (e.g., *sym2* and *sym37* in pea and *rj1* in soybean) (7, 20, 21), but resembles gene-for-gene resistance against plant pathogens. In this report, we describe the positional cloning of *Rj2* and *Rfg1*, the two soybean genes identified several decades ago that restrict nodulation with two distinct groups of rhizobial strains (16). In particular, the *Rj2* gene restricts nodulation of soybean with specific *Bradyrhizobium japonicum* strains such as USDA122 (17), whereas *Rfg1* prevents nodulation with certain fast-growing *Sinorhizobium fredii* strains such as USDA257 (18). We show that *Rj2* and *Rfg1* are allelic genes encoding a member of the Toll-interleukin receptor/nucleotide-binding site/leucine-rich repeat (TIR-NBS-LRR) class of plant resistance proteins (22). This finding reveals a common recognition mechanism underlying symbiotic and pathogenic host–bacteria interactions and bodes well for the existence of their cognate avirulence genes derived from rhizobia. Our study suggests that establishment of a root nodule symbiosis requires the evasion of plant immune responses triggered by rhizobial effectors.

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Results and Discussion

Positional Cloning of *Rj2* and *Rfg1*. The *Rj2* locus was previously mapped to the soybean linkage group J (chromosome 16) (23–25). We carried out fine mapping of the locus using an F_2 population derived from the cross between L76-1988 and L82-2024 (hereafter referred to as L76 and L82, respectively). L76 (*Rj2*/*Rj2*, *rfg1*/*rfg1*) restricts nodulation with *B. japonicum* USDA122 but not with *S. fredii* USDA257, whereas L82 (*rj2*/*rj2*, *Rfg1*/*Rfg1*) can nodulate with USDA122 but not USDA257 (Fig. 1 *A* and *B*). From a total of 1,258 F_2 plants inoculated with USDA122, 296 plants nodulated, which fits the 3:1 (nonnodulation to nodulation) ratio ($\chi^2 = 1.45$, df = 1, $P = 0.23$), consistent with restriction of nodulation by USDA122 being controlled by a single dominant gene (17). Genotyping of the 296 nodulated individuals (*rj2*/*rj2*) using single-nucleotide polymorphism (SNP) markers allowed us to locate the *Rj2* locus within a 47-kb genomic region (Fig. 1*C*). The 47-kb genomic sequence of Williams 82 (*rj2*/*rj2*) contains at least 10 predicted genes (Glyma16g33690–Glyma16g33800) (25). One of these, Glyma16g33780, encodes a member of the TIR-NBS-LRR class of disease resistance proteins. This gene was considered as a strong candidate of the *rj2* allele.

