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Studies on *Agrobacterium rol* Gene Homologues in *Nicotiana rustica* L., *N. plumbaginifolia* viv. and their Hybrid

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Abstract: Present study tends to investigate the existence of the bacterial oncogenes- *rol* genes of *Agrobacterium rhizogenes* Riker., in *Nicotiana rustica, N. plumbaginifolia* and their hybrid. These *Nicotiana* species and hybrid were normal morphologically under field conditions without any neoplastic growth. So, the habituation studies were carried out to identify the presence of *rol* genes by culturing the leaf discs on hormone supplemented Murashige and Skoog medium and then shifting to MS basal medium. Habituation showed phytohormone independent growth response even after shifting to MS basal medium indicating the presence of *rol* genes. Then, Polymerase Chain Reaction analysis showed amplification of *rol A, B* and *C* genes in *N. rustica*, *rol A* and C in *N. plumbaginifolia* and the hybrid. The amplified genes were sequenced and deposited in GenBank. The homology between the novel genes was compared with the *rol* genes of *A. rhizogenes* as well as the other plants species containing *rol* gene homologues using Pairwise sequence alignment search tool. Results were discussed in terms of the occurrence of bacterial genes in plants, the extent of their expression and the probable reasons for silencing of the foreign genes.

Key words: Phytohormonal habituation, morphogenesis, gene expression, *rol* genes, sequence alignment, variations

INTRODUCTION

Nicotiana is one of the common genera containing the bacterial oncogenes of *Agrobacterium rhizogenes* (Joshua *et al*., 2009). Their occurrence indicates the ancient horizontal transfer of T-DNA of the Ri plasmid from *A. rhizogenes* to the plant genome (Christey and Braun, 2005). The integration of T-DNA into the *Nicotiana* genome is through insertion at double stranded breaks by nonhomologous end joining (Chilton and Que, 2003). Expression of the cellular *rol* gene homologues present in plants alter endogenous auxin/cytokinin balance causing severe developmental abnormalities, neoplastic growths, morphological and physiological abnormalities like dwarfed phenotype, reduced apical dominance, smaller, wrinkled leaves, increased rooting, altered flowering and reduced fertility (Casanova *et al*., 2005). Normally, the plants holding *rol* genes show habituation or phytohormone independent growth on MS (Murashige and Skoog, 1962) basal medium. Differences were observed in the extent of expression of Ri plasmid genes of *A. rhizogenes* which was dependent on the position of incorporation of these genes into the plant kingdom (Mirza, 2005).

Cellular *rol* gene expression results in tumorigenesis, several morphological abnormalities and phytohormone independent growth under culture conditions in *N. glauca*×*N. langsdorffii* hybrid (Suneetha *et al*., 2006, 2009). Expression of *rol* genes is employed in floriculture in improving the ornamental and horticultural traits (Casanova *et al*., 2005) like rose (Souq *et al*., 1996), lily (Mercuri *et al*., 2003) and carnation plants. They are useful as potential activators of secondary metabolism (Bulgakov, 2008) in transgenic plants. *rol* genes expression resulted in 15 fold increase of anthraquinones in the transformed calli of *Rubia cordifolia* (Shkryl *et al*., 2008), production of 25 tropane alkaloids in transgenic *Datura innoxia* (Jousse *et al*., 2010) and in molecular breeding towards compact growth as studied in *Kalanchoe blossfeldiana* (Christensen *et al*., 2008).

The aim of this study is to understand the response of habituation in *N. rustica, N. plumbaginifolia, N. rustica* × *N. plumbaginifolia* hybrid under culture conditions; to identify the presence of *rol* genes in parents and the hybrid by doing PCR analysis; sequencing the genes; submission the sequences to Genbank of NCBI for accession numbers and pairwise

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sequence alignment for comparing the cellular *rol* gene sequences of the parents and the hybrid with the *rol* genes of *A. rhizogenes* and the other cellular homologues.

