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A Cultivar-Specific Interaction between *Rhizobium leguminosarum* bv. trifolii and Subterranean Clover Is Controlled by *nodM*, Other Bacterial Cultivar Specificity Genes, and a Single Recessive Host Gene

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Insertion mutagenesis identified two negatively acting gene loci which restrict the ability of *Rhizobium leguminosarum* bv. trifolii TA1 to infect the homologous host *Trifolium subterraneum* cv. Woogenellup. One locus was confirmed by DNA sequence analysis as the *nodM* gene, while the other locus, designated *csn-1* (cultivar-specific nodulation), is not located on the symbiosis plasmid. The presence of these cultivar specificity loci could be suppressed by the introduction of the *nodT* gene from ANU843, a related *R. leguminosarum* bv. trifolii strain. Other *nod* genes, present in *R. leguminosarum* bv. viciae (including *nodX*) and *R. meliloti*, were capable of complementing *R. leguminosarum* bv. trifolii TA1 for nodulation on cultivar Woogenellup. Nodulation studies conducted with F_2 seedlings from a cross between cultivar Geraldton and cultivar Woogenellup indicated that a single recessive gene, designated *rwt1*, is responsible for the Nod$^-$ association between strain TA1 and cultivar Woogenellup. Parallels can be drawn between this association and gene-for-gene systems common in interactions between plants and biotrophic pathogens.

Within the classical cross-inoculation groups of the *Rhizobium*-legume interaction (58), examples can be seen of more precise host-specific interactions at the plant cultivar level. Cultivar-specific nodulation has been documented for the symbiosis involving *Rhizobium leguminosarum* bv. trifolii and clover (30, 39), *R. fredii* and soybeans (2, 3, 9–12, 33, 46), and *R. leguminosarum* bv. viciae and peas (16, 40, 61, 62). Bacterial genes from the symbiosis plasmid (pSym) appear to control this cultivar specificity by acting as positive (2, 3, 13) or negative (33, 39) gene traits.

R. leguminosarum bv. trifolii TA1 can successfully induce nitrogen-fixing nodules on several subterranean clover cultivars, including Geraldton, Larisa, Mt. Barker, and Tallarook (30, 39). However, the ability of this strain to nodulate *Trifolium subterraneum* cv. Woogenellup is cold sensitive (30): at 28°C, strain TA1 forms an effective symbiosis with this cultivar; at 22°C, little or no sign of nodulation is apparent. The host genes which determine this incompatibility are not known. We recently showed that strain TA1 cannot nodulate cultivar Woogenellup because of the presence of negatively acting gene functions which restrict the host range of nodulation (39). In addition, the *nodT* gene from a related *R. leguminosarum* bv. trifolii strain, ANU843, acted as a dominant suppressor of these negatively acting gene functions and conferred the ability to nodulate cultivar Woogenellup.

This report presents a further analysis of the cultivar specificity genes in strain TA1 which act in a manner akin to the cultivar specificity avirulence genes well documented in plant-pathogen interactions (24, 26–28). We identified two distinct loci in strain TA1 which control cultivar specificity. One gene was identified by mutagenesis and DNA sequence analysis as *nodM*. The *nodM* gene from *R. leguminosarum* bv. trifolii ANU843 also acted to suppress nodulation by strain TA1 of cultivar Woogenellup. The other gene (*csn-1*) was identified by insertion mutagenesis with Tn*5*mob, was found to be unlinked to the nodulation gene region of strain TA1, and represents a novel host specificity determinant. In addition, we show that *nod* genes from other *Rhizobium* species are capable of suppressing the action of the negatively acting cultivar specificity genes in a manner akin to that recently reported for *nodT* from *R. leguminosarum* bv. trifolii ANU843 (39). These genes act as dominant suppressors, and it is clear that nodulation of a particular plant species is governed by a complex interaction of positively and negatively acting genes.

The segregation of host genes which may be specifically involved in cultivar specificity was determined by assaying F_2 seedlings from a cross between cultivar Geraldton and cultivar Woogenellup; the former is a cultivar which is effectively nodulated by strain TA1. We report that a single recessive host gene is involved in the cultivar-specific nodulation, indicating that a mechanism similar to, but more complex than, gene-for-gene cultivar specificity between plants and pathogens operates in the *Rhizobium*-legume symbiosis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial matings were done on TY agar or in TY broth (45), and triparental matings were done with the helper mobilizing plasmid pRK2013 (17). Conditions and media used for bacterial growth were described previously (45), except for GMM medium, which was used to support *Rhizobium* growth. GMM medium contained (milligrams per milliliter): KH_2PO_4, 540; K_2HPO_4, 690; $MgSO_4 \cdot 7H_2O$, 80; NaCl, 40; $CaCl_2 \cdot 2H_2O$, 40; $FeCl_3 \cdot 6H_2O$, 10; $MnSO_4 \cdot 4H_2O$, 10; H_3BO_3, 3; $ZnSO_4 \cdot 7H_2O$, 3; $Na_2MoO_4 \cdot 2H_2O$, 0.25; $CuSO_4 \cdot 5H_2O$, 0.25; $CoCl_2 \cdot 6H_2O$, 0.25; D-mannitol, 5,000;