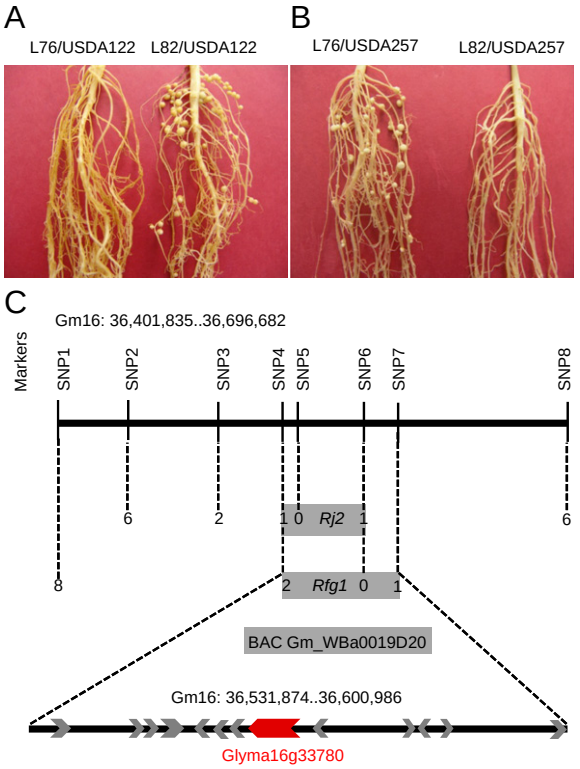


Fig. 1. Map-based cloning of *Rj2* and *Rfg1*. (*A*) Nodulation phenotypes of L76 (*Rj2*/*Rj2*, left) and L82 (*rj2*/*rj2*, right) by *B. japonicum* USDA122. (*B*) Nodulation phenotypes of L76 (*rfg1*/*rfg1*, left) and L82 (*Rfg1*/*Rfg1*, right) by *S. fredii* USDA257. (*C*) Fine mapping of *Rj2* and *Rfg1*. The *Rj2* locus was delimited to a 47-kb genomic region between markers SNP4 and SNP6, and the *Rfg1* locus was mapped to a 69-kb genomic region flanked by the markers SNP4 and SNP7. Numbers indicate the number of recombination breakpoints separating the marker from *Rj2* or *Rfg1* based on genotyping homozygous *rj2*/*rj2* or *rfg1*/*rfg1* segregants from the F_2 populations (296 plants for mapping *Rj2* and 323 plants for mapping *Rfg1*). Annotation of the 69-kb genomic DNA of Williams 82 (*rj2*/*rj2*, *Rfg1*/*Rfg1*) that covers both *Rj2* and *Rfg1* candidate gene regions identifies 13 putative genes. One of these, Glyma16g33780, was considered as a strong candidate of the *rj2*/*Rfg1* allele. A bacterial artificial chromosome (BAC) clone, Gm_WBa0019D20, from Williams 82 that covers the candidate gene regions is also indicated.

We performed de novo mapping of the *Rfg1* locus using an F_2 population derived from the cross between Williams 82 (*rj2*/*rj2*, *Rfg1*/*Rfg1*) and Peking (*rj2*/*rj2*, *rfg1*/*rfg1*). Preliminary mapping based on 200 F_2 individuals suggested that the *Rfg1* locus is tightly linked with the *Rj2* locus. Thus, we decided to finely map the *Rfg1* locus using an F_2 population derived from L76 and L82, taking advantage of the available molecular markers developed for mapping *Rj2*. Phenotyping a total of 1,350 F_2 plants inoculated with *S. fredii* USDA257 identified 323 nodulated plants. The ratio of nonnodulation to nodulation (1,027:323) statistically fits 3:1 ($\chi^2 = 0.83$, df = 1, $P = 0.36$), confirming that resistance to nodulation by USDA257 is conditioned by a single dominant gene (18). Fine mapping using the 323 nodulated F_2 plants (*rfg1*/*rfg1*) enabled us to delimit the *Rfg1* locus within a 69-kb region that encompasses the 47-kb *Rj2* candidate gene region (Fig. 1*C*). Because Glyma16g33780 is the only TIR-NBS-LRR type gene predicted from the 69-kb genomic region, we postulate that *Rj2* and *Rfg1* are allelic genes. This observation comes as an intriguing surprise, because the two genes were independently identified by their nodulation restriction with two phylogenetically distinct groups of rhizobial species (16–18).

Functional Validation of the Candidate Genes. We validated the candidate genes by complementation tests using *Agrobacterium rhizogenes*-mediated hairy root transformation (26). The experiments were conducted without antibiotic selection, and thus the resulting hairy roots contained both transgenic and wild type, which can be readily distinguished by examining the expression of the GUSPlus gene in the binary plasmid pCAMBIA1305.1, followed by analysis of transgene expression (Fig. 2*C*). As shown in Fig. 2, introduction of the candidate *Rj2* allele of L76 into L82 (*rj2*/*rj2*) resulted in complete block of nodulation on the transgenic roots inoculated with USDA122 (Fig. 2 *A* and *B*). From a total of 41 composite transgenic plants that possess both transgenic and wild-type roots, nodules were formed on the wild-type roots but never observed on the transgenic roots. The function of the candidate *Rj2* allele in L76 was further confirmed by RNAi-mediated gene silencing. Transformation of an RNAi construct into L76 led to significant reduction of the candidate gene transcripts (Fig. 2*F*) and enabled nodulation of the transgenic roots by USDA122 (Fig. 2 *D* and *E*). From a total of 30 composite plants, a range of 12–30 nodules were formed on the chimeric roots of 28 plants. Fluorescence microscopy confirmed that all of the nodules were from the roots that expressed the red fluorescent marker *DsRed1* in the binary plasmid pRedRoot II. Similarly, transferring the candidate *Rfg1* allele of Williams 82 into Peking (*rfg1*/*rfg1*) ($n = 27$ plants) and L76 (*rfg1*/*rfg1*) ($n = 22$ plants) abolished nodulation of the transgenic roots by USDA257, whereas nodulation was normal on the nontransgenic hairy roots (Fig. 3). Taken together, we conclude that *Rj2* and *Rfg1* are allelic genes encoding a member of the TIR-NBS-LRR class of plant disease resistance proteins.