MATERIALS AND METHODS

Plant material, callus induction and habituation: The parents *N. rustica*, *N. plumbaginifolia* and *N. rustica* × *N. plumbaginifolia* hybrid (Dr. T.V.R. Lakshmi Andhra University, 2006-2008) were grown in botany farm of Andhra University, Visakhapatnam. Leaves collected from 3 months old plants of *N. rustica*, *N. plumbaginifolia* and the hybrid were surface sterilized with ethanol and 0.1% mercuric chloride. Leaf discs were cultured on MS basal medium at 25°C and 16 h light/8 h dark photoperiod. Subculturing was done for every 21 days and the results were recorded.

To elicit the response of habituation, the leaf discs of *N. rustica*, *N. plumbaginifolia* and the hybrid were initially cultured either on MS+BAP (5 mg L^{-1}) or MS+IAA (3 mg L^{-1}) medium. The cultures were grown at 25°C and 16 h light/8 h dark photoperiod. Twenty one days old cultures from both the hormonal media were shifted to MS basal medium and the results of 45 days old cultures were recorded.

DNA isolation, amplification and sequencing: Genomic DNA was extracted from fresh tender leaves of *N. rustica*, *N. plumbaginifolia* and the hybrid using Dellaporta method of plant DNA extraction (Dellaporta *et al*., 1983). The extraction procedure includes the reaction with extraction buffer (1 M Tris HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl, 10 μM β-mercaptoethanol, 1% Polyvinyl pyrrolidone), 20% SDS at 65°C, precipitation of protein with 5 M potassium acetate, precipitation of DNA with iso-propanol and suspension of crude DNA in resuspension buffer 1 (100 mM EDTA and 250 mM Tris HCl). The DNA was purified using RNase, phenol:chloroform:isoamylalcohol (25:24:1), precipitated with 3 M sodium acetate, chilled ethanol and resuspended in resuspension buffer 2 (50 mM EDTA and 100 mM Tris HCl). The concentration and purity of DNA were estimated using UV-VIS spectrophotometer (Systronics, Model-117).

DNA amplification reaction was carried out in Appendorf's master cycler using the genomic DNA of *N. rustica*, *N. plumbaginifolia* and the hybrid as templates and 18mer sequences of *rol A, B* and *C* genes of *A. rhizogenes* as primers (Biotech Desk Pvt. Ltd.), Taq DNA polymerase and its buffer with $MgCl_2$ and dNTP mix (Bangalore Genei Pvt. Ltd.).

The primer sequences are as follows

- *rolA* primers - Forward: 5'-GGAATTAGCCGGACTAAA-3';
- Reverse: 5'-AGGTCTGAATTTTCACGT-3';
- *rolB* primers - Forward: 5'-CAAATTGCTATTCCTTCC-3'
- Reverse: 5'-TTACTGCAGCAGGCTTCA-3'
- *rolC* primers - Forward: 5'-GTCGAGGATGTGACAAGC-3'
- Reverse: 5'-GCCGATTGCAAACTTGCA-3'

Thermal profile was as follows: Initial denaturation at 94°C for 4 min, Denaturation at 94°C for 1 min, Annealing at 59°C for 1 min and Extension at 72°C for 1.5 min - this reaction was repeated for 30 cycles; Final extension at 72°C for 10 min. Amplified fragments of DNA were separated on gel electrophoresis using 1.2% agarose gel and the size of the fragments were measured with reference to 100 bp ladder.

Specific PCR amplified fragments of the size of *rol A*, *B* and *C* genes were gel eluted from low melting agarose gel (1%) using elution buffer (1 M Tris-Cl, 0.5 M EDTA; pH 8.0), precipitated using 10 M ammonium acetate, dissolved in 0.1 M Tris-Cl (pH 8.0) and were sequenced utilizing the services of MWG Biotech Pvt. Ltd., Bangalore, India. The sequences were extracted in Fasta format using Chromas software downloaded from http://www.technelysium.com.au/chromas.html

Genbank submission and pairwise sequence alignment: The sequences of cellular *rol* genes present in *N. rustica*, *N. plumbaginifolia* and the hybrid were submitted to GenBank of NCBI (National Centre for Biotechnology Information) using BankIt tool (www.ncbi.nlm.nih.gov/websub/tool) for further evaluation of authenticity, novelty of the sequences and for accession numbers.

