Acta virologica **55**: 283 – 284, 2011 doi:10.4149/av_2011_03_283

LETTER TO THE EDITOR

Nucleocapsid protein gene mediated resistance against groundnut bud necrosis virus in tomato using sense and antisense constructs

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Received September 13, 2010; accepted August 22, 2011

Keywords: GBNV; N gene; sense; antisense; transgenic tomato; resistance

Spotted wilt (also known as bud blight) is caused by groundnut bud necrosis virus (GBNV) and is emerging as a serious problem affecting tomato (*Lycopersicon esculentum* Mill) cultivation in the Indian subcontinent. Field symptoms of the disease include extensive necrosis of buds and petioles, necrotic spots on leaves and concentric rings on fruits. The incidence of 19–34% was recorded (*1*). N gene as a transgene has been reported to confer a broad-spectrum resistance against Tospoviruses (*8, 12*). In the absence of natural resistance against GBNV in widely grown tomato varieties, the strategy of N gene-mediated resistance could be worth attempting against GBNV. N gene (831 bp) derived from GBNV isolate of Tamil Nadu (TN-Co) was amplified using a set of specific primers (HRP26: 5′-ATGTCTAACGT(C/T) AAGCA (A/G) CTC-3′and HRP28: 5′-TTACAATTCCAGCGA AGGACC-3′) derived from first and last 21 bases of the coding region of N gene of GBNV (*10*) and watermelon silver mottle virus (*13*). The amplified and confirmed full length N gene was initially cloned into pDrive vector (Qiagen, Germany) and then sub-cloned in both sense and antisense orientation into pBI121 (Clonetech laboratories, USA) and pBinAR (*16*) binary vectors respectively using *Xba*I and *Bam*HI sites. Then it was mobilized into the disarmed *Agrobacterium tumefacients* strain LBA 4404 (*6*) separately by triparental mating procedure (*5*), using the helper plasmid pRK2013 (*3*). Presence of N gene construct was confirmed by colony PCR and sequencing. Three popular tomato varieties *viz.,* Co3, Pusa Early Dwarf and Pusa Ruby and the explants *viz*., cotyledons (7 days old) and leaves (35 days old) explants were used for *Agrobacterium*-mediated transformation. Regeneration protocol was initially standardized for three varieties (data not shown). For callusing and shootings, the effective plant growth regulator combination such as IAA (0.0002 mg/ml) and kinetin (0.0002 mg/ml) for Co3, and indole butric acid (0.002 mg/ml) and 6-benzylaminopurine (0.002 mg/ml) were used for Pusa Early Dwarf and Pusa Ruby varieties, and IAA 0.0002 mg/ml was used for rooting of all varieties. The explants were co-cultivated with 48 hr-grown *Agrobacterium* culture (OD_{600}= 0.4) (with and without construct) and incubated for two days in the dark at 25°C (*4*). After co-cultivation, tomato explants were washed gently by half strength MS medium (*17*) and air dried on sterile filter paper. The explants were transferred to selection callusing medium containing augumentin (0.5 mg/ml) and kanamycin (0.1 mg/ml) and incubated at 25°C in 16hrs photoperiod. Well developed calli were after two weeks transferred to selection shoot medium with augmentin (0.4 mg/ml) and kanamycin (0.1 mg/ml). After another two weeks, when regenerated plantlets reached 2–3cm in height, they were cut off and placed on a selection rooting medium (*4*) with augmentin (0.1 mg/ml) and kanamycin (0.05 mg/ml). The plantlets were removed from the culture and transferred to sterilized soil mix (vermiculite and sand 1:1 ratio) in pot (size 95 × 100cm) and 100 ml of half strength MS media was poured in to the soil mix (*4*). After 10 days, the plants were transferred to a large size (195 × 180cm) pot and maintained in phytotron at 25°C in 12 hrs day/12 hrs night photoperiod. Southern hybridization

