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Studies on Presence and Response of *Agrobacterium rol* Genes in Three Varieties of Tobacco

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Abstract: Present study was undertaken to identify the presence of *rol* genes of *Agrobacterium rhizogenes* in *Nicotiana tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi and their response towards hormonal shifts in culture. These three species of *Nicotiana* appear normal under field conditions and the leaf discs produced shoots on cytokinin containing Murashige and Skoog medium. These shoots when shifted to MS basal medium, *N. tabacum* var. Samsun showed rooty morphogenesis and the growth of the shoots continued for *N. undulata* but the growth stopped and the discs turned brown in *N. tabacum* var. Xanthi. Similar results with rooty morphogenesis were observed on MS supplemented with auxin medium. Genomic DNA of these three *Nicotiana* species when used for PCR analysis with *rol A*, *B*, *C* and *D* gene primers of *A. rhizogenes*, *N. tabacum* var. Samsun showed amplification of *rol A*, *B*, *C* and *D* genes, *N. undulata* showed amplification of *rol A* and *C* genes and no specific amplification was observed for *N. tabacum* var. Xanthi. The results were discussed in terms of the correlation between the presence of different combination of *rol* genes in *N. tabacum* var. Samsun and *N. undulata* and their response for habituation on hormone free medium with differential morphogenesis on hormonal shifts.

Key words: Habituation, hormonal shifts, morphogenesis, phytohormone independent growth, *rol* genes

INTRODUCTION

Transformation of T-DNA of the Ri plasmid from *Agrobacterium rhizogenes* to plants results in the development of hairy roots at the site of infection (Christey and Braun, 2005). The presence of cellular T_L-DNA genes in tobacco plants indicate ancient horizontal transfer of genes between plants and an ancestor *A. rhizogenes* (Suzuki *et al*., 2002; Aoki, 2004). The probable regions of Ri plasmid integration were T_L-DNA (the left stretch of the T-DNA) and T_R-DNA (the right stretch of the T-DNA) of *A. rhizogenes* (White *et al*., 1985; Forsbach *et al*., 2003). The plasmid carries two regions essential for tumor induction, namely the T region and the vir region. During infection the T region is transferred to the plant cell, where it becomes stably integrated in one of the host chromosomes and vir region act as mediator for expression of T-DNA leading to the production of the plant hormones auxin and cytokinin, as well as the synthesis of specific amino-acid derivatives termed opines (Regensburg-Tuïnk and Hooykaas, 1993).

The extent of expression of Ri plasmid genes of *Agrobacterium* was dependent on the position of incorporation of these genes into the plant kingdom (Mirza, 2005). Expression of the *rol* genes in plants alters several of the plant's developmental processes and affects their architecture. In general, these plants show a dwarfed phenotype, reduced apical dominance, smaller, wrinkled leaves, increased rooting, altered flowering and reduced fertility (Cosanova *et al*., 2005) and results in the phytohormone independent growth on MS (Murashige and Skoog, 1962) basal medium. Introduction of *rol* genes of *A. rhizogenes* into the genome of *N. debeneyi* resulted in intense earlier root formation (Aoki and Syono, 2000). Expression products of *rol* genes act as modulators of plant growth and cell differentiation. They are potential activators of secondary metabolism (Bulgakov, 2008).

The aim of this study is to understand the response of the leaf discs, rooty and shooty cultures on phytohormone independent MS basal medium; to identify the presence of different combinations of *rol* genes in *N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi by doing PCR analysis; to discuss the results in terms of presence of different combinations of *rol* genes in two different species of *Nicotiana* and their varied response to habituation by hormonal shifts.

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MATERIALS AND METHODS

Plant material: Seeds of *N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi were brought from Central Tobacco Research Institute, Rajahmundry. They were grown in botany farm of Andhra University, Visakhapatnam.

Callus induction: Leaves collected from 3 months old plants of *N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi were surface sterilized with ethanol and 0.1% mercuric chloride. Leaf discs were cultured on MS basal medium. The cultures were grown at 25°C and 16 h light/8 h dark photoperiod. Subculturing was done for every 21 days and the results were recorded.

Morphogenesis: To elicit morphogenesis in cultured leaf discs, the discs of *N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi were cultured on MS+BAP (5 mg L^{-1}) and MS+IAA (3 mg L^{-1}). 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. The data is given in the Table 1-3.