Expression and Allelic Polymorphisms of the *Rj2*/*Rfg1* Locus. *Rj2* and *Rfg1* alleles are constitutively expressed in both roots and leaves, and their expression in the root does not markedly change on rhizobial inoculation (Fig. 4). Both *Rj2* (L76) and *Rfg1* (L82) transcripts are predicted to encode a protein of 1052 aa, consisting of an N-terminal TIR domain, a centrally located NBS domain, and at least eight degenerate LRRs C-terminal to the NBS domain (Fig. 5*A*). Sequence comparison identified a total of seven amino acid substitutions between the two allelic products (E452K, I490R, Q731E, E736N, P743S, E756D, and R758S) (Fig. 5*B*). These polymorphisms occur in both NBS and LRR domains and define the genetic basis of specificity differences between the L76 and L82 alleles.

To gain a further understanding of allelic diversity and specificity at the *Rj2*/*Rfg1* locus, we tested a set of 21 soybean lines for

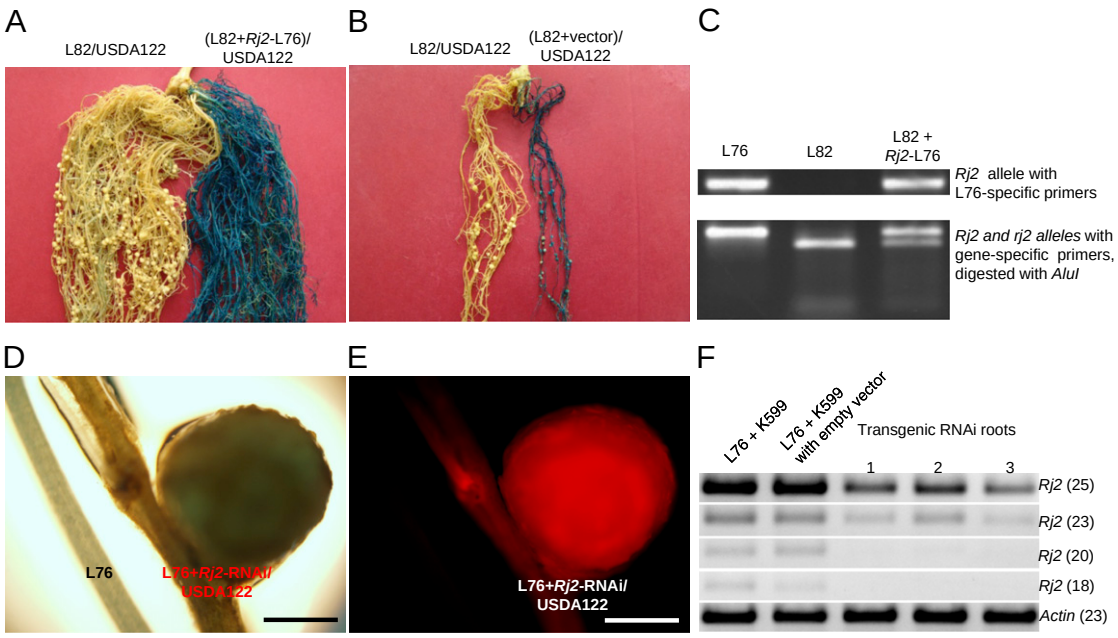


Fig. 2. Functional validation of *Rj2*. (*A*) Introduction of *Rj2* (L76) into L82 (*rj2*/*rj2*) led to block of nodulation on the transgenic roots by USDA122 (*Right*), but nodulation was normal on the wild-type roots (*Left*). (*B*) Vector control experiment showing nodulation on the roots transformed with the empty vector pCAMBIA1305.1 (*Right*). (*C*) Expression of the *Rj2* allele of L76 in L82 transgenic roots. The transgenic roots were first identified by GUS staining of a small portion of the root segments. Both allelic-specific (*Upper*) and gene-specific (*Lower*) primers were used for reverse-transcriptase (RT)-PCR analyses. The alleles amplified from the gene-specific primers were distinguished by digestion with AluI, a polymorphic restriction site derived from a single-nucleotide variation. (*D*) Bright-field picture of a wild-type (*Left*) and a nodulated transgenic root (*Right*) expressing an *Rj2* RNAi construct and a *DsRED1* reporter gene. (*E*) The same roots as shown in *D* using filter settings to visualize *DsRED1* expression. (Scale bars, 1 mm.) (*F*) Down-regulation of *Rj2* in three random transgenic roots expressing the RNAi construct. Fluorescence microscopy was used to identify the transgenic roots expressing the red fluorescent marker *DsRed1*. RNAs were isolated from hairy roots induced by *A. rhizogenes* strain K599. The number in parentheses indicates the cycle number of the RT-PCR. The soybean (*Glycine max*) *Actin* gene was used as a control.