The statistical significance of matches between the *rol* genes of *A. rhizogenes* and cellular *rol* genes of *N. rustica*, *N. plumbaginifolia* and the hybrid were studied using the BLAST (Basic Local Alignment Search Tool) tool available under NCBI (www.ncbi.nlm.nih.gov/BLAST).

RESULTS

The parents *N. rustica*, *N. plumbaginifolia* and their hybrid were normal morphologically and physiologically under field conditions. The hybrid developed resembled *N. rustica* in the shape of the leaf, flower, pod and the seed.

Response of *N. rustica* and PCR analysis: Increase in the leaf disc size and callus initiation was observed on MS basal medium. Shooty callus growth observed on MS supplemented with BAP (5 mg L^{-1}) within 21 days showed shooty growth with few roots after shifting to MS basal medium. Similarly, white callus with roots was observed when the leaf discs were cultured on MS supplemented with IAA (3 mg L^{-1}) within 21 days which continued even after shifting to MS basal medium (Table 1).

N. rustica showed amplification with *rolA* (240 bp band), *rolB* (780 bp band) and *rolC* (490 bp band) sets of primers. The following were the Genbank accepted sequences with their accession numbers of *N. rustica:*

Cellular *rolA* gene present in *Nicotiana rustica*

```
LOCUS GU132537 237 bp DNA

ORIGIN
 1 ctcggctgcc agacttcgga gtatatcgct cgtctgttct aagcttgtta ggcgtgcaaa
 61 ggccaagagg aaggccaaac gggtatcccc gggcgaacgc gaccatcttg ctgagccagc
 121 caatctgagc accactcctt tggccatgac ttcccaagcc cgaccgggac gttcaacgac
 181 ccgcgagttg ctgcgaaggg accctttgtc gccggacgga aaaatttcag acctaag
//
```

Cellular *rolB* gene present in *Nicotiana rustica*

```
LOCUS GU182969 775 bp DNA linear
ORIGIN
 1 ggttgcacag cttcatgacg ccctcctcgc cttcctgatc aggccccgag gagtcgcagg
 61 gttaggtctg gctccggtga ggaggcggcc ggacgtgata tcccgagggc atttttggtg
 121 aattgtgtgg tgccgcaagc tacaacatca taggggcggt tttcagtccc tcgccgcaga
 181 aagaaggtgc aagctacctc tctcccgtaa acgttggtca cttttaactc cagcaagtga
 241 atgaacaagg aacttgcgaa aatggcgatg aagcattcta aatcaggttc ctccgtgcgg
 301 ctgtgcggcc aagcaaggtt gtgaacacgg agcatctcct ggagggcgag ctcgctccga
 361 tatggttgaa tcgttgtcgc cagcacggcc tccattccaa atgtaatgga ttgttccttc
 421 agcactttct gcatcttctc gcgagaaaga tagacaaata catgttggtc gttttctcga
 481 gccagatccg gctgactaac aaacatagga ggatgatagc agactttgtt cttcaagagc
 541 tcagctagtt gtttaagtat atatatcggt ggagagtttt ccttcaaatc tagcactgca
 601 agagcccatc gtttctggaa atgcaggagg ggtttgctat agtcacggct atagattgca
 661 aaagcaaatc ggatcccctc gaataggttt atctggctcc atgctggagt gagatctact
 721 ggttgaaatc gtgaggaata aacccctttt ttgggaatgg ggtagacata ccttt
//
```

Cellular *rolC* gene present in *Nicotiana rustica*

```
LOCUS GU182971 487 bp DNA linear
ORIGIN
 1 caatagtact agaccctgca tgacgcctca gatgtagcgt ataccttgt atcgtagccg
 61 ggctggataa tcaatcgtat ggatattgac taagaaggag ggtcggtggg ccacgggctg
 121 cgtgtgcctc tacgtcgact gcccgacgat gatgcgtctc gcttctatgg agggtccttg
 181 ccttacaatt ggatgcaagg cgcgctcctc accaagcttc ccccgtaccg gcatgatgtg
 241 actgttcgat gaggtcgtaa cagggctcac acaagcatca ggttttttcg gttacgcgga
 301 tcctatgcgg agcgcctact tcgctgcatt ttctttccct gggcgtgtca tcaagctgaa
 361 tgagcagatg gagctaactt cgacaaaggg aaagtgtctg acattcgacc tctatgccag
 421 cacccagctt aggttcgaac ctggtgagtt ggtgaggcat ggcgagtgag tttttcaaaa
 481 ccgcaaa
//
```

