Horizontal gene transfer and mutation: Ng*rol* genes in the genome of *Nicotiana glauca*

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Ng*rol* genes (Ng*rolB*, Ng*rolC*, NgORF13, and NgORF14) that are similar in sequence to genes in the left transferred DNA (TL-DNA) of *Agrobacterium rhizogenes* have been found in the genome of untransformed plants of *Nicotiana glauca*. It has been suggested that a bacterial infection resulted in transformation of Ng*rol* genes early in the evolution of the genus *Nicotiana.* Although the corresponding four *rol* genes in TL-DNA provoked hairy-root syndrome in plants, present-day *N. glauca* and plants transformed with Ng*rol* genes did not exhibit this phenotype. Sequenced complementation analysis revealed that the Ng*rolB* gene did not induce adventitious roots because it contained two point mutations. Single-base site-directed mutagenesis at these two positions restored the capacity for root induction to the Ng*rolB* gene. When the Ng*rolB,* with these two base substitutions, was positioned under the control of the cauliflower mosaic virus 35S promoter (P35S), transgenic tobacco plants exhibited morphological abnormalities that were not observed in P35S-Ri*rolB* plants. In contrast, the activity of the Ng*rolC* gene may have been conserved after an ancient infection by bacteria. Discussed is the effect of the horizontal gene transfer of the Ng*rol* genes and mutations in the Ng*rolB* gene on the phenotype of ancient plants during the evolution of *N. glauca*.

Various features of living organisms elicit our interest in a better understanding of biodiversity. Many biologists agree that the diversification of creatures originated as a consequence of mutations introduced during their evolution. Horizontal gene transfer is a mutational process for transfer of nucleotides between organisms (1). Horizontal gene transfer is a mechanism for the acquisition of novel capability within a generation and is considered important in the divergence and adaptation of bacterial populations. Horizontal transfer of genes is also observed between other kingdoms. A typical example of such transfer was found in the interaction of *Agrobacterium* species with plants. Soil microorganisms in the genus *Agrobacterium* elicit morphological responses in certain plant species by transferring one or two small regions of DNA [transferred DNA (T-DNA)] from its plasmid to the host plant's genome (2). *Agrobacterium rhizogenes* is responsible for the formation of adventitious roots known as "hairy roots." The agropine-type strain of *A. rhizogenes* has two distinct T-DNA regions, left transferred DNA (TL-DNA) and right transferred DNA, in its Ri plasmid. Four loci involved in root induction have been identified from an analysis of TL-DNA by insertional mutagenesis (3). They are designated *rolA*, *rolB*, *rolC*, and *rolD*. These genes correspond to ORFs 10, 11, 12, and 15 among the 18 ORFs of TL-DNA (4). Of these, the *rolB* locus is thought to be the most important for hairy-root induction (3, 5–7). Studies of transgenic plants that carry various combinations of the TL-DNA genes have shown that two other ORFs, ORF13 and ORF14, also play significant roles in the induction of roots on carrot disks and tobacco leaf segments (6, 7). Whole plants regenerated from hairy roots exhibit a characteristic abnormal phenotype known as "hairy-root syndrome" (8). These plants stably transmit TL-DNA genes to their progeny.

Sequences similar to TL-DNA have been found in untransformed plants of the genus *Nicotiana* (9, 10). A sequence similar to TL-DNA was obtained from the genome of *Nicotiana glauca.* This region contained four ORFs that corresponded to *rolB*, *rolC*, ORF13, and ORF14 (11, 12). These ORFs were designated Ng*rolB*, Ng*rolC*, NgORF13, and NgORF14. Expression of the Ng*rol* genes has been detected in the genetic tumors of hybrids between *N. glauca* and *Nicotiana langsdorffii* (12–14). The reading frames of TL-DNA in *A. rhizogenes* are referred to herein as Ri*rol* loci, to distinguish them from the corresponding plant genes. Previous reports have suggested that an ancient infection of *Nicotiana* by an *A. rhizogenes*-like ancestor resulted in the horizontal transfer of T-DNA genes to their genomes (10, 11, 15). It was proposed that the horizontal transfer of these Ng*rol* genes might have affected the phenotype of *N. glauca* (11, 15–17), but little is known about the role of these genes. We are therefore interested in the effects of these Ng*rol* genes on plants. We considered the following questions: (*i*) whether the function of Ng*rol* genes has been conserved in plants after the ancient integration event, and (*ii*) why *N. glauca* plants do not exhibit characteristics of hairy-root syndrome. The differences in function between the genes of plants and bacteria are analyzed, and the history of Ng*rol* genes in the evolution of *N. glauca* is discussed.