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Reference or source
Strains		
E. coli NM522	(*lac-pro*) F′ *lacZ*M15 *lacI hsd-5*	31
Rhizobium sp. strain		
TA1	Wild-type *R. leguminosarum* bv. trifolii strain	30
ANU794	Sm^r derivative of TA1	21
ANU329	Sp^r derivative of TA1	22
ANU7941	ANU794 *nodM*::Tn*5*mob	This study
ANU7942	ANU794 *nodM*::Tn*5*mob	This study
ANU7943	ANU794 *csn-1*::Tn*5*mob	This study
ANU815	ΔpSym derivative of ANU794	22
ANU843	Wild-type *R. leguminosarum* bv. trifolii strain	45
ANU845	ANU843; pSym cured	20
CC2480a	Wild-type *R. leguminosarum* bv. trifolii strain	30
UNZ29	Wild-type *R. leguminosarum* bv. trifolii strain	30
Plasmids		
pBS	SK−; sequencing vector	Stratagene
pIJ1089	Contains 30 kb of DNA in pLAFR1 spanning *nodTNMLEFDABCIJ* of *R. leguminosarum* bv. viciae 248	23
pIJ1095	Contains 29 kb of DNA in pLAFR1 spanning *nodTNMLEFDABCIJX* of *R. leguminosarum* bv. viciae TOM	34
pIJ1230	pIJ1095 *nodX*::Tn*5*	34
pLAFR3	Broad-host-range cloning vector derived from pLAFR1; Tc^r	50
pMP180	Contains *nodDFELMNT* region of *R. leguminosarum* bv. viciae 248	7
pMP180::D2	pMP180 *nodD*::Tn*5* Km^r	60
pMP180::E1	pMP180 *nodE*::Tn*5* Km^r	60
pMP180::F4	pMP180 *nodF*::Tn*5* Km^r	60
pMP528	pMP180 *nodT*::Tn*phoA* Km^r	7
pMP578	pMP180 *nodT*::Tn*phoA* Km^r	7
pMP562	pMP180 *nodM*::Tn*phoA* Km^r	7
pMP574	pMP180 *nodM*::Tn*phoA* Km^r	7
pMP510	pMP180 *nodL*::Tn*phoA* Km^r	7
pMP589	pMP180 *nodL*::Tn*phoA* Km^r	7
pRK2013	Mobilizing helper plasmid; Km^r	17
pRmSL26	Contains 19.5 kb of *R. meliloti nod* DNA spanning *nodDABCIJ* in pLAFR1; Tc^r	42
pRt032	Contains 14 kb of ANU843 *nod* region in pKT240; Cb^r	48
pRt0328	pRt032 *nodN58*::MudII1734 Km^r	J. McIver
pRt032::822	pRt032 *nodM822*::MudI1734 Km^r	36
pRt032::m570	pRt032 *nodM nod-box*::MudII1734 Km^r	M.A. Djordjevic
pRt032::m114	pRt032 *nodA*::MudII1734 Km^r	44
pRt032::C10	pRt032 *nodC*::Tn*5* Km^r	20
pRt032::932	pRt032 *nodD*::MudI1734 Km^r	36
pRt032::C7	pRt032 *nodE*::Tn*5* Km^r	20
pRt032::m344	pRt032 *nodR*::MudII1734 Km^r	J. McIver
pRt032::B15	pRt032 *nodI*::Tn*5* Km^r	20
pRt032::G5	pRt032 *nodJ*::Tn*5* Km^r	20

[a] Sm, Streptomycin; Sp, spectinomycin; Tc, tetracycline; Km, kanamycin.

sodium glutamate, 500; thiamine HCl, 0.4; and biotin, 0.2. Bacto-Agar (15 g/liter; Difco) was added for solid medium, and the pH was 6.8 before autoclaving. Antibiotics were used for selection purposes at the following concentrations (milligrams per liter): for *Escherichia coli*, kanamycin, 50; tetracycline, 10; carbenicillin, 100; and spectinomycin, 50; for *Rhizobium* species, kanamycin, 150; tetracycline, 2; carbenicillin, 90; spectinomycin, 150; and streptomycin, 150.

DNA manipulations. DNA isolations, ligations, electrophoresis, and hybridizations were done as described by Maniatis et al. (43). Nonstringent hybridizations were done at 50°C. All enzymes were obtained from Boehringer Mannheim Biochemicals and New England BioLabs and used as recommended by the suppliers. DNA probes were ^{32}P labeled with random primers (59). The plasmid vector pBS SK− (Stratagene) was used in cloning for sequencing. Single-stranded DNA for sequencing was isolated as described by Gray et al. (32). Sequencing reactions were done with a Sequenase kit (US Biochemical Corp., Cleveland, Ohio) in accordance with the manufacturer's instructions. The protocol provided with this kit is an adaptation of the dideoxy chain termination method (47) which uses a modified T7 DNA polymerase (54). A specific Tn*5* primer (5′-CGT TCAGGACGCTACTT-3′; a gift from J. Watson, Commonwealth Scientific and Industrial Research Organization, Canberra, Australia) was used to sequence the Tn*5*mob insertion sites.