their symbiotic compatibility with USDA122 and USDA257, with a particular focus on previously known *Rj2*/*Rj2* and *rfg1*/*rfg1* genotypes (Fig. 5*B*). Both *Rj2* and *rfg1* alleles occur with a low frequency in surveyed soybean varieties (16). Of the 21 genotypes, 9 carry an *Rj2* (*rfg1*) allele that allows for nodulation with USDA257 but not with USDA122; 7 possess an *rj2* (*Rfg1*) allele that permits nodulation with USDA122 but not with USDA257; and the remaining 5 contain an *rj2* (*rfg1*) allele that enables nodulation with both USDA122 and USDA257. We did not identify the *Rj2* (*Rfg1*) allele type that prohibits nodulation with both strains.

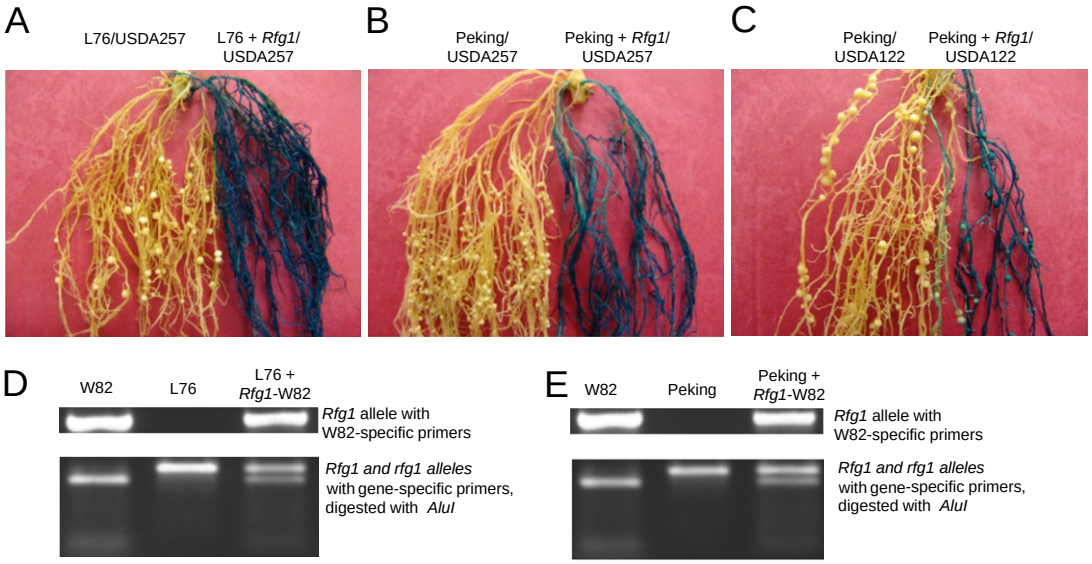


Fig. 3. Functional validation of *Rfg1*. Transferring *Rfg1* (Williams 82) to L76 (*A*) and Peking (*B*) resulted in block of nodulation on the transgenic roots by USDA257. (*C*) Control experiment showing that the transgenic *Rfg1* roots of Peking can nodulate with USDA122. (*D*) Expression of the *Rfg1* allele of Williams 82 (W82) in L76. (*E*) Expression of the *Rfg1* allele of W82 in Peking.

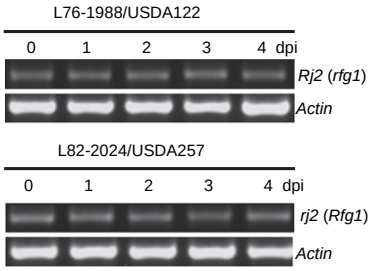
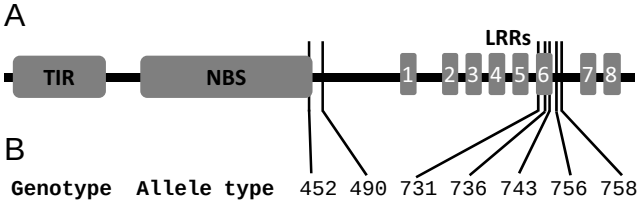


Fig. 4. Constitutive expression of the *Rj2* (*rfg1*) and *rj2* (*Rfg1*) alleles in the roots of L76 and L82, respectively. RT-PCR was performed using the gene-specific primers (25 cycles). Roots at 0, 1, 2, 3, and 4 d postinoculation (dpi) were collected for RNA isolation.

These results suggest that soybean plants carrying an *Rj2* or *Rfg1* allele commonly show contrasting specificity with USDA122 and USDA257 and thus can form symbiotic interactions with at least one of the two strains. We expect that *Rj2* (*Rfg1*) alleles would be rare, if they do exist, in natural populations.