Materials and Methods

Nucleic Acid Analysis. Ng*rol* genes were subcloned from clone λNg31 (kindly provided by M. P. Gordon and E. W. Nester, University of Washington), which included the TL-DNA-similar region from the genome of *N. glauca* (10). A plasmid, pNBC-1314, was constructed by subcloning the *Spe*I-*Xho*I fragment of 7,718 bp of λNg31 at the unique *Xba*I site into the T-DNA region of the binary vector pMM454-Km (kindly provided by M. Sekine, Nara Institute of Science and Technology, Japan) (Fig. 1*A*). Similarly, pNB was constructed by subcloning the *Spe*I-*Sal*I fragment of 2,972 bp in pMM454-Km. Ri*rol* genes were subcloned from plasmid pLJ1, which contained the TL-DNA region of pRiHRI of *A. rhizogenes* strain HRI (2). Construction of plasmids pNC1314, pRBC1314, pRB, and pRC1314 was described previously (6, 18). A binary vector pHTS6.1 was also used for the establishment of transformants (19).

ORF and promoter cassettes of *rolB* homologues were constructed for the promoter-swap experiment. Each cassette was fused and subcloned into the modified binary vector pMM454-KmRi13, which included a *Cla*I-*Hin*dIII 3,229-bp fragment of pLJ1 at the *Cla*I/*Hin*dIII site in the T-DNA region of pMM454-Km beforehand. A fragment containing the 5′ region of the Ri*rolB* ORF was amplified by PCR from pLJ1 with the

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Abbreviations: P35S, cauliflower mosaic virus 35S promoter; TL-DNA, left transferred DNA; T-DNA, transferred DNA; RB, the ORF cassette of Ri*rolB;* NB, the ORF cassette of Ng*rolB*; pRB, a plasmid harboring the Ri*rolB* locus in its T-DNA region; pNB, a plasmid harboring the Ng*rolB* locus in its T-DNA region; T0, primary transformants; T1, progeny plants of T0.

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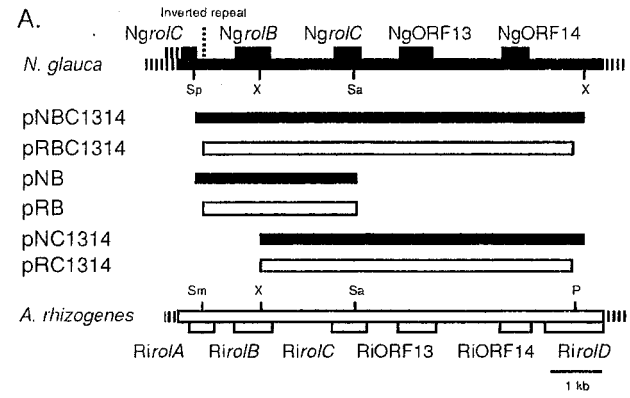

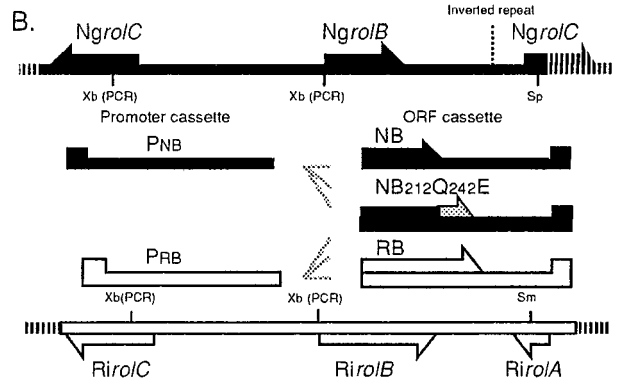

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   1                                                  52
Ri MDPKLLFLPRFQPVDLTPAWSQINLFEGIRFAFAIYSRDYSKPLLHFQKRWA
Ng  ASQSQ H     RN    GK    SKE QS  MT  EV   T  DY
   53                                                 104
Ri LAVLDLKENSPPIYILKQLAELLKNKVCYHPPMFVSQPDLARENDQHVFVYL
Ng DVIF  E K LRM             I        E       R  R  I
  105                                                 156
Ri SREKMQKVLKEQSITFGMEAVLATTIQPYRSELALQEMLRVHNLAWPHSRTE
Ng          E     V                D  V        R    R M
  157                                                 208
Ri EPDLECFIAIFASSLFIHLLELKVTNVYGREVACTFFLRRGTENRPYDVVAC
Ng  R           T     TT     L     D    V  AST         F
  209                                                 259
Ri GTTQFTKNALGISRPAASSPEPDLTLRLSGPDQEGEEGVMKPAAVNLKKEA*
Ng   * SE   V P L  P           W L  *S      S I       *
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Fig. 1. (*A*) Schematic representation of constructs. A restriction map of the Ng*rolB*-NgORF14 region (*Top*, solid line) is compared with the Ri*rolB*-RiORF14 region (*Bottom*, open box). The various fragments and chimeric constructs are indicated by solid bars (Ng*rol* genes) and open boxes (Ri*rol* genes). P, *Pvu*I; Sa, *Sal*I; Sm, *Sma*I; Sp, *Spe*I; X, *Xho*I. (*B*) Promoter and ORF cassettes. PNB, Ng*rolB* promoter cassette; PRB, Ri*rolB* promoter cassette; NB, cassette harboring the coding and 3′ untranslated region of Ng*rolB*; $NB_{212}Q_{242}E$, NB cassette under the modifications of site-directed mutagenesis; RB, cassette harboring the coding and untranslated region of Ri*rolB*; Xb (PCR), new restriction sites for *Xba*I that were created by PCR. (*C*) Comparison of the amino acid sequences deduced from the Ri*rolB* and Ng*rolB* genes (4, 11). Amino acids encoded by Ng*rolB* that are the same as those encoded by Ri*rolB* are not shown. Asterisks indicate termination codons. Arrows indicate the termination codons that were changed by site-directed mutagenesis. The restored region in the amino acid sequence encoded by $Ngrol B_{212}Q_{242}E$ is indicated by a box.