Tn*5*mob mutagenesis. *E. coli* containing the suicide vector pSUP5011 (49) was used to introduce Tn*5*mob into strain ANU794. Overnight TY broth cultures were mixed by vacuum filtration onto a nitrocellulose filter, which was then incubated on a TY agar plate at 28°C for 3 h. Appropriate

dilutions of the transconjugant cells were made and plated out on selective media containing kanamycin to generate single colonies. Since strain TA1 induces slowly forming, poorly developed nodules on cultivar Woogenellup, mutants with enhanced nodulation ability were screened by direct observation of the roots of inoculated plants. About 15,000 potential Tn*5*mob-induced mutants were inoculated onto 150 individual cultivar Woogenellup plants in pools containing about 300 colonies. An agar plate assay involving three cultivar Woogenellup seedlings per plate was used, as this procedure allowed direct observation of the plant roots. Large, red-pigmented, rapidly induced nodules were found on the tap roots on 7 of 150 plants. Bacterial isolates from these were screened for the appropriate genetic markers, checked by molecular analysis, and reinoculated onto cultivar Woogenellup plants to confirm the enhanced nodulation phenotype. Nodulation was always directly compared with that on plants inoculated with strain TA1, ANU794, or ANU329 (negative control) and strain ANU843 (positive nodulation control).

Chromosomal mobilization of Tn*5*mob mutations. To confirm that Tn*5*mob was responsible for the Nod$^+$ phenotype, we introduced the chromosome-mobilizing plasmid RP4-4 (Tc^r Km^s derivative of RP4; 32a, 49) separately into ANU794-derived mutant strains. Purified Km^r Tc^r transconjugants were mated with a Sp^r derivative of strain TA1 (strain ANU329) to allow for reciprocal recombination between wild-type and mutated gene regions. Final selection for Sp^r Km^r Tc^s transconjugants isolated new strains which contained the respective Tn*5*mob insertions in the ANU329 background but which did not contain the RP4-4 plasmid. Molecular characterization of these derivatives was done to confirm that recombination had occurred.

Plant assays. Plants were assayed with an agar plate assay (45) or a Magenta jar assay (Magenta Corp., Chicago, Ill.) under nitrogen-free conditions. Magenta jar assays were adapted from the technique of Bender et al. (4). Approximately 10^8 *Rhizobium* cells from an overnight BMM broth culture (optical density at 600 nm, 0.27) were resuspended in 10 ml of F medium in a sterile petri dish, and germinated seedlings were exposed to the inoculum for 1 h to permit bacterial attachment before being planted at nine seedlings per jar. Each jar was covered with a plastic bag to maintain vermiculite sterility, with a hole in the top of the bag to allow gas exchange. Plants in both types of assay were grown for 3 to 4 weeks in a growth chamber with fluorescent and incandescent lights (400 microeinsteins per m^2 per s) and a root temperature of 22°C. Humidity was 60%, and a light-dark cycle of 17 h-7 h was used. Clover seeds were sterilized and germinated as previously described (45).

F_2 generation seedlings from a cross between cultivar Geraldton and cultivar Woogenellup were obtained from P. Nichols and W. Collins, Western Australia Department of Agriculture. Pollen from cultivar Woogenellup was used to fertilize cultivar Geraldton flowers, and the resulting F_1 plants were screened for characteristics of the petiole, leaf, and flower to ensure that genuine hybrids had been constructed. The F_2 generation was produced by allowing the isolated F_1 plants to self-pollinate.

RESULTS

Isolation of mutants of strain ANU794 which induced an enhanced nodulation response on cultivar Woogenellup. To identify possible negatively acting genes that condition the inability of strain TA1 to induce normal nodulation on cultivar Woogenellup, we mutagenized strain ANU794 (Sm^r derivative of strain TA1) with Tn*5*mob. Seventeen mutants which induced large, red-pigmented tap root nodules on cultivar Woogenellup (Fig. 1) were isolated from the nodules. After purification and retesting for the enhanced nodulation phenotype, genomic DNA was isolated from these mutants, restricted with *Eco*RI (as Tn*5*mob contains no *Eco*RI sites), and hybridized to a Tn*5*-specific probe. Two distinct classes of mutant were identified. In the class I mutants, the Tn*5* probe hybridized to an 18-kb *Eco*RI fragment, while in the class II mutants, hybridization was observed with a 10-kb *Eco*RI fragment. The pattern of banding in restriction profiles of ethidium bromide-stained total DNA was indistinguishable from that of the parental strain. This result, together with the retention of the genetic markers of strain ANU794 (Sm^r), verified that the mutants were indeed derivatives of ANU794.

