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R. Batchvarova · V. Nikolaeva · S. Slavov
S. Bossolova · V. Valkov · S. Atanassova
S. Guelemerov · A. Atanassov · H. Anzai

Transgenic tobacco cultivars resistant to *Pseudomonas syringae pv. tabaci*

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Abstract Six oriental cultivars of tobacco (*Nicotiana tabacum* L.) were evaluated for transformation and foreign gene expression. Leaf-disc explant tissue was transformed with *Agrobacterium tumefaciens* strain LBA4404 carrying the plasmid pARK21, which contains NPTII gene and *ttr* (tabtoxin resistance) gene conferring the resistance to *Pseudomonas syringae* pv. *tabaci*. The disease resistance of regenerated plants and segregation of this trait up to R_7 progeny were investigated in a greenhouse and under field conditions. Our results indicated that the resistance to *Pseudomonas syringae* pv. *tabaci* introduced by transformation is heritable.

Key words Tobacco · *Pseudomonas syringae* pv. *tabaci* · Transformation · Resistance · Inheritance

Abbreviations *NPTII* Neomycin phosphotransferase · *IAA* indole-3-acetic acid · *BAP* 6-benzylaminopurine

Introduction

In last decade, genetic transformation has become one of the main tools for obtaining plants resistant to different diseases (De Block et al. 1987; De Greef et al. 1989; Daub et al. 1994). *Agrobacterium*-mediated genetic transformation as a method for introducing novel genes into plants has become routine for a number of plant species. Now more effort is being focused on the expression of the introduced genes, confirming resistance to viral, bacterial and fungal diseases, insects and herbicides, in order to improve crop protection (Powell-Abel et al. 1986; Loesch-Fries et al. 1987; Stalker et al. 1988; Graybosch et al. 1989; De Haan et al. 1992; Lindbo and Dougherty 1992).

Wildfire disease is one of the main plant protection problems of tobacco breeding (Lucas 1975). Very little success has been achieved using chemical control. An alternative means to controlling this plant disease is the development of resistant cultivars. The introduction of genes providing resistance to wildfire disease in different tobacco cultivars is one of the approaches for achieving this goal. The pathogenic toxins might be one of the more attractive targets for the genetic engineering of plant disease resistance (Yoneyama and Anzai 1993).

The causal pathogen of wildfire disease bacterium *Pseudomonas syringae* pv. *tabaci* produces tabtoxin (Turner and Debbage 1982). This toxin causes characteristic chlorotic halos on tobacco leaves during wildfire disease development. The nontoxigenic mutants of the bacterium cause angular leaf spot disease without chlorisis (Turner and Tana 1974). The tabtoxin upon cleavage in the plant liberates tabtoxin-β-lactam, which is an inhibitor of glutamine synthetase and leads to the accumulation of ammonia and plant cell death. The bacteria remain alive because they naturally carry the tabtoxin resistance gene (*ttr*). This gene has been cloned (Anzai et al. 1989) and shown to confer resistance to wildfire when introduced into tobacco plants. In the construct used by Anzai et al. (1989) the *ttr* gene is linked to the 35S promoter, *npt*II and *nos* terminator. The same construct was used in our experiments to introduce the *ttr* gene into Bulgarian tobacco cultivars.

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R. Batchvarova (✉) · A. Atanassov
The De Montfort University, Norman Borlaug Centre for Plant Science, The Institute of Genetic Engineering, 2232 Kostinbrod-2, Bulgaria

V. Nikolaeva · S. Slavov · S. Bossolova · V. Valkov
S. Atanassova
Institute of Genetic Engineering, 2232 Kostinbrod-2, Bulgaria

S. Guelemerov
Bulgartabac-Holding Group, Sofia, Bulgaria

H. Anzai
Meiji Seika Kaisha, Pharmaceutical Research Center, Yokohama 222, Japan

The purpose of this work was to investigate the expression of the *ttr* gene in economically important oriental tobacco cultivars and to follow the inheritance and expression of wildfire resistance.

Materials and methods

Plant material

In vitro tobacco plants were used in the transformation experiments. Six oriental tobacco cultivars were represented, 'Nevrokop 1146', 'Plovdiv 50', 'Melnik 812', 'Rodopska Yaka', 'Rila 88', 'Rila 89', all originating from different tobacco-growing areas in Bulgaria.

