UDC 633.71
Тутун/Tobacco, Vol.54, Nº 9-10, 207-216, 2004
Институт за тутун - Прилеп, Р. Македонија

ISSN 0494-3244

УДК: 633.71-248.114.4:632.938
Изворен научен труд

TRANSGENIC TOBACCO WITH COMPLEX RESISTANCE TO BACTERIAL AND VIRAL DISEASES AND THE HERBICIDE GLEAN®

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INTRODUCTION

Tobacco (*Nicotiana tabacum*) is susceptible host to viral, bacterial and fungal pathogens (TSWV, PVY, CMV, *Ps. syringae pv. tabaci, Phytophthora parasitica* Dast var. *nicotianae*) and parasites (broomrape). They significantly decrease quality and quantity of tobacco production.

The most effective and economical method to control plant diseases is to develop resistant commercial tobacco cultivars. A number of approaches have been used for this purpose (selection, mutagenesis, genetic transformation).

The integration and expression of foreign genes in plants by genetic transformation is now a routine procedure for many species. Genetic transformation has been used for the production of transgenic crop plants with herbicide tolerance (16), resistance to fungal (2), viral (17) and bacterial (12) diseases.

Recent developments in plant genetic are focused on the integration of multiple transgenes into the plant genome and coordinate expression of these transgenes.

A number of tobacco transformations for introduction of genes conferring resistance to biotic stress were performed in the AgroBioInstitute.

By introduction of *Np* gene, CP gene and replicase gene, transgenic lines from different tobacco cultivars with increased and complete resistance to TSWV, PVY and CMV respectively were obtained. The inheritance of transgenes was investigated in the next generations and it was proved that resistance to TSWV, PVY and CMV is stable.

High level of resistance to *Pseudomonas syringae* p.v. *tabaci* was obtained in transgenic tobacco plants expressing the *ttr* gene. Stable inheritance and expression of transgene was followed up to R9 generation.

Broomrape (*Orobanche ramosa* L.) is an obligate root parasite of tobacco. The basic idea for broomrape control is to apply the herbicides in the field with herbicide resistant tobacco. Several tobacco cultivars were successfully transformed with bar gene conferring resistance to the herbicide Basta, ahas3R and P450 2C9 genes for resistance to herbicide Glean, and P450 1A1 gene for resistance to Tolurex®. The genes were introduced into tobacco plants by *Agrobacterium tumefaciens* mediated transformation. Under greenhouse conditions the transgenic plants showed resistance to Basta®, Glean® and Tolurex® in doses 3 to 10 times higher then normally applied in the field.

The aim of our work was to combine genes conferring resistance to pathogens and the herbicide Glean® in one tobacco line by sexual crosses. We chose four different transgenic tobacco lines from cultivar Nevrokop 1146 resistant to TSWV (14), PVY (8), P. *syringae* pv. *tobacco* (1) and the herbicide Glean® (15) respectively created in the AgroBioInstitute.

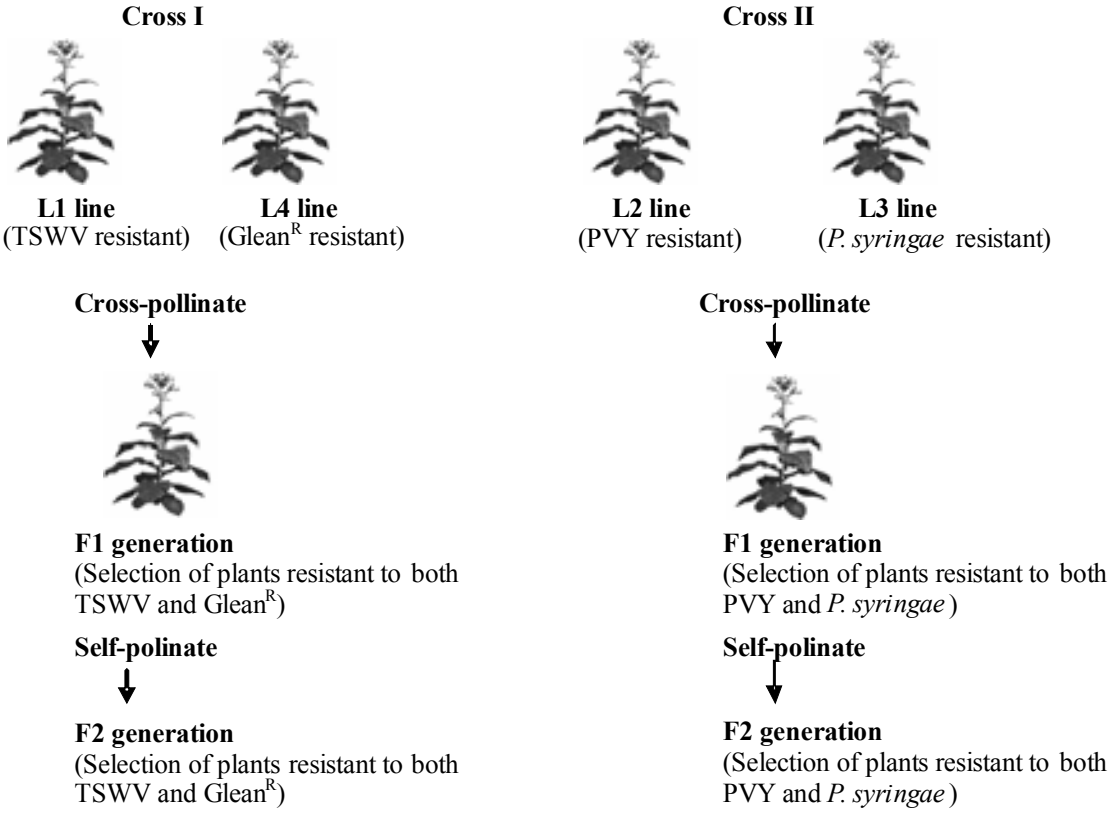

Fig. 1 Schematic presentation of the cross I and the cross II.