The *Eco*RI Tn*5*mob-containing DNA fragments were

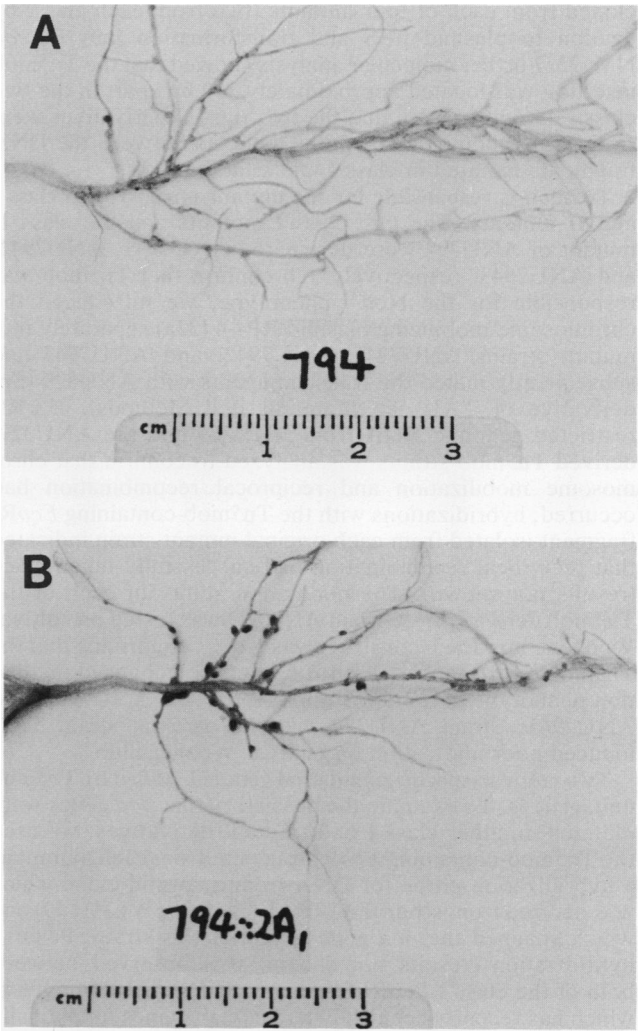

FIG. 1. Nodulation of cultivar Woogenellup by strain ANU794 and by a Tn*5*mob-induced mutant (later designated ANU7941; see the text). (A) Poor lateral root nodulation induced by strain ANU794. (B) Enhanced nodulation ability typical of a Nod$^+$ Tn*5*mob-induced mutant, with extensive nodulation of tap and lateral roots. Plants were grown in Magenta jars for 4 weeks at 22°C.

cloned from each of four mutants (two from each class) by ligation to plasmid pBS and transformation into *E. coli* NM522. Further molecular analysis showed that the Tn*5*mob insertion was located approximately 150 bp apart in the two class I derivatives and that the two class II derivatives were siblings. No homology could be detected between the DNA fragments mutated in class I and II derivatives.

Tn*5*mob is responsible for the mutant phenotype in class I and II mutants. The two class I mutants and the class II mutant of ANU794 were designated ANU7941, ANU7942, and ANU7943, respectively. To confirm that Tn*5*mob was responsible for the Nod$^+$ phenotype, we introduced the chromosome-mobilizing plasmid RP4-4 (32a) separately into mutant strains ANU7941, ANU7942, and ANU7943 and subsequently mated the transconjugants with ANU329 (Sp^r derivative of TA1; see Materials and Methods). *Eco*RI-restricted genomic DNA from ANU329 and the ANU329-derived Tn*5*mob strains was analyzed to confirm that chromosome mobilization and reciprocal recombination had occurred; hybridizations with the Tn*5*mob-containing *Eco*RI fragment isolated from each original mutant strain indicated that reciprocal recombination had successfully taken place (results not shown). The nodulation ability of each of the Tn*5*mob derivatives of strain ANU329 was tested on cultivar Woogenellup. Each construct was Nod$^+$, confirming that the Tn*5*mob insertion was responsible for the enhanced nodulation phenotype of mutant strains ANU7941, ANU7942, and ANU7943. Strain ANU329, like its parental strain TA1, induced poor nodulation on cultivar Woogenellup.

Two cultivar-specific nodulation genes identified by Tn*5*mob mutagenesis. To examine the possibility that *nod* genes were mutated in either class I or II mutant derivatives, we used the Tn*5*mob-containing *Eco*RI fragment from each mutant as a hybridization probe for DNA from a cosmid clone which was derived from strain TA1 DNA (cosmid pWLH1; 39) and which spanned the *nod* gene region of this strain. Positive hybridization (results not shown) was observed between both of the class I *Eco*RI fragments and an *Eco*RI fragment which has recently been shown to contain genes functionally and structurally homologous to ANU843 *nodABCIJD FERLMN* (39). As expected, the class II *Eco*RI fragment did not hybridize to the *nod* region of strain TA1. A 1.7-kb *Hin*dIII fragment containing 1.2 kb of the Tn*5*mob arm and flanking DNA sequences of the class II mutant was isolated and subsequently hybridized to genomic DNA from *R. leguminosarum* bv. trifolii CC2480a and ANU843 (both capable of nodulating cultivar Woogenellup), ANU794 and UNZ29 (both incapable of nodulating cultivar Woogenellup), and ANU845 (pSym cured) and ANU815 (ΔpSym). Positive hybridization was observed to all of these strains (Fig. 2), indicating that the mutation in the class II mutant is not pSym located. We designate this novel locus *csn-1* (cultivar-specific nodulation). Preliminary sequence data (not shown) indicate that the 0.5 kb of *Rhizobium* DNA in the 1.7-kb *Hin*dIII probe is gene specific. Hence, it appears that the presence of this *csn-1* gene region does not directly correlate with the ability of a strain to nodulate cultivar Woogenellup. However, DNA sequencing of the corresponding locus in the strains tested may indicate subtle differences between nodulation-restricted and -unrestricted strains.