Plant transformation

Agrobacterium tumefaciens strain LBA 4404 with plasmid pARK 21 carrying the *ttr* and nptII genes was used for transformation. The bacterium was grown overnight in liquid YEB (10 g peptone, 10 g yeast extract, 5 NaCl per litre) supplemented with 100 mg/l rifampicin and 100 mg/cl kanamycin to an OD of 0.8 at 550 nm wavelength and then used in co-cultivation experiments.

Plant transformation was done by the leaf-disc method (Horsch et al. 1985). Leaf pieces originating from 8-week-old in vitro plants were immersed in the *A. tumefaciens* suspension for 10 min and then placed on solidified MS (Murashige and Skoog 1962) basic medium supplemented with 2 mg/l BAP, 0.2 mg/l IAA, 20 mg/l adenine and 250 mg/l L-glutamine for a 24-h co-cultivation period. Afterwards the leaf explants were transferred to the same medium supplemented with 500 mg/l cephataxim and 100 mg/l kanamycin. Green explants were subcultured every 2 weeks for 2 months onto a fresh medium containing 250 mg/l cephataxim and 100 mg/l kanamycin. The transformed regenerants were selected and transferred for rooting onto MS basic medium including vitamins and 100 mg/l kanamycin. All in vitro experiments were maintained at 25°C and under a 16/8-h light/dark period. Regenerated transformed plants were adapted and grown in 15-cm diameter plastic pots in soil.

Test for resistance

All R_0 regenerated plants were tested in vivo for resistance to *P. syringae* pv. *tabaci* under greenhouse conditions. The inoculum was prepared from three Bulgarian isolates, races 0, 1 and 2 of the bacterium, and a strain from ATCC − 48117. Bacteria were grown on nutrient dextrose agar (NDA, Difco nutrient agar supplemented with 10 g/l Difco dextrose and 5 g/l Difco yeast extract; Difco laboratories, USA) at 28°C in the dark for 24 h. Bacteria were washed and suspended in sterile water. The inoculum concentration was adjusted spectrophotometrically to 10 cells/ml. Six-week-old plants with four to six fully expanded leaves were pricked with a needle, sprayed with the bacterial suspension and grown at high humidity in a greenhouse at 20°–25°C.

In addition a detached leaf bioassay was performed on the same plants in order to confirm the results from the greenhouse test. Tobacco leaves were cut, inoculated by bacterial suspension with the concentration mentioned above and kept in petri dishes at high humidity, 28°C and under light (12 h). After 10 days plants without any symptoms were considered to be resistant.

Wildfire resistant R_0 transformations with a phenotype typical of the respective cultivars were chosen and selfed. Their progenies were tested for wildfire resistance by the same inoculation methods as described above under greenhouse and field conditions. The percentage of resistance was estimated as a ratio of resistant to total number of inoculated plants of each line. About 200 plants of each progeny were tested. Only resistant plants were selfed, and their seeds were used in next generation.

NPTII assay and DNA hybridization

DNA from several resistant to *P. syringae* pv. *tabaci* R_0 plants was isolated according to Dellaporta et al. (1983). The presence of the *npt*II gene was determined by dot blot assay (McDonnell et al. 1987). For Southern blot analyses 10 μg plant DNA was digested with the *Hind*III restriction enzyme and separated onto 0.8% agarose gel. DNA was blotted onto GeneScreen Plus membrane (DuPont) and hybridized with [^{32}P]-dCTP-labelled *ttr* gene. The probe was isolated from the plasmid pARK 21 by restriction with the *Eco*RI and *Xba*I endonucleases (Maniatis et al. 1982). DNA isolated from nontransformed plants from the same cultivars was used as a control.