DNA isolation: DNA was isolated from fresh tender leaves of *N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi using Dellaporta method of plant DNA extraction. According to Dellaporta *et al.* (1983) fresh tender leaves of 1-3 g were harvested and ground to very fine powder with liquid N_2. The powder was transferred to a 30 mL autoclaved polypropylene tube, 15 mL of 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) was added and mixed. One milliliter of 20% SDS was added, mixed by inverting and incubated at 65°C for 10 min. Five milliliter of 5 M potassium acetate was added, mixed gently and incubated at 0°C for at least 20 min. The tubes were then centrifuged at 12,000 rpm for 20 min at 4°C in a refrigerated high speed centrifuge (Remi C_{24}). The supernatant was transferred into a tube containing 10 mL of iso-propanol and incubated at -20°C for 20 min. The tubes were centrifuged at 12,000 rpm for 15 min, the supernatant was discarded and the crude DNA pellet was dissolved in 0.7 mL of resuspension buffer 1 (100 mM EDTA and 250 mM Tris HCl).

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

Type of medium	Discs cultured	Responded	Percentage of response	Type of response
MS	250	194	78	Greenish white callus
MS+BAP	250	240	96	Callus with shoots
BAP to MS	200	172	86	Whole plant
MS+IAA	250	235	94	Callus with roots
IAA to MS	200	164	82	Whole plant

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

Type of medium	Discs cultured	Responded	Percentage of response	Type of response
MS	250	168	67	Greenish white callus
MS+BAP	250	220	88	Callus with shoots
BAP to MS	200	200	100	Shooty growth continued
MS+IAA	250	229	92	Callus with roots
IAA to MS	200	200	100	Rooty growth continued

Table 3: Response of the leaf discs of *N. tabacum* var. Xanthi on MS basal medium and their habituation on hormonal shifts

Type of medium	Discs cultured	Responded	Percentage of response	Type of response
MS	250	Nil	Nil	Browning of leaf disc
MS+BAP	250	98	39	Callus with few shoots
BAP to MS	200	Nil	Nil	Browning of callus
MS+IAA	250	105	42	White callus
IAA to MS	200	Nil	Nil	Browning of callus

Purification of DNA: The contents of the crude extract were transferred into sterile 1.5 mL eppendorf tubes. To this DNA solution 4 μL of RNASE was added and incubated for 1 h at room temperature. Equal volume of phenol:chloroform (1:1) was added to the supernatant, mixed and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was recovered. Then equal volume of phenol:chloroform:isoamylalcohol (25:24:1) was added to the supernatant, mixed and centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant was recovered. To the supernatant 75 μL of 3 M sodium acetate and 500 μL of chilled ethanol were added, mixed by inverting and incubated at -20°C for 30 min. The precipitate was washed with 70% ethanol and left to air dry. Then it was resuspended in 100 μL of 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, India). To store for longer time the DNA fibers were left in ethanol at -20°C.

PCR analysis: DNA amplification was carried out using the genomic DNA of *N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi as templates and *rol A*, *B*, *C* and *D* genes of *A. rhizogenes* sets of primers. The primer sequences are as follows:

***rol A* primers:**
Forward: 5'-GGAATTAGCCGGACTAAA-3'
Reverse: 5'-AGGTCTGAATTTTCACGT-3'

***rol B* primers:**
Forward: 5'-CAAATTGCTATTCCTTCC-3'

Reverse: 5'-TTACTGCAGCAGGCTTCA-3'

***rol C* primers:**
Forward: 5'-GTCGAGGATGTGACAAGC-3'
Reverse: 5'-GCCGATTGCAAACTTGCA-3'

***rol D* primers:**
Forward: 5'-TCGGCAGTAGCTCTCAAC-3'
Reverse: 5'-ATTGCCAGTATGGCTTCA-3'

Amplification was carried out in Appendorf's master cycler. Thermal profile was as follows:

- Initial denaturation at 94°C for 4 min
- Denaturation at 94°C for 1 min, Annealing at 50°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 by gel electrophoresis using 1.2% agarose gel. 100 bp ladder was used to calculate the base pair length of *rol* genes. The results were recorded.

RESULTS

Response of *N. tabacum* var. Samsun: Callus initiation was observed on MS basal medium (Fig. 1a). Green multiple shoots were grown on MS+BAP (5 mg L^{-1}) and white clusters of roots were grown on MS+IAA (3 mg L^{-1}). But when the shooty callus grown on MS+BAP was shifted to MS basal medium rooty morphogenesis was observed (Fig. 1b). Similarly, the rooty callus grown on MS+IAA, when shifted to basal MS basal medium shooty morphogenesis was observed (Fig. 1c). This type of growth was continued on MS medium on further subcultures without hormonal requirements. Response of *N. undulata*: Greenish white callus growth was observed from the veinal region of the leaf discs when cultured on MS basal medium (Fig. 2a). Green multiple shoot growth on MS+BAP (5 mg L^{-1}) and white clusters of root growth on MS+IAA (3 mg L^{-1}) were observed. But when the shooty callus and rooty callus were shifted to MS basal medium their growth continued (Fig. 2b, c).