DNA sequence analysis demonstrates that Tn*5*mob is located in *nodM* in class I mutants. *Hin*dIII digestion of the two *Eco*RI fragments from the two class I derivatives was used to subclone the end of each Tn*5*mob arm and flanking DNA from strain ANU794 into the sequencing vector pBS SK−. A Tn*5* primer (see Materials and Methods) was used to

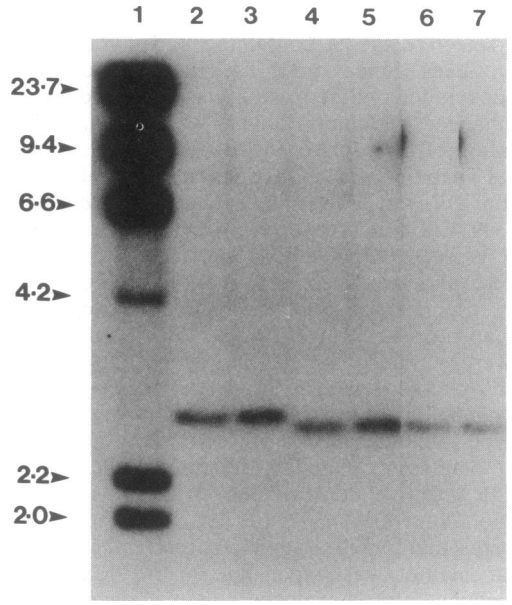

FIG. 2. Hybridization of the *csn-1* gene region to genomic DNA from various *R. leguminosarum* bv. trifolii strains. Lanes: 1, lambda size markers (kilobases); 2, ANU843; 3, ANU845 (pSym cured); 4, ANU794; 5, ANU815 (ΔpSym); 6, CC2480a; 7, UNZ29. Genomic DNA was restricted with *Eco*RI before hybridization. A 1.7-kb *Hin*dIII fragment (containing 1.2 kb of the arm of Tn*5*mob) internal to the Tn*5*mob-containing *Eco*RI fragment from the ANU794-derived class II mutant (later designated ANU7943; see the text) was used as a probe. Preliminary sequence data indicated that the 0.5 kb of *Rhizobium* DNA in this probe is gene specific (data not shown). Strains ANU843 and CC2480a proficiently nodulated cultivar Woogenellup, but strains ANU794 and UNZ29 did not.

sequence the DNA flanking the site of insertion of Tn*5*mob. A comparison of the sequences obtained to known *nod* gene sequences revealed that both of these mutants were affected in the *nodM* gene (Fig. 3). The sequence of the *nodM* gene of ANU843 is 96% homologous to that of the *nodM* gene of ANU794 over the 308 nucleotide bases sequenced in the vicinity of the insertion sites of the two class I derivatives (Fig. 3). The mutations in each of the class I derivatives are 174 bases apart and occur in the 5′ end of the TA1 *nodM* gene.

The *nodM* genes in strains ANU843 and TA1 are functionally identical. As an adjunct to the DNA sequence analysis, several genetic complementation tests were done to determine whether the *nodM* genes in strains ANU843 and TA1 are functionally identical. Previous results (19, 39, 48) had shown that the strain ANU843-derived plasmid pRt032, which contains *nodABCIJDFERLMN*, is capable of conferring nodulation ability on a range of clover species (*T. repens*, *T. pratense*, and *T. subterraneum*). Despite this capability, plasmid pRt032 fails to confer nodulation ability on cultivar Woogenellup to strains TA1 and ANU815 (ΔpSym). Derivatives of plasmid pRt032 containing insertion mutations in various *nod* genes (Table 1) were introduced into strain ANU815. Only plasmid pRt032::822, which contains a MudI1734 insertion in *nodM*, was capable of fully restoring nodulation ability on cultivar Woogenellup in the ANU815 background (Table 2). Plasmid pRt032::m570 also restored nodulation ability (Table 2). Plasmids pRt032::C7 (mutated in *nodE*) and pRt032::932 (mutated in *nodD*) partially restored nodulation ability (Table 2). This result indi-