following primers: 5′-GCTCTAGAATGGACCCCAAATT-GCTA-3′ and 5′-TCGGATCCCCTCGAA-3′. The ORF cassette of Ri*rolB* (RB) was constructed by subcloning this PCR-amplified fragment into the Ri*rolB* sequence. A fragment that included part of the Ng*rolB*-coding region was generated by PCR from λNg31 with the following primers: 5′-GCTCTAGAATG-GCTTCCCAATCGCAATTC-3′ and 5′-GTCCCTTTCTCGA-GCCAAATC-3′. The ORF cassette of Ng*rolB* (NB) was constructed by introducing this fragment into the Ng*rolB* sequence. The promoter cassette PNB, with the promoter sequence of the Ng*rolB* gene, was amplified from λNg31 with the following primers; 5′-CATTCTAGAGTGAGTTGGA-3′ and 5′-ATCTCTAGATTCTCACCCGGCTCG-3′. By using the same primers, the promoter cassette PRB, with the promoter sequence of the Ri*rolB* gene, was generated from pLJ1.

The chimeric genes cauliflower mosaic virus 35S promoter (P_{35S})-Ng*rolB* and P_{35S}-Ri*rolB* included the Ng*rolB* and Ri*rolB* coding regions, respectively, under the control of P_{35S} (20). The NB and RB cassettes were used to construct these chimeric genes. These cassettes were also positioned under the chimeric promoter for high-level expression (21). A chimeric gene P_{35S}-glucuronidase (GUS), which included the GUS gene ORF under the control of P_{35S}, was used as the negative control.

The following oligonucleotide primers were synthesized for site-directed mutagenesis of Ng*rolB*: 5′-GGCACTACACAGT-TCAGCG-3′ and 5′-CTTGACCAGGAAAGCGAGG-3′. Nucleotide substitutions were made (Mutan-K kit, Takara Shuzo, Kyoto, Japan) by using the λNg31 sequence as the template. The resulting mutagenized Ng*rolB* sequence was used to construct chimeric genes under the control of the above-mentioned promoters.

The P_{35S}-Ng*rolC* gene was constructed as previously described in the construction of P_{35S}-Ri*rolC* (22). A fragment consisting of the Ng*rolC* gene containing the restriction site *Hpa*I was generated by PCR from the *N. glauca* genome with the following two primers: 5′-CCTACTTTGTTAACATGGC-3′ and 5′-GTCGGGCAGTCGACGTA-3′. The PCR-generated fragment of the 5′ region of Ng*rolC* was digested with *Hpa*I/*Sal*I and subcloned into pHTS6.1 (19), which previously harbored a partial sequence of Ng*rolC*.

Northern analysis was carried out with some tissues of plants by using antisense RNA probes as described (13).

Inoculations of Leaf Segments to Induce Hairy Roots. *Agrobacterium tumefaciens* EHA101 (23) that harbored constructs derived from pMM454-Km was tested for its ability to induce the formation of roots on tobacco-leaf segments. Inoculation was performed as described previously (6).

Molecular Evolutionary Analysis. Molecular evolutionary analysis of the Ng*rolB* and Ri*rolB* genes was performed with the ODEN computer program (Natural Institute of Genetics, Mishima, Japan). Numbers of nucleotide substitutions per site (K_n) were estimated by the method of Tajima and Nei (24). Numbers of synonymous (K_s) and nonsynonymous (K_a) substitutions per site were estimated by the method of Nei and Gojobori (25).

Results

Comparison of Function Between Ri*rol* and Ng*rol* Genes. The function of Ng*rol* genes was analyzed by transforming leaf segments of *N. tabacum* with *A. tumefaciens* that harbored either pRBC1314 or pNBC1314 (Fig. 1*A*). Almost all of the leaf segments inoculated with pRBC1314 developed vigorous roots that had the features of hairy roots (Fig. 2*A*). No extensive development of roots, however, appeared on the segments infected with *A. tumefaciens* that harbored pNBC1314. Phenotypes of regenerated plants that had been transformed with these genes were then studied. Whole plants resulting from introduction of the region, including the four Ri*rol* genes, displayed some abnormal phenotypic features, such as internodes of reduced length and wrinkled leaves (data not shown). Roots grew rapidly with a highly branched pattern and reduced geotropism. These features are characteristic of hairy-root syndrome (8). In contrast, transgenic plants that