We sequenced the alleles (cDNAs) of the 21 genotypes. Pairwise comparisons of these allelic products revealed that the aforementioned seven amino acid substitutions between L76 and L82 represent a minimum difference observed between an *Rj2* (*rfg1*) and an *rj2* (*Rfg1*) allele; all other polymorphisms are also present within the two allelic groups and thus are not associated with allelic specificity. As shown in Fig. 5*B*, the nine *Rj2* (*rfg1*) alleles share a similar protein haplotype at the seven sites (E452, I490, Q731, E736, P743, E756, and R/L758), whereas the six *rj2* (*Rfg1*) alleles share an identical protein haplotype at the seven sites (K452, R490, E731, N736, S743, D756, and S758).



Genotype	Allele type	452	490	731	736	743	756	758
L76-1988	Rj2(rfg1)	E	I	Q	E	P	E	R
PI209340	Rj2(rfg1)	E	I	Q	E	P	E	R
PI548473	Rj2(rfg1)	E	I	Q	E	P	E	R
Hardee	Rj2(rfg1)	E	I	Q	E	P	E	R
CNS	Rj2(rfg1)	E	I	Q	E	P	E	R
PI157469	Rj2(rfg1)	E	I	Q	E	P	E	L
PI230974	Rj2(rfg1)	E	I	Q	E	P	E	L
PI548447	Rj2(rfg1)	E	I	Q	E	P	E	L
PI200522	Rj2(rfg1)	E	I	Q	E	P	E	L
Peking	rj2(rfg1)	K	R	Q	E	P	E	L
Sooty	rj2(rfg1)	K	R	Q	E	P	E	L
Kingwa	rj2(rfg1)	K	R	Q	E	P	E	L
Davis	rj2(rfg1)	K	R	Q	E	P	E	L
Emerald	rj2(rfg1)	K	R	Q	E	P	E	L
L82-2024	rj2(Rfg1)	K	R	E	N	S	D	S
Williams 82	rj2(Rfg1)	K	R	E	N	S	D	S
Hill	rj2(Rfg1)	K	R	E	N	S	D	S
Williams	rj2(Rfg1)	K	R	E	N	S	D	S
Jack	rj2(Rfg1)	K	R	E	N	S	D	S
McCall	rj2(Rfg1)	K	R	E	N	S	D	S
TN4-86	rj2(Rfg1)	K	R	E	N	S	D	S

Fig. 5. Amino acid polymorphisms among the three allelic groups of Rj2 (rfg1), rj2 (rfg1), and rj2 (Rfg1). (*A*) Domain structure of the TIR-NBS-LRR protein showing the seven substitution sites between the Rj2-L76 and Rfg1-L82 proteins. (*B*) Allelic comparisons of the 21 soybean genotypes at the seven sites.

Intriguingly, the five *rj2* (*rfg1*) alleles are chimeric of the above two allelic types (K452, R490, Q731, E736, P743, E756, and R758). Therefore, the allelic diversity and specificity likely resulted from intragenic recombination events, even though we do not yet know the ancestral alleles. Sequence comparisons between the *Rj2* and *rj2* alleles (regardless of the *Rfg1* specificity) and between the *Rfg1* and *rfg1* alleles appear to suggest that the *Rj2* function requires a haplotype containing E452 and I490, whereas the *Rfg1* function requires a haplotype consisting of E731, N736, S743, D756, and S758 (Fig. 5*B*). This inference remains to be tested by sequencing more alleles and by domain swaps and functional validation experiments.

Incompatible Rhizobial Strains Can Induce Root Hair Curling but Fail to Infect the Host Carrying the Cognate Resistance (*R*) Genes. Numerous rhizobial genes have been implicated in species-specific or genotype-specific nodulation (27–29). Our discovery that host *R* genes are involved in determination of symbiotic specificity is in line with the finding that many rhizobial strains possess a T3SS that delivers effectors into the host cells to modulate host range (11). Some rhizobial effectors are homologous to those secreted by bacterial pathogens, suggesting a similar recognition mechanism between symbiotic and pathogenic host–bacteria interactions (11, 30).

In the incompatible interactions controlled by the *Rj2* or *Rfg1* genes, the rhizobial strains were able to induce root hair curling and occasionally cause nodule primordium formation (Fig. 6 *A* and *B*), but cortical cell division ceased at an early stage due to a lack of infection thread formation (Fig. 6 *C*–*F*) (31). The failure of infection-thread growth was likely caused by defense responses triggered by the recognition of yet unknown rhizobial effectors by the host *R* genes (Fig. 6 *G* and *H*).