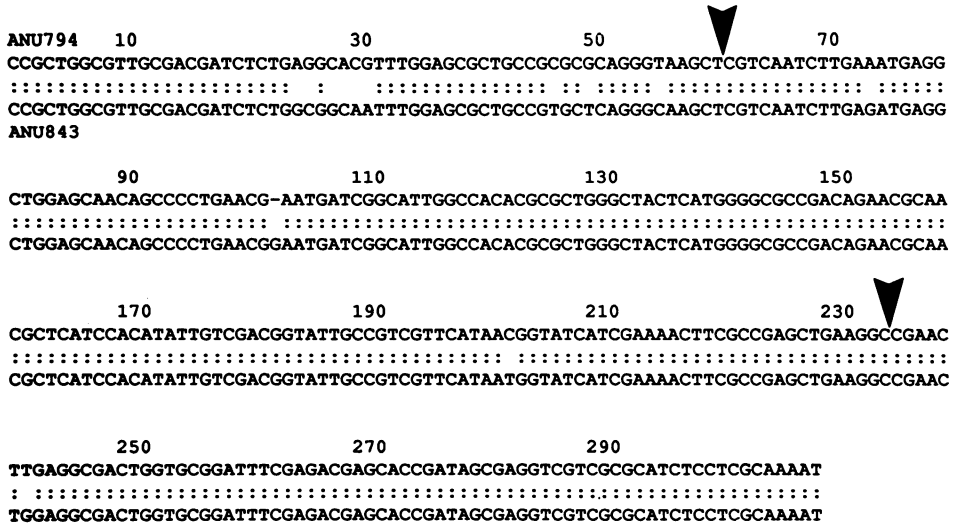

FIG. 3. Comparison of the DNA sequences of portions of the *nodM* genes of strain ANU843 and two ANU794-derived mutants (later designated ANU7941 and ANU7942; see the text). Three hundred eight bases of *nodM* sequence flanking the sites of Tn*5*mob insertion (arrowheads) in mutant strains ANU7941 (top arrow) and ANU7942 (bottom arrow) are shown. Bases homologous between strain ANU843 and ANU794 *nodM* genes are joined by dots. Total homology over the region sequenced was 96%. Sequence data for strain ANU843 were provided by J. J. Weinman (58a).

cates that the *nodE* gene probably acts in a negative manner in conjunction with other genes in this background. The restoration of nodulation ability by plasmid pRt032::932 is most likely due to the presence of a second copy of *nodD* retained in the ANU815 (ΔpSym) background (39). Plasmid pRt032, containing insertion mutations in gene *nodA*, *nodC*, *nodI*, *nodJ*, *nodR*, or *nodN* (*nodN* lies downstream of *nodM* in the same operon), failed to enhance the ability of strain ANU815 to nodulate cultivar Woogenellup (Table 2), suggesting that none of these genes acts in a negative manner to restrict the nodulation of this cultivar.

Nodulation genes from foreign *Rhizobium* species are capable of suppressing the activity of strain TA1 *nodM*. Previous results (39) indicated that the *nodT* gene from *R. leguminosarum* bv. trifolii ANU843 can confer cultivar Woogenellup nodulation ability to strain TA1. Nodulation genes from other *Rhizobium* species were also found to be capable of complementing strain TA1 to Nod$^+$ on cultivar Woogenellup. These included *nod* gene-containing clones from *R. meliloti* (on plasmid pRmSL26) and *R. leguminosarum* bv. viciae 248 (on plasmids pIJ1089 and pMP180) and TOM (on plasmids pIJ1095 and pIJ1230 [*nodX*::Tn*5*]) (Table 1). All five plasmids were capable of complementing strain TA1 for cultivar Woogenellup nodulation in a manner akin to that imparted by the introduction of *R. leguminosarum* bv. trifolii *nodT* (similar to that depicted in Fig. 1). However, differential results were obtained when the effects of these introduced plasmids in the TA1 and ANU815 backgrounds were measured. The introduction of cosmids pIJ1095 and pIJ1230 into strain ANU815 led to Nod$^+$ and Nod$^-$ results on cultivar Woogenellup, respectively, whereas the introduction of either cosmid into strain TA1 led to a Nod$^+$ result. This result showed that the *nodX* gene was essential for the ability of plasmid pIJ1095 to confer cultivar Woogenellup nodulation ability to strain ANU815 but that other genes (besides *nodX*) interacted with pSym-located genes in the background of strain TA1 to effect the nodulation phenotype.

Further analysis was conducted on the ability of *R. leguminosarum* bv. viciae-derived plasmids pMP180 and pIJ1089 to confer cultivar Woogenellup nodulation ability to strain TA1. Neither of these plasmids contains the *nodX* gene. The results showed that the *R. leguminosarum* bv. viciae *nodDEFLMT* genes are not individually involved in complementation, as pMP180 constructs containing mutations in any of these genes (pMP180::D2, pMP180::E1, pMP180::F4, pMP510, pMP589, pMP562, pMP574, pMP528, and pMP578) were still able to confer cultivar Woogenellup nodulation ability. The *nodN* gene on pMP180 and pIJ1089 may therefore be responsible for complementing the nodulation failure of strain TA1 on cultivar Woogenellup; it is also possible that several *nod* genes act together. Interestingly, this result suggests that the *R. leguminosarum* bv. viciae NodT is not functionally homologous to that of *R. legumi-*

TABLE 2. Ability of *nod* gene constructs derived from strain ANU843 to confer cultivar Woogenellup nodulation ability to strain ANU815

Construct	Gene affected	Nodulation ability[a]
pRt032		−
pRt032::m114	*nodA*	−
pRt032::C10	*nodC*	−
pRt032::932	*nodD*	+ (29)
pRt032::C7	*nodE*	+ (13)
pRt032::B15	*nodI*	−
pRt032::G5	*nodJ*	−
pRt032::m344	*nodR*	−
pRt0328	*nodN*	−
pRt032::m570[b]	*nodM nod-box*	+ (60)
pRt032::822	*nodM*	+ (91)

[a] +, Nod$^+$ response (average percent nodulated plants). −, Nod$^-$ response. Results are from at least two repeat plant assays testing 10 to 15 plants each. Plants were grown on agar plates at 22°C for 3 weeks.

[b] The m570 mutation is located in the *nod-box* sequence 5′ to the *nodMNX* operon.

nosarum bv. trifolii despite the fact that it is 60% homologous (51).