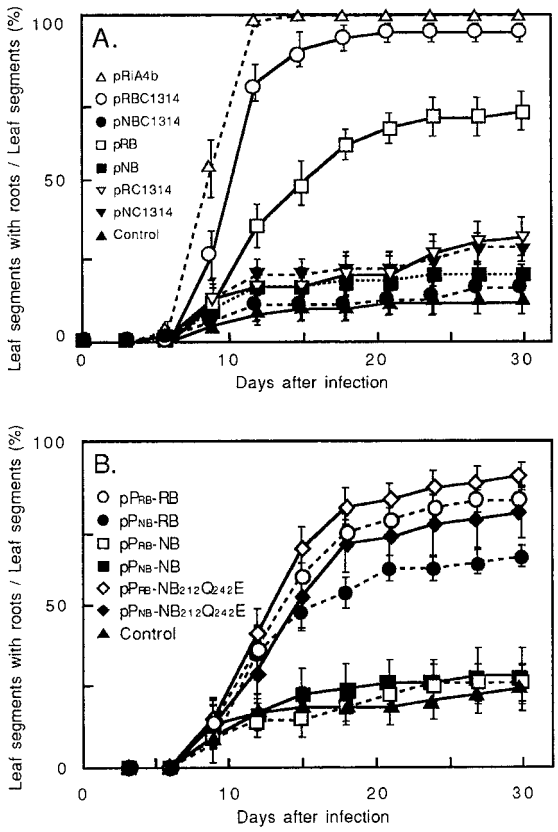

Fig. 2. Root Induction by Ng*rol* or Ri*rol* genes. The frequency of root production was determined by calculating the number of segments that formed roots as a percentage of the total number of segments in each flask. Binary vector pMM454-Km was used as the control. (*A*) Leaf segments were infected with *A. tumefaciens* harboring constructs of Ng*rol* and Ri*rol* genes. Sixty leaf segments in ten flasks were used for each construct. Bars indicate SE ($n = 10$). (*B*) Leaf segments were inoculated with the constructs of *rolB* homologues. Fifty-four leaf segments in nine flasks were examined for each construct. Bars indicate SE ($n = 9$).

harbored Ng*rol* genes did not exhibit any of these traits of hairy-root syndrome.

Functions of Ng*rolB* and Ri*rolB* Genes in Root Induction. Deletion analysis of the Ri*rolB*-RiORF14 region revealed that the Ri*rolB* locus is indispensable for adventitious root induction (6). To compare the functions of the Ri*rolB* and Ng*rolB* genes in root induction, constructs pRB and pNB were introduced into tobacco-leaf segments (Fig. 1*A*). When pRB was introduced into *N. tabacum*, significant induction of hairy roots was observed on leaf segments (Fig. 2*A*; refs. 5, 6). pNB, however, was unable to induce more root formation than the control (pMM454-Km). pNC1314 and pRC1314 were unable to induce roots as vigorously as did pNB.

Exchange of Promoter Regions Between Ng*rolB* and Ri*rolB* Genes. Nagata *et al.* (14) performed fluorometric and histochemical analyses of transgenic plants that harbored a GUS-reporter gene under the control of the promoters of the Ng*rolB* and Ri*rolB* genes. They showed that the pattern of expression of the Ng*rolB* gene was the same as that of the Ri*rolB* gene in terms of tissue specificity, but that the promoter of Ng*rolB* was 2- to 3-fold less active than that of Ri*rolB*. They suggested that the low level of activity of Ng*rol* promoters might be one of the reasons that *N. glauca* plants do not display symptoms of hairy-root syndrome.

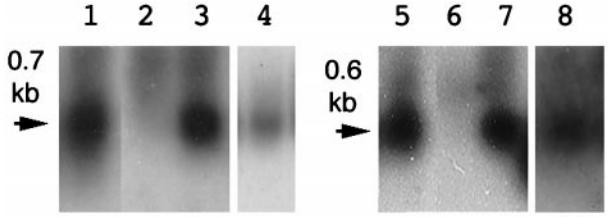

Fig. 3. RNA gel blot analysis of Ng*rolB* (lanes 1–4) and Ng*rolC* (lanes 5–8) in plant tissues. Lanes 1 and 5, genetic tumor of the hybrids of *N. glauca* and *N. langsdorffii*. Lanes 2 and 6, leaf tissue of *N. glauca*. Lanes 3 and 7, stem tissue of *N. glauca*. Lanes 4 and 8, callus tissue of *N. glauca* cultured in MS medium supplemented with 1-mg liter^{-1} kinetin. Poly(A)$^+$RNA (3 μg for lanes 1–3 and 5–7, and 10 μg for lanes 4 and 8) was subjected to electrophoresis. The probes for analysis of expression of the Ng*rolB* and Ng*rolC* were a 1.1-kbp *Pst*I-*Hin*dIII fragment and a 0.8-kbp *Hin*cII-*Eco*RI fragment of pLJ1, respectively (13).