Role of a T3SS Mutant of *S. fredii* USDA257 in Modulation of Genotype-Specific Nodulation. We further examined the effects of a T3SS mutant of USDA257, called DH4, on nodulation properties of nine selected soybean genotypes that represent all three described allelic types (Table 1). The DH4 mutant disrupted the *RhcU* function and failed to secrete any effector proteins (32). This experiment revealed that the T3SS mutant gained the ability to nodulate the soybean genotypes carrying an *Rfg1* allele and maintained the ability to nodulate the plants carrying an *rfg1* allele. The mutant strain can increase, decrease, or have no effect on nodule numbers in comparison with the wild-type strain depending on the genetic background (Table 1). Similar results were also reported for other rhizobial strains such as *Rhizobium* sp. NGR234 (29). These observations suggest that, in contrast to bacterial pathogens, a rhizobial T3SS and its secreted effectors are not required for rhizobial infection and nodulation, but possibly function as a facilitator superimposed on the Nod-factor signaling pathway. In the absence of recognition by the host *R* genes, the T3SS effectors may play a positive role in facilitating rhizobial infection, but function negatively if perceived by the host *R* genes. Thus, our study supports the hypothesis that symbiosis development requires the evasion of plant immune responses triggered by rhizobial effectors (11, 13). Nod factors and surface polysaccharides have been postulated to play a role in suppression of defense responses in compatible hosts induced by microbe-associated molecular patterns (MAMPs) (11–14, 33), but they appear unable to overcome the effector-triggered immunity (ETI) to establish a symbiosis (34).

Evolution of Nodulation Restriction *R* Genes. Although very few legume species have been thoroughly surveyed for their symbiotic variability, dominant genes that restrict nodulation with specific rhizobial strains have been frequently identified in the tested species (35). Unlike most *R* genes conferring strain-specific disease resistance, a single *Rj2* or *Rfg1* allele could restrict nodu-

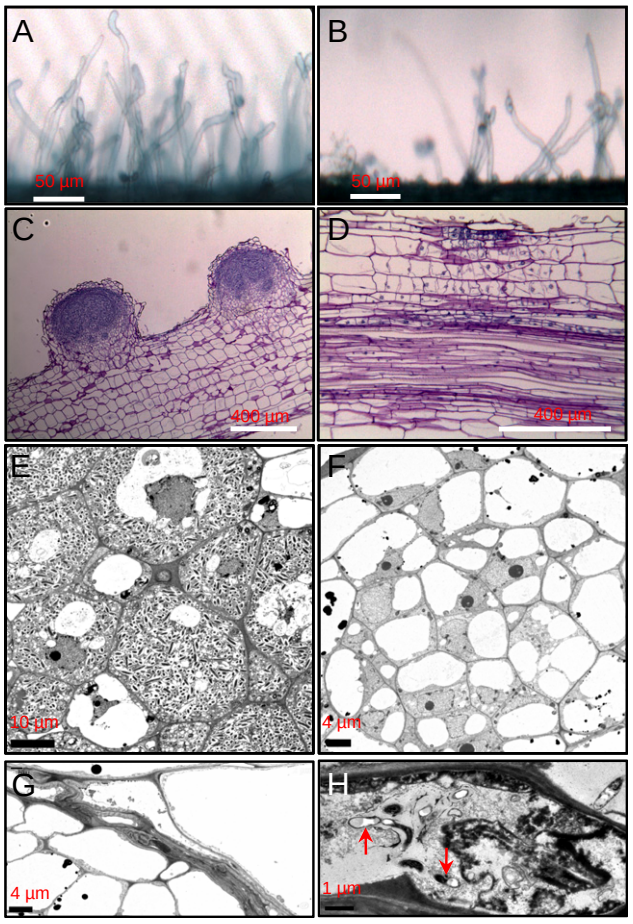


Fig. 6. Root responses of L82 and L76 inoculated with *B. japonicum* USDA122. (*A* and *B*) USDA122 induces root hair curling on both compatible (L82, *A*) and incompatible (L76, *B*) hosts. Photographs were taken 3 d after inoculation. (*C*) At 8 d after inoculation, USDA122 initiates numerous nodule primordia on the roots of L82. (*D*) In contrast, only occasionally does USDA122 cause cortical cell divisions on the roots of L76. (*E*) Transmission electron microscopy of young nodules of L82 revealed the presence of abundant bacteria inside each cell. (*F*) Even though USDA122 occasionally induced cell division on the roots of L76, these cells did not contain bacteria. (*G* and *H*) Interestingly, the outermost epidermal cells exhibited secondary thickening (*G*) and sometimes enclosed few bacteria (*H*). The appearance of these bacteria was unusual and appeared to be undergoing disintegration (arrows). Dark staining components were readily seen in such cells presumably due to the hypersensitive response of L76 roots to USDA122.