Table 1: Response of the leaf discs of *N. rustica* on MS basal medium and their habituation after hormonal shifts

Type of medium	Discs cultured	Responded	% of response	Type of response
MS	250	168	67	Callus initiation
MS+BAP	250	220	88	Shooty callus
BAP to MS	200	200	100	Shoots with few roots
MS+IAA	250	229	92	Rooty morphogenesis
IAA to MS	200	200	100	Rooty growth continued

Response of *N. plumbaginifolia* and PCR analysis: A very slow growth of callus initiation was observed from the leaf disc on MS basal medium. Callus initiation from the veinal region was observed on MS with BAP (5 mg L^{-1}) within 21 days which showed a fast growth of multiple shooty morphogenesis upon shifting to MS basal medium. Similarly, white rooty callus growth was observed when the leaf discs were cultured on MS with IAA (3 mg L^{-1}) within 21 days which proliferated after shifting to MS basal medium (Table 2).

N. plumbaginifolia showed amplified fragments only for *rolA* (250 bp band) and *rolC* (480 bp band)sets of primers. The following were the Genbank accepted sequences with their accession numbers of *N. plumbaginifolia:*

Cellular *rolA* gene present in *Nicotiana plumbaginifolia*

```
LOCUS GU132539 247 bp DNA linear
ORIGIN
 1 ctcggctggc cagacttcgg agtatatcgc tcgtctgttc taagcttgtt aggcgtgcaa
 61 aggccaagag gaaggccaaa cgggtatccc cgggcgaacg cgaccatctt gctgagccag
 121 ccaatctgag caccactcct ttggccatga cttcccaagc ccgaccggga cgttcaacga
 181 cccgcgagtt gctgcgaagg gaccctttgt cgccggacgt gaaaattcaa gacctaggtt
 241 ttcttgc
//
```

Cellular *rolC* gene present in *Nicotiana plumbaginifolia*

```
LOCUS GU182973 478 bp DNA linear
ORIGIN
 1 cgtactgcta gaccatgaag acgcctcaaa tgagcgtaac ccttgatcga gccgggtgag
 61 aatcaatcga tggatattga cgaagaagga gggtcggtgg gccacgggct gctgtacctc
 121 tacgtcgact gcccgacgat gatgctctgc ttctatggag ggtccttgcc ttacaattgg
 181 atgcaaggcg cactcctcac caaccttccc ccgtaccagc atgatgtgac tctcgatgag
 241 gtcaatagag ggctcaggca agcatcaggt tttttcggtt acgcggatcc tatgcggagc
 301 gcctacttcg ctgcatttct ttccctgggc gtgtcatcaa gctgaatgag cagatggagc
 361 taacttcgac aaagggaaag tgtctgacat tcgacctcta tgccagcacc cagcttaggt
 421 tcgaacctgg tgagttggtg aggcatggcg agtgattttt aaaaacgcca caacacca
//
```

Response of *N. rustica* × *N. plumbaginifolia* and PCR analysis: Callus initiation was observed on MS basal medium, green shooty callus was grown on MS with BAP (5 mg L^{-1}) within 21 days and a very fast growth of multiple shoots with few roots was observed when shifted to MS basal medium. Similarly, white rooty callus growth was observed on MS with IAA (3 mg L^{-1}) which continued on shifting to MS basal medium (Table 3).