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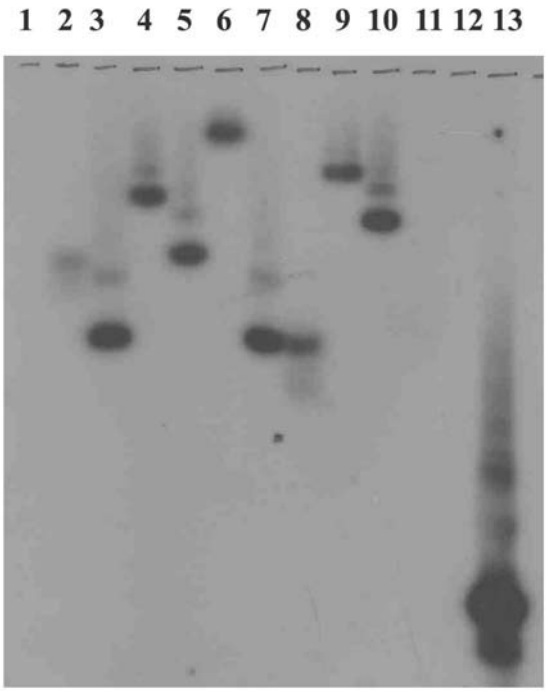
Abbreviations: GBNV = groundnut bud necrosis virus; IAA = 3-indole acetic acid; N gene = nucleocapsid gene



Figure

***Bam*HI restricted genomic Southern blot analysis of transgenic lines**
Lanes 1 and 11: untranformed plants; Lane 2: Co3S1; Lane 3: Co3S2; Lane 4: Co3S12; Lane 5: PRS1; Lane 6: PRS3; Lane 7: PRA1; Lane 8: PRA2; Lane 9: PRA4; Lane 10: PRA5; Lane 13: Positive control.

(*15*) was performed after isolation of genomic DNA (*7*) and restriction digestion with *Bam*HI. For Northern blot analysis, total RNA was isolated from Southern-positive plants using TRIZOL reagent (Invitrogen, USA. Purified total RNA (20 µg) was separated by formaldehyde gel electrophoresis, blotted, and hybridized to an N gene probe following standard procedure (*9*). Direct antigen coated enzyme linked immunosorbent assay was used to detect expression of N protein in putative transformants using polyclonal antiserum to GBNV (*2*). Southern-positive T0-plants were evaluated for resistance to GBNV by three-fold mechanical inoculation with sap from GBNV-infected tomato plants using 0.01 M potassium phosphate buffer pH 7.0 and carborundum (600 meshes) as an abrasive (*11*). Untransformed plants were used as control and inoculated plants were examined for symptom development.

PCR product of expected size (ca. 800 bp) was observed, cloned and sequenced (GenBank Acc. No. AY463968). N gene constructs in *sense* and *antisense* orientation were mobilized separately into *Agrobacterium* strain LBA4404 and then transferred into tomato. Of the 35 PCR-positive transformants, only nine were Southern-positive. Transgenic lines Co3S2, PRA1, PRA5 possessed single insert of the transgene, whereas Co3S1, Co3S12, PRS1, PR S3, PRA2, and PRA4 possessed double inserts of the transgene (the figure lane 2, 4, 5, 6, 8, and 9). In Northern blot analysis and ELISA, all transformants except for CO3S2, PRA2 and PRA4 were positive. (data not shown). These results are in agreement with earlier findings (*14, 15*). Transgenic lines with a single insertion of the N gene would be useful in breeding program because it is easier to breed for a trait controlled by a single dominant gene. Putative Southern-positive transgenic lines were highly resistant to GBNV infection, showing no symptoms upon inoculation with the TN-Co isolate. The usefulness of N gene mediated resistance against GBNV in tomato would depend on the stability of the gene through generations and evaluation of the transgenic lines against GBNV isolates from different locations.

Acknowledgements. This work was supported by the grant no 8827 Indian Agricultural Research Insitute, New Delhi and Council of Scientiic and Industrial Rsearch, New Delhi.

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