lation by multiple rhizobial strains (16). The wide prevalence of such genes in natural populations suggests that they are unlikely to be under negative selection. It is possible that some rhizobia and pathogenic bacteria use a common set of "virulence" effectors to facilitate invasion of the host, providing the host with a dilemma between forming symbiosis and mounting disease resistance. Eventually, natural selection would maintain both alleles to balance benefits and costs, depending on ecological conditions. A second scenario would be that some host genotypes have evolved *R* genes to selectively interact with certain strains but exclude others. For example, the predominance of the *Rj4* allele in soybeans may be attributed to its role in protecting the host from nodulation with many *Bradyrhizobium elkanii* strains that produce rhizobitoxine, a phytotoxin that causes foliar chlorosis on susceptible host plants (16).

Conclusions. We report here that NBS-LRR type disease resistance genes are involved in determination of symbiotic specificity in soybean, through positional cloning of the *Rj2* and *Rfg1* genes that restrict nodulation with specific strains of *B. japonicum* and *S. fredii*, respectively. Our discovery is consistent with recent reports describing rhizobial T3SS and its secreted effectors that play an important role in modulation of host range. It remains to be seen whether multiple or only a few *R* gene loci are involved in determination of nodulation specificity and whether these loci are conserved in legumes. The *Rj2*/*Rfg1* locus exhibits highly conserved synteny with the orthologous regions in *M. truncatula* (AC156629) and *L. japonicus* (AP010004) that also contain the closest *Rj2*/*Rfg1* homologs. This observation may facilitate testing whether rhizobial recognition specificity has an ancient origin during legume evolution. Our finding may also offer unique strategies to enhance symbiotic nitrogen fixation in crop legumes. For example, the nodulation-restrictive *R* genes may be manipulated so that a host can deterministically interact with rhizobial inoculants with high nitrogen-fixing efficiency and exclude those indigenous strains that are highly competitive but with very low nitrogen-fixing efficiency (16). It will also be interesting to know whether these types of *R* genes shape the diversity of the rhizobial community in the rhizosphere. Much remains to be learned about how an *R* gene conditions the beneficial plant–microbe interactions in natural ecosystems.

Materials and Methods

Plant Materials and Nodulation Assay. The F_2 mapping populations were derived from the crosses between L76-1988 (*Rj2*/*Rj2*, *rfg1*/*rfg1*) and L82-2024 (*rj2*/*rj2*, *Rfg1*/*Rfg1*) and between Williams 82 (*rj2*/*rj2*, *Rfg1*/*Rfg1*) and Peking (*rj2*/*rj2*, *rfg1*/*rfg1*). Plants were grown in sterile vermiculite in a growth chamber programmed for 12 h light at 26 °C and 12 h dark at 23 °C. Roots of 1-wk-old seedlings were inoculated with *B. japonicum* USDA122 or *S. fredii* USDA257. Nodulation phenotypes were recorded 2 wk postinoculation.

Complementation Tests and RNAi-Mediated Gene Silencing. For *Rfg1*, we used the *Rfg1* (*rj2*) allele of Williams 82 for complementation tests. Briefly, genomic DNA of the BAC clone Gm_WBa0019D20 of Williams 82 was digested with PstI and BmgBI to obtain a 10.9-kb fragment that contains the ~4.9-kb coding region plus ~4.0 kb upstream of the start codon and ~2.0 kb downstream of the stop codon. The genomic fragment was introduced into the binary vector pCAMBIA1305.1 through blunt end cloning. For *Rj2*, we developed a genomic construct consisting of a synthetic L76-1988 allele on the basis of its allelic sequence of Williams 82. The genomic construct described above was digested with EcoRI to delete a ~2.2-kb fragment that contains all of the polymorphic sites detected between the coding sequences of Williams 82 and L76-1988. The corresponding genomic fragment of L76-1988 was then amplified by PCR and ligated into the digested pCAMBIA1305.1 vector using the In-Fusion Advantage PCR Cloning kits (Clontech). For silencing of *Rj2*, an RNAi construct was generated by cloning a 405-bp inverted-repeat sequence of the NBS-encoding region into the pRedRoot II vector that contains the red fluorescent marker *DsRed1*.