Plasmid pRmSL26 is known to encode *nodD1* and *nodABCIJ* (15, 29, 37, 55) and *fixF* (1). When pRmSL26 DNA was probed with fragments specific for *nodX* and *nodT* genes, no positive hybridization was detected, even under nonstringent conditions, although faint hybridization was observed to *R. leguminosarum* bv. trifolii *nodM* and *nodN* gene-specific fragments (17a). It has not been determined which gene(s) on plasmid pRmSL26 confers cultivar Woogenellup nodulation ability to strain TA1.

Analysis of the F_2 generation of cultivar Geraldton × cultivar Woogenellup. The segregation of the plant genes involved in the cultivar-specific interaction between strain TA1 and cultivar Woogenellup was examined. A total of 72 F_2 generation seedlings from a cross between cultivar Geraldton and cultivar Woogenellup were inoculated with strain TA1, and the nodulation phenotype was examined in a Magenta jar assay. While strain TA1 induced nodules on tap and lateral roots of some F_2 plants (cultivar Geraldton-like nodulation response), other F_2 plants were not nodulated or were only poorly nodulated on lateral roots (cultivar Woogenellup-like nodulation response) (Fig. 4). The ratio of F_2 plants nodulated in a manner similar to that of cultivar Geraldton to F_2 plants with a nodulation profile similar to the strain TA1-cultivar Woogenellup phenotype was calculated to be 53:19, or approximately 3:1. This ratio indicates that a single recessive gene, which we propose to call *rwt1* (resistance of Woogenellup to strain TA1), conditions the cultivar-specific incompatibility between strain TA1 and cultivar Woogenellup.

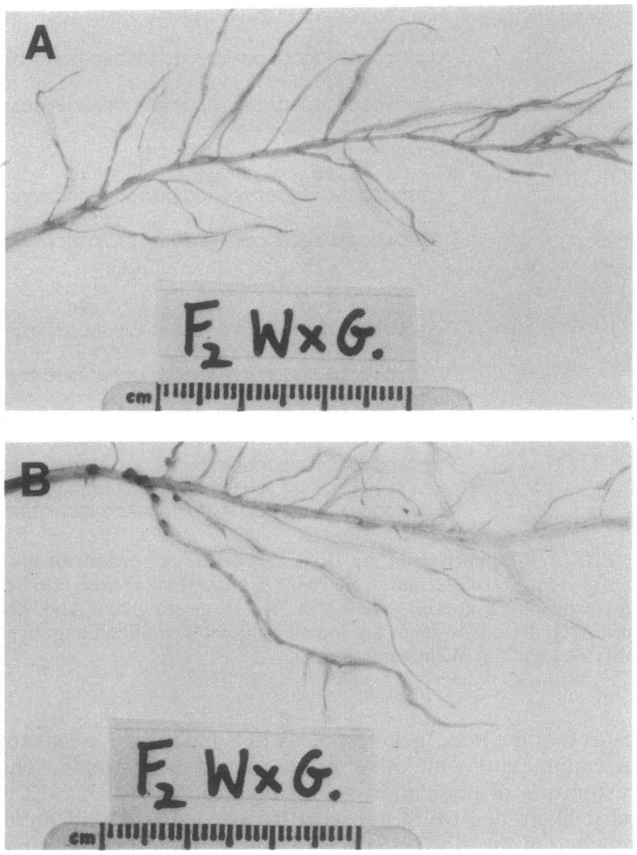

FIG. 4. Nodulation phenotypes of F_2 cultivar Geraldton × cultivar Woogenellup plants inoculated with strain TA1. (A) Cultivar Woogenellup-like nodulation response by F_2 plants, showing virtually no nodule induction. (B) Cultivar Geraldton-like nodulation response by F_2 plants, showing nodulation of tap and lateral roots. The ratio of the former to the latter was calculated to be 1:3. Nodulation profiles of control cultivar Woogenellup and cultivar Geraldton plants inoculated with strain TA1 were similar to those depicted in panels A and B, respectively. Plants were grown in majenta jars for 3 weeks at 22°C.

DISCUSSION

The mutagenesis of TA1 derivative strain ANU794 clearly indicated the presence in this strain of negatively acting genes which suppress the nodulation of cultivar Woogenellup. This report presents evidence for the first time that a *nod* gene, *nodM*, is involved in restricting host range in a cultivar-specific manner. Another novel, negatively acting cultivar specificity locus, *csn-1*, was also identified and is located outside pSym. Although the mechanistic basis for the inability of strain TA1 to nodulate cultivar Woogenellup is unknown, it is interesting that recent evidence has been presented that the *nodM* gene product functions as a glucosamine synthetase (43a). It is therefore possible that the action of gene products, including NodM and Csn1, finely modifies the surface chemistry or molecular signals exuded by strain TA1 such that an incompatible response is generated with cultivar Woogenellup (18, 38).