The effect of promoter activity of these two genes on root induction was examined by exchanging the Ng*rolB* and Ri*rolB* promoters. The construct pPNB-RB fuses the promoter region of Ng*rolB* with the Ri*rolB*-coding region in pMM454-KmRi13 (Fig. 1*B*). The positive control, pPRB-RB, contains the Ri*rolB* gene under the control of its own promoter. Both pPNB-RB and pPRB-RB were able to elicit root formation (Fig. 2*B*). Root induction was not notably enhanced after inoculation with pPRB-NB or pPNB-NB. Therefore, the exchange of promoters did not significantly affect the functions of the coding regions of Ri*rolB* and Ng*rolB* genes. Slight depression of the frequency of root induction was, however, detected after transformation with pPNB-RB when compared with pPRB-RB. A difference in the activity of *cis*-acting sequences between Ri*rolB* and Ng*rolB* as shown by Nagata *et al.* (14) may cause this depression of root induction.

Transcription of Ng*rol* Genes in *N. glauca*. An explanation for the normal phenotype of *N. glauca* presented by Sinkar *et al.* (26) is that the Ng*rol* genes in *N. glauca* might not be transcribed in plant tissues, allowing plants to display the normal phenotype. Northern analysis was performed on some tissues of *N. glauca*. Transcripts of the Ng*rolB* and Ng*rolC* genes were detected in the stem tissue of *N. glauca* as well as the genetic tumor of hybrids of *N. glauca* and *N. langsdorffii* (Fig. 3). Callus tissues derived from *N. glauca* also contained the transcripts of these genes. In contrast, no signal was observed in the extract from leaf tissues. Recently, some reports showed that *trolC*, *torf13–1*, and *torf13–2* homologues of TL-DNA genes in the genome of *N. tabacum* are also transcribed in regulated patterns in plant tissues (16, 27).

Molecular Evolutionary Analysis. A comparison of the sequence of Ng*rolB* with that of Ri*rolB* revealed a difference between these genes in the lengths of their coding regions (Fig. 1*C*; ref. 11). *rolB* homologues of strains of *A. rhizogenes* other than the agropine-type strain also have coding regions longer than that of the Ng*rolB* gene (28–30). Although the ORF of Ng*rolB* starts at the corresponding base of Ri*rolB*, differences in bases at sites 633 and 723 bp from the initiation codon generate early-termination codons. The first termination codon reduces the ORF of Ng*rolB* by 144 bp from that of Ri*rolB*. We then compared the features of nucleotide-sequence differences between Ng*rolB* and Ri*rolB* genes. Numbers of nucleotide substitutions per site (K_n) were calculated for four different regions: the 5′ noncoding region, the coding region of 633 bp, the next region of 144 bp, and the 3′ noncoding region (Table 1). This 144-bp region is a 3′ untranslated region of Ng*rolB* gene that corresponds to a 3′ coding sequence in the Ri*rolB* gene. This region has a small K_n value that may indicate the presence of functional constraints

Table 1. A comparison of K_n, K_s and K_a values in Ng*rolB* and Ri*rolB* genes

	5′UTR*	ORF region†	144-bp portion‡	3′ UTR§
K_n	0.339 ± 0.021	0.197 ± 0.020	0.187 ± 0.042¶	0.362 ± 0.044
K_s		0.341 ± 0.058	0.267 ± 0.100	
K_a		0.153 ± 0.019	0.128 ± 0.038	
K_a/K_s		0.449	0.479	

*5′ untranslated region between *rol*B and *rol*C homologues (1,207 bp).
†ORF region of Ng*rolB* and Ri*rolB* gene (633 bp).
‡3′ untranslated region of Ng*rolB* gene that corresponds to 3′ coding sequence of Ri*rolB* gene (144 bp).
§3′ untranslated region from the site of Ri*rolB* termination codon to the site of the end of the left arm of the cellular T-DNA (318 bp; ref. 11)
¶K_n is significantly higher in the 5′ and 3′ UTRs than in the 144-bp portion, but is not significantly different between the ORF and 144 regions ($P < 0.01$; *t*-test).

against nucleotide changes. We can see that the K_n value in the 144-bp region is smaller than those in the 5′ and 3′ noncoding regions, but is much the same as that in the ORF region (Table 1). Frequencies of synonymous (K_s) and nonsynonymous (K_a) nucleotide substitutions and K_a/K_s ratios were also calculated. A K_a/K_s ratio <1 may result from the elimination of most nonsynonymous changes through natural selection. When the coding region and the 144-bp region were examined, K_s exceeded K_a for both the regions. These results revealed that the 144-bp region following the ORF of Ng*rolB* might have been conserved as a coding sequence for amino acids. Two single-base substitutions can change these two termination codons to amino acids. The resulting 48-aa extension is similar to the corresponding sequence of the Ri*rolB* protein. These results indicate that two point mutations of the Ng*rolB* gene occurred during the evolution of *N. glauca*.