Table 1. Nodulation properties of USDA257 and a T3SS mutant (DH4) on different soybean genotypes

	Average nodule no. per plant (n = 10)								
Allelic type	*Rj2* (*rfg1*)			*rj2* (*Rfg1*)			*rj2* (*rfg1*)		
Genotype	L76-1988	PI548473	Hardee	McCall	Williams 82	L82-2024	Peking	Sooty	Kingwa
USDA257	37.9 ± 8.1	24.8 ± 6.2	23.4 ± 5.1	0	0	0	39.0 ± 14.0	52.4 ± 8.4	45.7 ± 9.2
DH4	35.0 ± 8.7	34.1 ± 9.1	37.5 ± 9.6	28.7 ± 11.6	31.8 ± 7.5	12.1 ± 3.5	25.5 ± 4.9	28.4 ± 3.1	21 ± 8.0

Hairy Root Transformation of Soybean. *A. rhizogenes*-mediated hairy root transformation was performed on the basis of the protocol described by Kereszt et al. (26). Briefly, the *A. rhizogenes* strain K599 that contains a binary vector was injected into the cotyledonary node of 1-wk-old seedlings. The infected seedlings were maintained in sterile vermiculite in a growth chamber with 90% humidity. Two to 3 wk after infection, when hairy roots were well developed at the infection site, the main roots were removed, and the composite plants were inoculated with the rhizobial strains USDA122 or USDA257. Nodulation assays were performed 2 wk after inoculation. The transgenic roots were identified through GUS staining of a small portion of the hairy roots in the cases of complementation tests or through detection of the red fluorescent marker *DsRed1* using a fluorescent microscope.

Analysis of Gene Expression by RT-PCR. Total RNA was isolated by the Qiagen Plant RNeasy mini kit. Two micrograms of RNA was used to perform RT reactions using M-MLV reverse transcriptase (Invitrogen) in a 20-μL reaction mixture. Two microliters of the RT reaction was used as a template in a 20-μL PCR solution. The PCR primers were as follows: *Rj2*-L76 specific, 5′ATGGCAATTCTGTTATGGAAGACTC3′ and 5′CTGGCCTTCCATTAGCTTTG3′; *Rfg1*-Williams 82-specific, 5′ATGACAATTCTGTTATGGAAGAGTT3′ and 5′CTGGCCTTCCATTAGCTTTG3′; *Rj2/Rfg1* specific primers, 5′GGCACCTCATACGGACTGTT3′ and 5′AGGCTCAGAAGTTTTCCACCT3′; and Gm-*Actin*, 5′GAGCTATGAATTGCCTGATGG3′ and 5′CGTTTCATGAATTCCAGTAGC3′.

Anatomical Analysis. Soybean seeds were surface sterilized and germinated on 1% water agar at 30 °C for 3 d. The roots of the seedlings were dipped into bacterial suspensions (10^9 cells/mL) and transferred to autoclaved plastic growth pouches that were premoistened with nitrogen-free nutrient solution. The location of the root tip of each seedling was marked on the surface of the pouch. The plants were grown for 3 d at 24 °C under a 12-h photoperiod. Primary roots extending 5 cm above and 5 cm below the root tip mark were dissected, stained with 0.01% methylene blue, and examined for root hair curling under a dissecting microscope using bright field illumination. Soybean roots harvested at 5 and 8 d after inoculation were cut into small pieces and immediately fixed in 50% ethyl alcohol, 5% glacial acetic acid, and 10% formaldehyde for 24 h at 4 °C. The tissues were dehydrated in a graded ethanol series followed by a graded series of xylene. Following this, the roots were infiltrated with several changes of paraffin at 60 °C and embedded in paraffin. Embedded tissues were sectioned (10 μm thick) with a microtome, stained with hematoxylin and eosin, and examined with bright-field optics.

Electron Microscopy. Soybean roots inoculated with *B. japonicum* USDA122 were dissected into 2- to 4-mm pieces with a double-edge razor blade and fixed immediately in 2.5% glutaraldehyde buffered at pH 7.2 with 50 mM sodium phosphate buffer. The tissues were fixed at room temperature for 4 h, washed extensively with five changes of phosphate buffer, and post-fixed with 2% aqueous osmium tetroxide for 1 h. Following this, the tissues were rinsed with several changes of buffer, dehydrated in a graded acetone series, and infiltrated with Spurr's resin. Thin sections were cut with a diamond knife, collected on uncoated 200-mesh copper grids, and stained with 0.5% uranyl acetate and 0.4% lead citrate. Stained sections were examined with a JEOL 1200 EX transmission electron microscope at 80 kV.

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