N. rustica×*N. plumbaginifolia* showed amplified fragments for *rolA* (290 bp band) and for *rolC* (480 bp band) sets of primers. The following were the Genbank accepted sequences with their accession numbers of the hybrid:

Table 2: Response of the leaf discs of *N. plumbaginifolia* on MS basal medium and their habituation after hormonal shifts

Type of medium	Discs cultured	Responded	% of response	Type of response
MS	250	100	50	Slight callus initiation
MS+BAP	250	125	50	Callus initiation
BAP to MS	200	100	50	Multiple shoots
MS+IAA	250	200	92	Rooty morphogenesis
IAA to MS	200	200	100	Rooty growth continued

Table 3: Response of the leaf discs of *N. rustica* x *N. plumbaginifolia* on MS basal medium and their habituation after hormonal shifts

Type of medium	Discs cultured	Responded	% of response	Type of response
MS	250	175	70	Callus initiation
MS+BAP	250	220	88	Green callus
BAP to MS	200	200	100	Shooty and rooty callus
MS+IAA	250	225	90	Rooty morphogenesis
IAA to MS	200	190	95	Rooty growth continued

Cellular *rolA* gene present in *Nicotiana rustica* × *Nicotiana plumbaginifolia*

```
LOCUS GU132538 283 bp DNA linear
ORIGIN
 1 ctgcggcatg gcccagtacc ttcggaagta ttatcgctcg tctgttctaa gcttgttagg
 61 cgtgcaaagg ccaagaggaa ggccaaacgg gtatccccgg gcgaacgcga ccatcttgct
 121 gagccagcca atctgagcac cactcctttg gccatgactt cccaagcccg accgggacgt
 181 tcaacgaccc gcgagttgct gcgaagggac cctttgtcgc cggacggaaa attacagacc
 241 ttaagtttgt gttcttcact tgtttgtgaa atgggctttc att
//
```

Cellular *rolC* gene present in *Nicotiana rustica* × *Nicotiana plumbaginifolia*

```
LOCUS GU182972 480 bp DNA linear
ORIGIN
 1 cgatgagctg ctagcctgag aacgctcaat gagcgtaacc cttgatcgag ccgggtgaga
 61 tcatcgatgg atattgacga agaaggaggg tcggtgggcc acgggctgct gtacctctac
 121 gtcgactgcc cgacgatgat gctctgcttc tatggagggt ccttgcctta caattggatg
 181 caaggcgcac tcctcaccaa ccttcccccg taccagcatg atgtgactct cgatgaggtc
 241 aatagagggc tcaggcaagc atcaggtttt ttcggttacg cggatcccta attgccggga
 301 ggcggcccta ctttcgctgc attttctttc cctgggcgtg tcatcaactt gaatgagcag
 361 atggagctaa ctcgacaaag ggaaagtgtc tgacattcga cctctatgcc agcacccagc
 421 ttaaggttcg aacctggtga aattggtgaa gccatgccaa ggattttttt aaaaaaaata
//
```

Table 4: Genbank accession numbers for the cellular *rol* gene sequences

Source of cellular	*rolA/B/C* gene	*rol* gene Accession No.
N. rustica	rolA	GU132537
N. plumbaginifolia	*rolA*	GU132539
N. rustica×*N. plumbaginifolia*	*rolA*	GU132538
N. rustica	*rolB*	GU182969
N. rustica	*rolC*	GU182971
N. plumbaginifolia	*rolC*	GU182973
N. rustica×*N. plumbaginifolia*	*rolC*	GU182972

List of novel cellular *rol* gene sequences with their accession numbers were given in Table 4.

Pairwise sequence analysis of cellular *rol* genes with *A. rhizogenes rol* genes: The cellular *rolA* genes of *N. rustica* and *N. plumbaginifolia* showed 97% and *N. rustica* × *N. plumbaginifolia* hybrid showed 98% identity with *r olA* gene of Ri plasmid Agropine strain A4 of *A. rhizogenes*. The other cellular homologue present was *rolA* gene of *A. thaliana* showing 97% identity with the cellular *rolA* genes of present study. The cellular *rolB* gene of *N. rustica* showed 99% identity with the *rolB* gene of Ri plasmid Agropine strain A4 of *A. rhizogenes*. Cellular *rolB* was also identified in *N. glauca* showing 83% identity with *rolB* genes of *N. rustica*. Very few variations of addition and deletion mutations were observed in pairwise sequence alignment with reference to *rolA* and *B* genes of *A. rhizogenes*. The cellual *rolC* genes of *N. rustica*, *N. plumbaginifolia* and the hybrid showed 84, 99 and 84% identity, respectively with the *rolC* gene of *A. rhizogenes* Agropine type strain A4. The cellular *rolC* homologues were also identified in *N. debneyi*, *N. glauca* and *N. cordifolia* which showed 80-84% identity with the *rolC* genes of *N. rustica*, *N. plumbaginifolia* and the hybrid. More number of variations of addition, deletion and substitutions were observed in cellular *rolC* than *rolA* and *B* genes studied earlier.