Results and discussion

Transformation efficiency

Co-cultivation of tobacco leave with *A. tumefaciens* took 24 h, and the first plantlets appeared 8–10 weeks later. During this period no regeneration was observed from control nontransgenic tobacco leaf explants placed on selective medium. Different cultivars gave different numbers of rooted plants on MS basic medium with kanamycin (Table 1). The regenerated plants were considered to be transformants. When the transformation efficiencies of the tobacco cultivars were composed, three groups could be determined; cvs

Table 1 Efficiency of the transformation of tobacco cultivars with the construct carrying the *ttr* gene (*Km* kanamycin)

Cultivar	Number of initial leaf discs cocultivated with *A. tumefaciens*	Number of rooted regenerants on selective medium with Km	Transformation efficiency (%)	Number of resistant R_0 plants
1. Plovdiv 50	57	11	19.3	7
2. Nevrokop 1146	56	12	21.4	2
3. Rila 88	60	20	33.3	6
4. Rila 89	60	21	35.0	5
5. Melnik 812	60	20	33.3	6
6. Rodopska Yaka	60	24	40.0	13

'Plovdiv 50' and 'Nevrokop 1146' showed the lowest percentages of transformation efficiency (about 20%), and cv 'Rodopska Yaka' the highest (40%). The rest of the cultivars formed a middle group with a transformation efficiency of 33–35%. Cultivars 'Rila 88' and 'Rila 89' are genetically similar and originate from closely proximal areas, and they showed the same efficiency of transformation. All donor in vitro plant material and transformation experiments were maintained under the same conditions, which means that the differences in the number of regenerated plants from the different tobacco cultivars are due to the specific potential of each tobacco cultivar for integrating the foreign gene.

Wildfire resistance of the transformations

Chlorosis appeared on tobacco leaves of the controlled nontransformed plants 2–3 days after inoculation. Typical symptoms of wildfire disease (necrotic lesions surrounded by yellow halo) were observed about 10 days later. Most of the selected in vitro Kanamycin-resistant plants appeared to be susceptible to wildfire disease. From a total of 108 selected in vitro kanamycin-resistant transformants isolated from all of the cultivars, only 39 were resistant to *P. syringae* pv. *tabaci* when tested in vivo. The percentage of resistant R_0 plants (which did not develop any lesions) varied among the cultivars (Table 1). Similarly to the case of transformation efficiency, cv 'Nevrokop 1146' had the lowest percentage of resistant R_0 transformants (only 2 were resistant), and cv 'Rodopska Yaka' the highest percentage. The other three cultivars,'Rila 88', 'Rila 89' and 'Melnik 812' formed again a middle group with 5–6 resistant R_0 transformants from the 20–21 regenerants tested. An exception was cv 'Plovdiv 50', which gave the smaller number of R_0 plants regenerated on kanamycin (11), but most of these (7) were resistant to *P. syringae* pv. *tabaci*. All control nontransgenic plants originating from the same cultivars and used as a donor plant material were totally susceptible.

These experiments did not enable us to determine if the wildfire susceptible kanamycin-resistant R_0 transformants "possess" the foreign *ttr* gene. Their susceptibility to the disease could be due to the "silence" of the gene. Selection following generations continued only with those plants which showed wildfire resistance when tested in vivo. In vitro selection on medium containing kanamycin was omitted.

Inheritance of resistance in progenies

In all of the cultivars wildfire resistance of R_1 progenies of transgenic plants was higher than that of the R_0 plants. For 'Nevrokop 1146' 86.06% of the R_2 progeny was resistant and 80% of its R_3 progeny was resistant

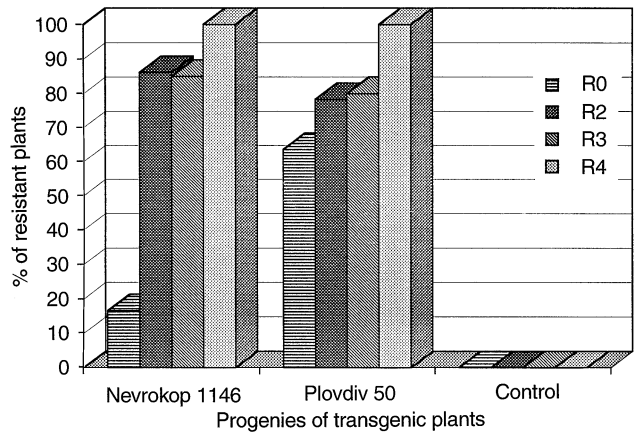

Fig. 1 Resistance of transgenic tobacco plants to *Pseudomonas syringae* pv. *tabaci*