Reversion of the Capacity for Root Induction by Site-Directed Mutagenesis. Two single base substitutions were made at sites 633 and 723 nt from the initiation codon of Ng*rolB* by site-directed mutagenesis. These substitutions changed the two termination codons (TAG and TAA) to a Gln (CAG) and a Glu (GAA), respectively. The putative reading frame of the resulting ORF, designated Ng*rolB*212Q242E (259 aa), closely matches that of Ri*rolB*. Tobacco-leaf segments were transformed with *A. tumefaciens* that carried the Ng*rolB*212Q242E construct under the control of the Ng*rolB* promoter in pMM454-KmRi13 (pPNB-NB212Q242E; Fig. 1*B*). More intense and earlier root formation was repeatedly observed after transformation with pPNB-Ng*rolB*212Q242E than with pPNB-NB (Fig. 2*B*). The ORFs of Ri*rolB* and Ng*rolB*212Q242E were equally effective in evoking root induction. The chimeric gene Ng*rolB*212Q242E under the control of the promoter of Ri*rolB* (PRB-NB212Q242E) was also able to elicit induction of roots. Thus, the restored region of 48 aa seems to be crucial for root induction of the Ng*rolB* gene.

Transformation with P35S-Ng*rolB*212Q242E. We further studied the function of the *rolB* homologues under the control of P35S. We used P35S because the growth-regulating function of these genes should become more clearly observable with high-level expression. More than six independent transformants were regenerated for each gene. Leaf disks inoculated with P35S-Ng*rolB*212Q242E exhibited reduced morphogenic capacity. Transformed plants grew slowly, the apex was distorted, the internodes were short, and the leaves exhibited wrinkled and epinastic growth (Fig. 4 *A* and *B*). Inflorescence had small numbers of irregularly shaped flowers with large sepals, wavy petals, and short pistils (Fig. 4*C*). Spontaneous self-pollination occurred normally, but production of seeds was reduced. These traits of P35S-Ng*rolB*212Q242E plants were also observed with another promoter for high-level expression (21). Transcripts of Ng*rolB*212Q242E were detectable in

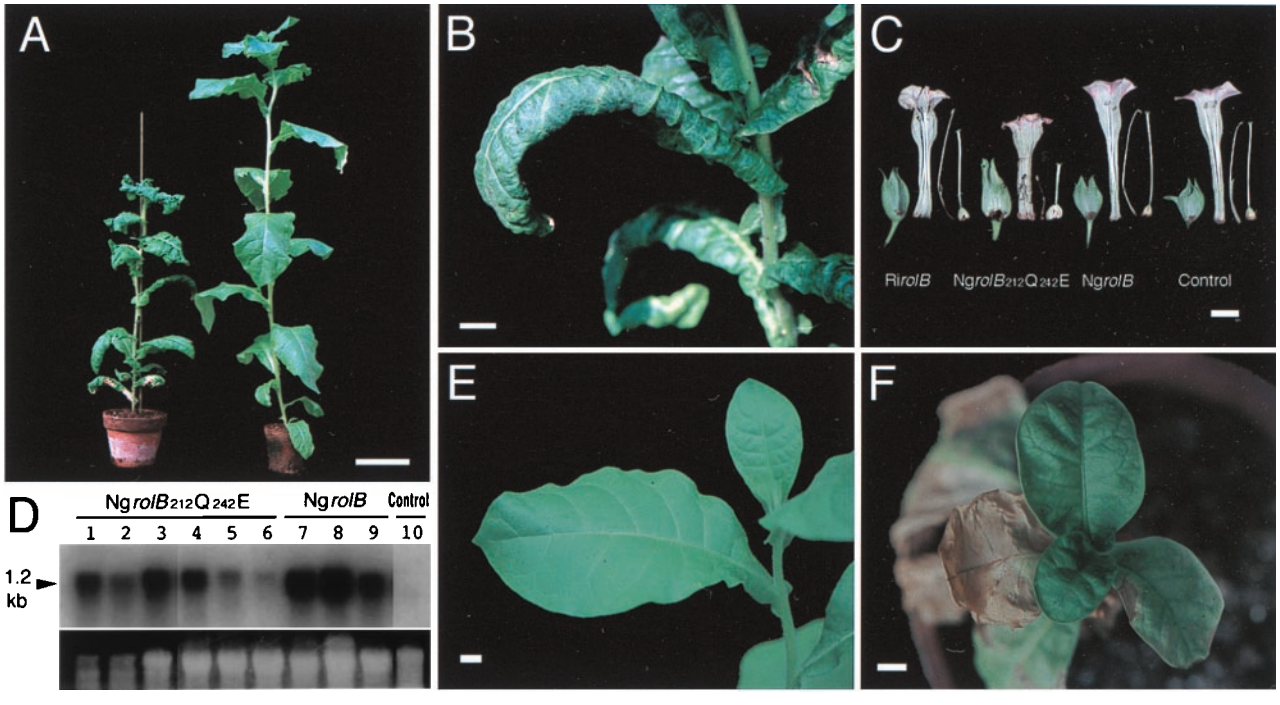

Fig. 4. Phenotypes of transgenic plants harboring *rolB* homologues. (*A*) A transgenic plant carrying the P35S-Ng*rolB*212Q242E chimeric gene (*Left*) and a control plant (*Right*). Bar = 10 cm. (*B*) Leaves of a Ng*rolB*212Q242E-overexpressing plant. (*E*) Leaves of a Ng*rolB*-overexpressing plant. (*F*) Leaves of a Ri*rolB*-overexpressing plant. (*C*) Comparison of floral leaves from transgenic plants. (*B, C, E,* and *F*) Bars = 1 cm. (*D*) Transcripts of the Ng*rolB*212Q242E (lanes 1–6) and Ng*rolB* (lanes 7–9) genes in transgenic plants. Lanes 1–3, T0 transgenic plants; lanes 4–9, T1 plants; lane 10, a control plant. The probe was a 0.8-kbp *Xba*I-*Sac*I fragment of λNg31. Fifteen micrograms of total RNA per lane was resolved on a formamide gel and blotted onto a nylon membrane.