Response of *N. tabacum* var. Xanthi: The leaf discs cultured on MS basal medium did not show any phytohormone independent growth (Fig. 3a). Callus initiation with one or two shoots on MS+BAP (5 mg L^{-1}) and white callus without any roots on MS+IAA (3 mg L^{-1}) were observed (Fig. 3b, c). But when the calli grown on auxin and cytokinin were shifted on to MS basal medium the growth did not continue and later they turned brown.

PCR analysis *of N. tabacum* var. Samsun, *N. undulata* and *N. tabacum* var. Xanthi: When the genomic DNA of *N. tabacum* var. Samsun was used as template amplified fragments of 260 bp band for *rol A*, 750 bp band for *rol B*,

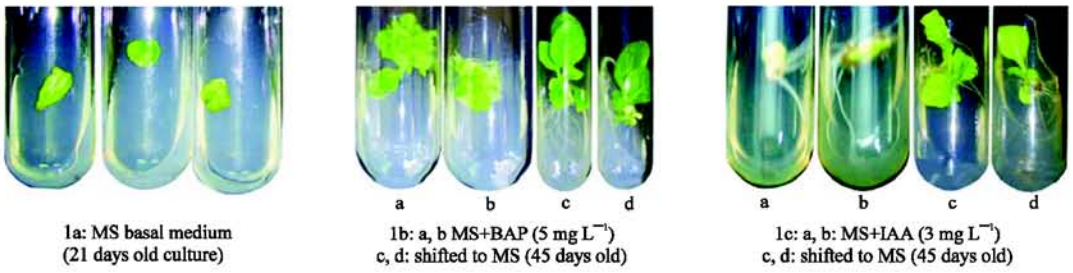


Fig. 1: Response from leaf discs of *N. tabacum* var. Samsun on MS basal medium and on hormonal shifts

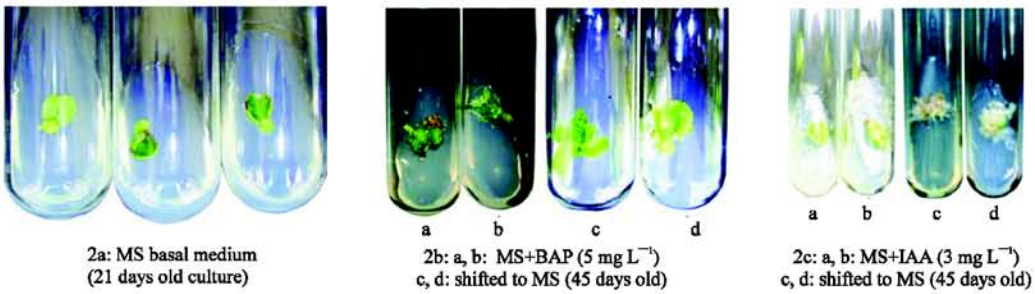
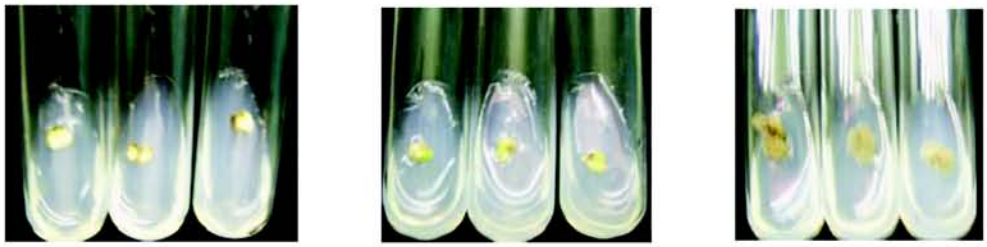
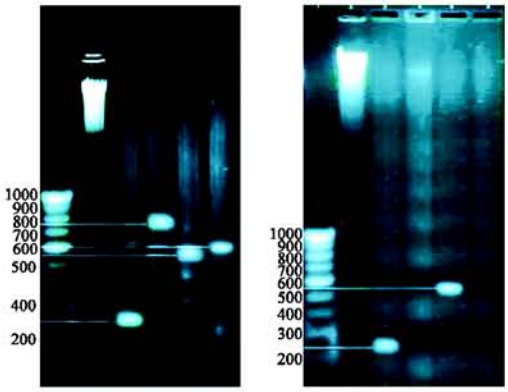