DISCUSSION

Plants have unique behavior of coexisting with microorganisms especially with *Agrobacterium rhizogenes*. Several members of the plant kingdom like *Kalanchoe*, *Petunia*, *Daucus* and 15 out of 42 species of *Nicotiana* so far studied were found to contain the TL-DNA genes (Intrieri and Buiatti, 2001). In the present study cellular *rol* genes were identified for the first time in *N. rustica*, *N. plumbaginifolia* and their hybrid *N. rustica* × *N. plumbaginifolia*.

Three types of expression levels were identified in plants carrying cellular *rol* genes (i) Silencing of cellular *rol* genes in field conditions and their expression under culture conditions showing phytohormone independent hormonal autonomy. As studied here the cellular *rol* genes expression was observed only under culture conditions showing enhanced culture response with organogenesis on hormonal shifts. The shooted calli (MS+BAP) developed few roots (MS alone) in *N. rustica*

and in the hybrid cultures but the rooted calli (MS+IAA) continued their growth on MS basal medium. On the other hand, in *N. plumbaginifolia* the shooty (MS+BAP) and rooty (MS+IAA) growth continued even after shifting to MS basal medium. The *rol* gene products of *rolA* (Sun *et al*., 1991), *rolB* (Estruch *et al*., 1991a) and *rolC* (Estruch *et al*., 1991b) alter the endogenous auxin/cytokinin balance, hence might be responsible for phytohormone independent growth under culture conditions. (ii) The cellular *rol* genes may remain permanently silent for generations both under field and culture conditions as studied in *N. glauca* (Suneetha *et al*., 2009). (iii) On the other hand, the expression of cellular *rol* genes result in tumorigenesis, morphological abnormalities in field and phytohormone independent growth under culture conditions as observed in *N. glauca* × *N. langdorffii* hybrid (Udagawa *et al*., 2004; Suneetha *et al.,* 2006).

The sequence of these genes viz., *rolA, B* and *C* in *N. rustica*; *A* and *C* genes in *N. plumbaginifolia* and the hybrid were highly homologous (>95% in *rolA* and *B;* ~80% homology for *rolC*) to the *rolA, B* and *C* genes present in Ri plasmid of *A. rhizogenes* strain A4. This represents the bacterial origin of the cellular *rol* genes present in plants and the donor strain could be strain A4 of *A. rhizogenes.* The entry of *rol* genes from *A. rhizogenes* to *Nicotiana* genome is through horizontal gene transfer (Aoki, 2004) from the bacterial plasmid to the plant genome. The other genes cellular *rol* genes include *rolA* of *Arabidopsis thaliana, rolB* of *N. glauca* and *rolC* of *N. glauca, N. cordifolia* and *N. debneyi.* Several alterations including additions, deletions and substitutions occurred in the cellular *rolA, B* and *C* gene homologues. The mutations were not the same in all the plant members. The types of mutations include addition and deletion of any one of the four nucleotides A, T, G or C; and substitutions of A to T, T to A, G to A, A to G, A to C, C to A, C to G, G to C, T to C, C to T, T to G, G to T types. *rolA* and *rolB* gene homologues showed very few mutations which are either additions or deletions. More number of mutations with several substitutions were observed with *rolC* gene. Addition and deletion of nucleotides may be due to the slippage in template or replicating strand when replication occurs in repetitive sequences (Griffiths *et al*., 1999).

Expression of prokaryotic *rol* genes present in the genome of different members of *Nicotiana* result in neoplastic growths under field or culture conditions. Silencing and expression of *rol* genes might be because of changes in the levels of DNA methylation (Suneetha *et al*., 2009), histone acetylation (Pokholok *et al*., 2005) or the addition, deletion and substitution mutations observed. The *rol* gene expression could be further utilized either to enhance the ornamental value of the plant or for the production of secondary metabolites.

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