A clear gene-for-gene match has been documented in many plant-pathogen interactions, in which a single dominant avirulence gene overrides the basic ability to form a compatible interaction only when the plant host possesses the corresponding (usually dominant) resistance gene (18, 24, 26–28). In contrast to well-documented gene-for-gene pathway interactions, in the interaction between *R. leguminosarum* bv. trifolii and cultivar Woogenellup, a clear gene-for-gene interaction occurs. Together with other nodulation gene products, the products of *nodM* and the *csn-1* locus probably act in the same pathway, since mutation of either gene allows cultivar Woogenellup nodulation to proceed. The action of both of these genes can be suppressed by the presence of the *nodT* gene (naturally deleted in strain TA1; 39) or other genes in foreign rhizobia (e.g., by *R. leguminosarum* bv. viciae *nodX* in certain backgrounds).

Previous results (39) indicated that a negatively acting gene encoded by the *nodABCIJTDFERLMN(X)* region of strain ANU843 (on plasmid pRI4003) prevented strain TA1 from nodulating cultivar Woogenellup at 22°C. This gene(s) was absent in plasmid pRI4001, containing *nodABCIJTD FERL*, and hence was located in the *nodMNX* region. The results presented here confirm that it is the *nodM* gene which acts in a negative manner in the nodulation of subterranean clover. The results from sequencing and complementation analysis also indicate that the *nodM* gene is functionally equivalent in *R. leguminosarum* bv. trifolii ANU843 and TA1. It is clear that while *nodM* does not play an obligatory role in nodulation (as evidenced by the lack of a definitive phenotype for *nodM* mutants; 6, 20), it is capable of interacting with the products of other nodulation genes to fine-tune the efficiency of nodulation on several host plants. In some cases, it appears that the *nodM* gene may act in a positive manner. For example, the *nodLMN* region of *R. leguminosarum* bv. viciae increases the efficiency of nodulating peas and *Vicia hirsuta* (52, 53) and, similarly, *nodM* of *R. leguminosarum* bv. trifolii increases the efficiency of nodulating red clover (39). Our results clearly indicate that

nodM of *R. leguminosarum* bv. trifolii also acts in a negative manner in the background of strain TA1 to restrict nodulation of subterranean clover cultivar Woogenellup at 22°C.

Negatively acting *nod* gene functions which act at the genus level have been demonstrated in other *Rhizobium*-legume interactions (8, 14, 15, 20, 25, 35), although cultivar specificity genes which restrict host range have only recently been reported, for example, in the symbiosis between *R. fredii* and soybeans (33). Tn*5*-induced mutants of *R. fredii* strain USDA257 acquired the ability to nodulate the soybean cultivar McCall, which the parent strain is unable to nodulate (9, 33). Although one of the mutations was located on pSym, no evidence of the involvement of specific *nod* genes was presented. The gene(s) mutated was proposed to be involved in preventing infection thread initiation or development in cultivar McCall (9). Similarly, Sadowsky et al. (46a) recently identified a genotype-specific nodulation determinant in *Bradyrhizobium japonicum* which restricts nodulation on certain soybeans.

Our results show that the *nodT* genes from *R. leguminosarum* bv. trifolii and *R. leguminosarum* bv. viciae are not functionally identical in that the *R. leguminosarum* bv. viciae *nodT* cannot confer cultivar Woogenellup nodulation ability. However, it appears that several genes in different *Rhizobium* species and biovars (encoded on plasmids pMP180, pIJ1095, pIJ1089, pIJ1230, and pRmSL26), including *nodX*, can act in a manner phenotypically similar to that of the *R. leguminosarum* bv. trifolii *nodT* gene. The *nodX* gene is responsible for conferring the ability of *R. leguminosarum* bv. viciae TOM to nodulate the primitive pea cultivar Afghanistan (13). Other *R. leguminosarum* bv. viciae strains, such as 248, which lack *nodX* cannot nodulate this pea cultivar and are limited to infecting North American or European pea lines (40, 61, 62). It is interesting to note that a gene downstream of *nodC* (probably *nodT*) has been shown to be important for the ability of ANU843 *nodE* mutants to nodulate cultivar Afghanistan peas (extended host range ability) (7, 20). These findings suggest that the products of *nodX* (*R. leguminosarum* bv. viciae) and *nodT* (*R. leguminosarum* bv. trifolii) act in a similar manner, possibly by enzymatically altering products which would otherwise impede the successful infection of the cultivar involved.

The results presented here show that a single recessive plant gene affecting the nodulation of cultivar Woogenellup, designated *rwt1*, is involved in the incompatibility between strain TA1 and cultivar Woogenellup. It is possible that the product of the clover host gene *rwt1* interacts with low-molecular-weight factors released from rhizobia after the induction of the *nod* genes (38). Similar host resistance genes which limit nodulation by specific *Rhizobium* strains have also been reported in symbioses between *R. leguminosarum* bv. viciae and peas (41) and between *B. japonicum* and soybeans (5, 56, 57). In contrast to the recessive nature of *rwt1*, strain-specific nodulation ability is determined by single dominant host genes in the interactions between pea cultivar Afghanistan and two particular strains of *R. leguminosarum* bv. viciae: the plant gene *Sym4* determines resistance to nodulation by strain 310a, and another host gene determines resistance to strain PRE (41).

It is clear from the results presented here that a fine balance of control exists between positive and negative functions provided by *nod* genes and other genes in bacteria and plant hosts and determines cultivar specificity in the *Rhizobium*-legume symbiosis. These types of cultivar-specific interactions may be more common in the *Rhizobium*-legume symbiosis than once thought.

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