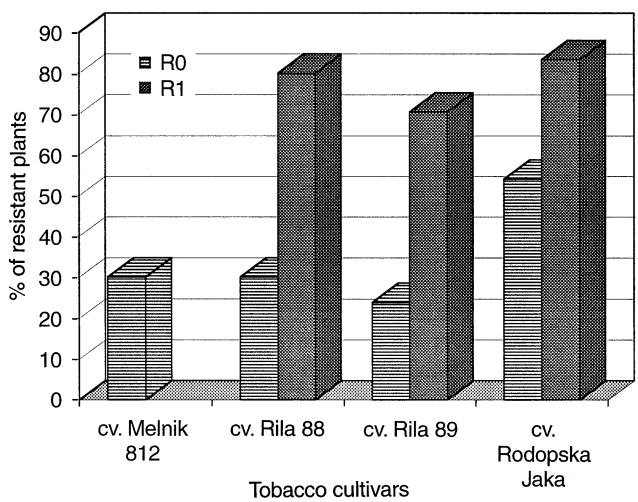

Fig. 2 Resistance of transgenic tobacco cultivars to *Pseudomonas syringae* pv. *tabaci* in R_0 and R_1 progenies. Control plants were totally susceptible

(Fig. 1). For 'Plovdiv 50' 78% of the R_2 plants was resistant and 90% of the R_3 plants. Totally resistant lines (100%) were obtained in the R_4 generation of those two cultivars, and this trait remained stable up to the R_7 generation. The experimental work was followed till that generation.

The results showed that resistant transgenic plants chosen in the R_1 and R_2 generations were heterozygous for *ttr* gene. The fact that the transgenic lines were fully resistant to *P. syringae* pv. *tabaci* from R_4 to the R_7 generations confirms that they are homozygous for the *ttr* gene.

Selection on other two cultivars, 'Rila 89' and 'Rodopska Yaka' led to an increased resistance in the R_1 progeny (Fig. 2). That fully resistant lines could be obtained in those two cultivars is promising for the next generations, as it was in the case with cvs 'Nevrokop 1146' and 'Plovdiv 50'.

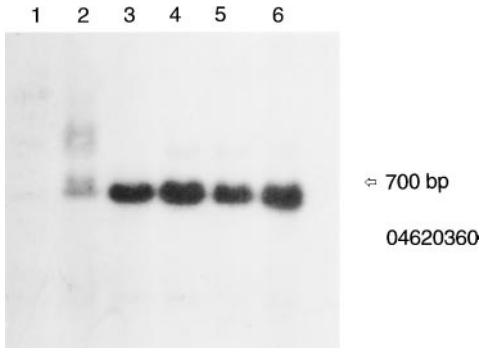

Fig. 3 Southern analysis of genomic DNA from transgenic tobacco plants. Plant DNA was digested with *Hind*III, separated on a 0.8% agarose gel, transfered to GeneScreen membrane and hybridized with the *ttr* gene fragment of pARK21. *Lane 1* Control, *lanes 2–6* transgenic plants

NPTII assays and DNA hybridization analyses of transgenic plants

Selected kanamycin-resistant R_0 plants were shown to be NPTII positive. A Southern blot analysis clearly demonstrated the integration of the *ttr* gene in NPTII positive and wildfire resistant R_0 transformants (Fig. 3). No hybridization signal was detected on control DNA extracted from nontransformed plants.

Thanutong et al. (1983) found that after in vitro selection of tobacco in the presence of the toxin of *Pseudomonas syringae* pv. *tabaci*, regenerated plants were all resistant to the pathogen. Our final results confirmed that using *Agrobacterium*-mediated transformation with the *ttr* gene that encodes the resistance to the tabtoxin provides the possibility of obtaining tobacco lines totally resistant to *Pseudomonas syringae* pv. *tabaci*. We have shown that engineered insensitive-to-toxin plants, are resistant to the respective pathogen. These are the first experiments with transgenic tobacco plants that follow the inheritance of resistance to *P. syringae* pv. *tabaci* up to the seventh generation. Results from laboratory and greenhouse experiments were confirmed in field conditions. This genetic engineering technique is of significant benefit and can reduce the period of breeding resistant tobacco lines to wildfire disease.

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