leaves of primary transformants (T0) and their progeny plants (T1) that exhibited anomalies (Fig. 4*D*). The level of transcription was weak in progeny of another line, evidencing mild phenotype with slightly distorted stems and wrinkled leaves (Fig. 4*D*, lane 6). By contrast, regenerated plants transformed with P35S-Ng*rolB* had a normal phenotype, as did their progeny (Fig. 4*E*). Established P35S-Ng*rolB* transgenic lines that exhibited stable normal morphology expressed the Ng*rolB* gene at varying levels. The size of the transcripts of P35S-Ng*rolB* was larger than that under the control of its own promoter. Enlargement of the transcript size was also reported when the Ri*rolB* gene was controlled by P35S (31). Overexpression of the Ri*rolB* gene was also examined in tobacco plants of six independent lines. As shown by Schmulling *et al.* (31), P35S-Ri*rolB* transformed plants had flat, round, and highly necrotic leaves (Fig. 4*F*). In contrast, P35S-Ng*rolB*212Q242E transgenic leaves were wrinkled, with epinastic growth, and were not necrotic. Roder *et al.* (32) reported that transformation with the Ri*rolB* gene under the control of a tetracycline-dependent promoter resulted in extremely stunted plants with necrotic and wrinkled leaves that did not develop a floral meristem. Although delay of growth and wrinkling of leaves were observed into the P35S-Ng*rolB*212Q242E plants, these plants developed inflorescence. We must not forget that, in addition to the generation of termination codons, additional mutations may have taken place in the Ng*rolB* sequence during the evolution of *N. glauca*. Much still remains to be done to understand the morphogenic activity of the *rolB* homologues, Ng*rolB*212Q242E and the ancient Ng*rolB* gene.

Transformation with P35S-Ng*rolC*. Analysis of the Ng*rolB* gene led us further into a consideration of the function of the other Ng*rol* genes. Comparison of the DNA sequences of Ng*rolC* with Ri*rolC* revealed that the reading frames of Ng*rolC* initiate and terminate at the corresponding bases of Ri*rolC*. Tobacco-leaf disks were transformed with the P35S-Ng*rolC* chimeric gene. More than 10 T0 regenerants showed a dramatically dwarfed phenotype, probably because of reduced internodal length (Fig. 5*A*). The P35S-Ng*rolC* transgenic leaves were lanceolate and pale green (Fig. 5*B*). Their floral organs were slender and small. Transgene expression was detected in transformants that showed the characteristic morphological alterations (Fig. 5*D*). No transcripts were detectable in leaves from a comparable T0 plant with a normal phenotype (Fig. 5*D*, lane 5). These characteristics are identical to the phenotype of the P35S-Ri*rolC* transgenic plants reported previously (22, 31). We have bred three independent P35S-Ng*rolC* and P35S-Ri*rolC* T0 plants that exhibited the same traits of abnormal leaf morphology (Fig. 5*C*). Their leaves had pale-green inner blades and wrinkled dark-green margins. These phenotypes may be caused by periclinal chimeric regeneration, as reported previously (22, 33).

Discussion

Analysis of the DNA sequence supported a model where members of the genus *Nicotiana* carry a portion of T-DNA from an ancient infection by the *A. rhizogenes*-like ancestor (10, 11). The acquisition of T-DNA may have altered the phenotype or selective advantage of the infected plants (11, 15–17). This alteration may explain the establishment and persistence of the homologues of *rol* genes in present-day plants. Alternatively, the T-DNA may have been trapped by chance survival of a regenerated plantlet (15). We are examining the possible role of the Ng*rol* genes in *N. glauca*.