Fig. 2: Response from leaf discs of *N. undlata* on MS basal medium and on hormonal shifts



3a: MS basal medium 3b: MS+BAP (5 mg L^{-1}) 3c: MS+IAA (3 mg L^{-1})

Fig. 3: Response from leaf discs of *N. tabacum* var. Xanthi on MS basal medium and on hormonal (21 days old culture)



4a: *N. tabacum* var. Samsun 4b: *N. tabacum*

Fig. 4: Gel electrophoresis showing PCR analysis on 1.2% agarose gel. Lane 1: 100 bp ladder; Lane 2: genomic DNA; Lane 3, 4, 5, 6: *rol A, B, C, D* sets of primers

540 bp band for *rol C* and 600 bp band for *rol D* sets of primers were observed (Fig. 4a). These amplified fragments of DNA were equivalent to the corresponding sizes of *rol A, B, C* and *D* genes of *A. rhizogenes*. This shows the presence of *rol A, B, C* and *D* genes in *N. tabacum* var. Samsun.

N. undulata showed amplified fragments only for *rol A* (260 bp band) and *rol C* (540 bp band) sets of primers (Fig. 4b). These amplified fragments of DNA were equivalent to the corresponding sizes of *rol A* and *C* genes of *A. rhizogenes* indicating the presence of *rol A* and *C* genes in *N. undulata*.

No specific amplification was observed with *rol A, B, C* and *D* sets of primers, which represents the absence of *rol* genes in *N. tabacum* var. Xanthi.

DISCUSSION

Presence of *rol* genes was identified for the first time in *N. tabacum* var. Samsun and *N. undulata* and their absence in *N. tabacum* var. Xanthi through tissue culture experiments and PCR analysis. *rol* gene homologous sequences were found to be present in several members of plant kingdom like *Kalanchoe, Petunia, Daucus* and 15 out of 42 species of *Nicotiana* so far studied (Frundt *et al*., 1998; Intrieri and Buiatti, 2001). *rol* genes of *A. rhizogenes* were integrated into the genome of *Nicotiana* species by horizontal transfer during the course of evolution (Aoki, 2004). The T-DNA can be integrated at different sites in the plant genome. The integration of T-DNA into the *Nicotiana* genome is through insertion at double stranded breaks by nonhomologous end joining (Chilton and Que, 2003). Integration of these genes into plant genome is not in the same combination for all the plants. Here, *rol A, B, C* and *D* genes were found to be present in *N. tabacum* var. Samsun and *rol A* and *C* genes in *N. undulata*.

The products of *rol* genes were known to interfere with phytohormone activities modulating plant growth and are potential activators of secondary metabolism. The combination of *rol A, B, C* and *D* genes work synergistically in growth, cell proliferation and root formation of plants (Spena *et al*., 1987; Aoki and Syono, 1999). The extent of the expression of *rol* genes in plants is varying they remained permanently silent in *N. glauca* and expressed in *N. glauca* x *N. langsdorffii* hybrid plants resulting in tumorigenesis, morphological and physiological abnormalities and phytohormone independent growth of cultures (Udagawa *et al*., 2004; Suneetha *et al*., 2006). Expression of *rol* genes in *Kalanchoe blossfeldiana* resulted in a compact plant with several morphological changes with respect to ornamental value such as plant height, number of lateral shoots, leaf size, leaf number, flower size and number of flowers (Christensen *et al*., 2008). Individual and combined expression of *rol A, rol B* and *rol C* genes resulted in increased biosynthesis of anthraquinones in transformed calli of *Rubia cordifolia* (Shkryl *et al*., 2008). Present experiment shows the silencing of *rol* genes under field conditions as the plants behaved normal without any morphological and physiological variations.

Phytohormone independent growth of the leaf discs and the habituation on hormonal shifts represent the expression of *rol* genes under culture conditions. Further, the presence of different combinations of *rol* genes was found to be responsible for their varied response under culture conditions. Presence of all four *rol A*, *B*, *C* and *D* genes caused enhanced response with organogenesis in *N. tabacum* var. Samsun and presence of only *rol A* and *C* genes in *N. undulata* was responsible for continued growth, the rooty or shooty depending on the hormone on which it was previously cultured. Further, the absence of *rol* genes in *N. tabacum* var. Xanthi resulted in the absence of phytohormone independent growth and habituation on hormonal shifts. Hence, we conclude that *rol* genes of *Agrobacterium* were present in different combinations in *N. tabacum* var. Samsun and *N. undulata*. They remained silent under field conditions whereas they were expressed under culture conditions resulting in phytohormone independent growth and habituation on hormonal shifts.

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