We suggest that *N. glauca* plants do not exhibit the phenotype of hairy-root syndrome because the Ng*rolB* sequence might have been truncated. An *A. rhizogenes*-like ancestor might have had the Ng*rolB* sequence with more amino acids than that of today in its plasmid. The ancient transformed plant harboring the complete Ng*rolB* gene might have formed hairy roots. Tepfer (8) referred to the observation that hairy roots of tobacco plants regenerated shoots when grown in pots, but roots of normal

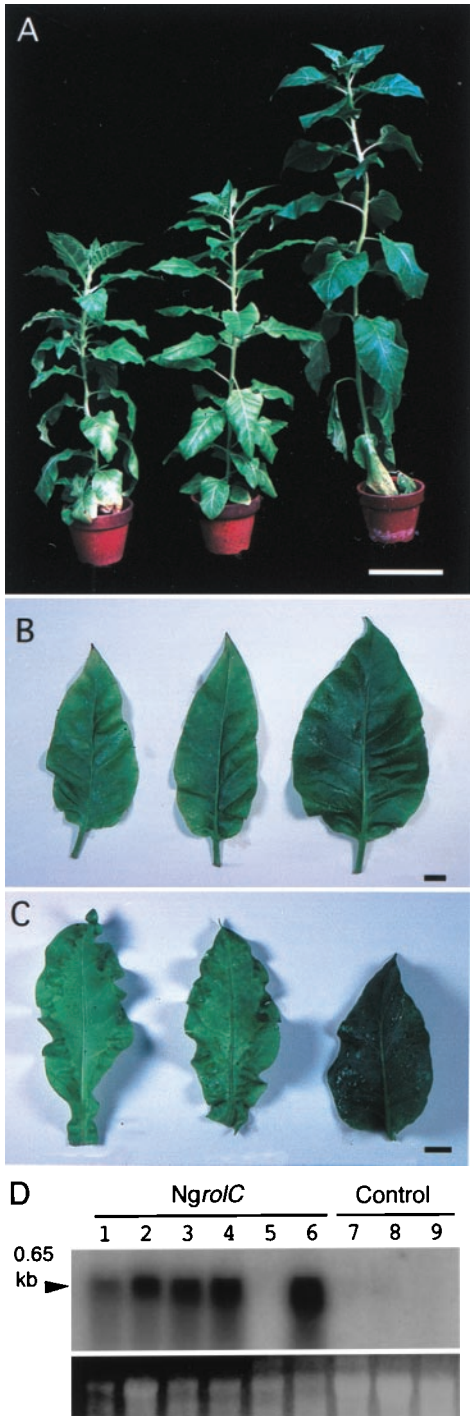

Fig. 5. Comparison of plants transformed with *rolC* homologues. (*A*) Whole plants harboring P35S-Ri*rolC* chimeric gene (*Left*) or P35S-Ng*rolC* gene (*Center*) and a control plant (*Right*). Bar = 10 cm. (*B*) Leaves from transgenic plants harboring P35S-Ri*rolC* (*Left*) or P35S-Ng*rolC* (*Center*) and from a control plant (*Right*). (*C*) Abnormal chimeric morphology of leaves from transgenic plants harboring P35S-Ri*rolC* gene (*Left*) or the P35S-Ng*rolC* gene (*Center*). A normal leaf of wild-type tobacco is shown as a control (*Right*). (*B* and *C*) Bars = 1 cm. (*D*) Transcripts of the Ng*rolC* in leaf tissues of transgenic plants. Lanes 1–5, T0 plants infected with *A. tumefaciens* harboring the P35S-Ng*rolC* gene; lane 6, a T1 plant; lanes 7–9, wild-type and control tobacco plants. The probe was a 1.1-kbp *Eco*RV-*Eco*RI fragment of λNg31.

plants did not regenerate shoots. Progeny of regenerated plants from the ancient hairy roots might have had the Ng*rol* genes in their genome. Subsequently, nonsense point mutations in the Ng*rolB* gene might have occurred, and the phenotype of hairy-root syndrome might have been lost. The present-day Ng*rolB* gene did not direct hairy-root formation. It is unlikely that a truncated Ng*rolB* gene was transformed to an ancestral plant.

The Ng*rolC* gene elicited biological response in transgenic tobacco plants when controlled by the P35S. This gene was transcribed in some tissues of *N. glauca*. Nagata *et al.* (14) have shown that the expression pattern of the promoter of the Ng*rolC* gene exhibited much the same tissue specificity as that of the Ri*rolC* promoter. We propose that the functional sequence of the Ng*rolC* gene may have been conserved after the ancient introduction from the bacterium. Transformation of Ri*rolC* has altered the resistance reactions of potato to pathogens and the flowering time of carrot (17, 34). Expression of Ng*rolC* may be connected with these phenotypes of *N. glauca*. We also reported that the functional sequences of NgORF13 and NgORF14 genes have been conserved after the ancient infection event as well as Ng*rolC* has been conserved (18). However, we cannot say for certain that the level of expression of these genes is sufficient for the appearance of their capacities (26). Further analysis of the expression pattern and level of Ng*rol* genes in tissues of *N. glauca* should help to clarify the physiological role of these genes.

The natural genetic transformation of *rol* genes might have generated novel variants that are progenitors of modern species (11, 15–17). The phenotype induced by *rol* genes could have adaptive significance for the plants (15–17). Phenotypic alterations caused by the expression of Ng*rol* genes might have contributed to the creation of biodiversity in the genus *Nicotiana*. Previous studies showed that several plants, including carrot and morning glory, also carry sequences similar to T-DNA (35, 36). These results raise the possibility that horizontal gene transfers, from bacteria to plants, have occurred during the evolution of some plant species. Further analysis of *rol*-homologues in plants might shed some light on the evolution of these plant species.

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