MATERIALS AND METHODS

Plant material

Four different transgenic lines originating from tobacco cultivar Nevrokop 1146 resistant to TSWV (L1 in R9 generation), PVY (L2 in R2), *Pseudomonas syringae* pv. *tabaci* (L3 in R9) and herbicide Glean® (L4 in R1) were used for the experiments.

The transgenic tobacco lines were sexually crossed to combine the traits in pairs (Fig. 1). In the cross I genes for resistance to TSWV and Glean® were combined (L1 x L4, L4 x L1). In the cross II genes for resistance to PVY and *P. syringae* pv. *tabaci* were combined (L2 x L3, L3 x L2).

Selected plants from F1 generation after tests for resistance to TSWV and Glean® for the cross I and tests for resistance to PVY and *P. syringae* pv. *tabaci* for the cross II were self pollinated.

Seven randomly selected numbers from the cross I were analyzed in F2 generation - I 57/7 and I 57/11 (originating from F1 generation of the cross I 57); I 12/10 and I 12/4 (from I 12); I 61/2 and I 61/19 (from I 61); and I 27/12 (from I 27). Totally 165 plants were tested for resistance to TSWV and Glean®.

From the cross II eight randomly selected numbers were chosen for analyses in F2 generation - II 10/26 and II 10/29 (originating from F1 generation of the cross II 10); II 44/15 and II 44/16 (from II 44); II 19/4 and II 19/19 16 (from II 19); II 15/20 and II 15/35 16 (from II 15). Totally 240 plants were tested for resistance to PVY and *P. syringae* pv. *tabaci.*

Tests for resistance:

Test for resistance to TSWV - The tobacco plants were mechanically inoculated at 2-3 leaf stage. The inoculum was prepared by grinding (1:5 w/v) of TSWV infected leaves on ice in buffer (pH 7.0) containing 0.1 M $Na_2HPO.12H_2O$, 0.1 M KH_2PO_4, 5% Na_2SO_3, 0.2% ascorbic acid, 2% PVP. The inoculated leaves were rinsed with water after 5 min. The resistant plants were selected after three weeks.

Test for resistance to chlorsulfuron (herbicide Glean®) - The herbicide was applied on the tobacco plants at vegetative stage 4-5 leaves. Sixty plants were planted on $4m^2$ and were sprayed with the herbicide in concentration 5 mg/m^2. The resistant plants were selected after 2 weeks.

Test for resistance to PVY - The tobacco plants were tested as described for TSWV. After three weeks the new growth leaves were analyzed for virus infection, using DAS-ELISA method (3).

Test for resistance to *Ps. syringae* pv. *tabaci* - Detached leaf bioassay was performed. The plants were inoculated with Bulgarian isolate of the *Ps. syringae* pv. *tabaci*. Bacteria was grown on nutrient dextrose agar at 28ºC in the dark for 24 h. Bacteria was suspended in sterile water (OD600=0.6). The tobacco leaves were placed in Petri dishes at high humidity, 28ºC and 12 h light. After 10 days resistant plants were selected.

PCR analyses

Genomic DNA was isolated from tobacco leaf tissue as described by Dellaporta (4).

PCR amplification was carried out by Ready to Go PCR Beats (Amersham Pharmacia Biotech INC).

For PCR amplification of the *Np* gene conferring resistance to TSWV the primers 5'-GGCAAAGACCTTGAGT-3'and 5'-CTTTGCTTTTCAGCAC-3' were used. The PCR program was 94 ºC for 5 min, 94 ºC for 30 sec, 60 ºC for 45 sec, 72 ºC for 1 min and 72 ºC for 5 min (33 cycles). The amplification fragment was 550bp.

Gene *ahas3R* confer resistance to herbicide Glean®. For the amplification of the 750bp gene fragment the primers 5'-ACGATGAGTTGTCCCTGCAG-3' and 5'-AGATCTCGTTCTCCCTTTCC-3' were used. The PCR program was 94 ºC for 3 min, 94 ºC for 1 min, 62 ºC for 1 min, 72 ºC for 1 min and 72 ºC for 5 min (30 cycles).

Gene ttr encoding resistance to *Ps. syringae* pv. *tabaci* is 723bp. CP gene encoding resistance to PVY is 1327bp. For the PCR analyses of these two genes the primers for their CaMV 35S promoter and nos terminator with following sequences: 5'-AAACCTCCTCGGATTCCATTG-3' and 5'-CCATCTCATAAATAACGTCATGCAT-3' were used. The program was 94 ºC for 5 min, 94 ºC for 30 sec, 57 ºC for 1 min, 72 ºC for 1 min and 72 ºC for 10 min (30 cycles).

Southern blot analyses

A total DNA was extracted from the leaf material according to Dellaporta (4). Twenty µg of DNA were digested with XbaI for the plants from the cross I and with EcoRI for the plants from the cross II. Digested DNA probes were fractioned by electrophoresis in 0.8 % agarose gels. The DNA were blotted to Hybond-N+ membranes (Amersham) and hybridized to a DIG labeled DNA probes. DNA fragments of the fourth transgenes were labeled with the specific primers for the transgenes by using PCR DIG Probe Synthesis Kit (Roche Diagnostics GmbH). Detection of signals was performed according to DIG Luminescent Detection Kit for Nucleic Acids (Roche Diagnostics GmbH).

RESULTS AND DISCUSSION

Cross I

Analyses of plants from F1 generation

To combine genes for resistance to TSWV and the herbicide Glean® sexual crosses between transgenic tobacco plants from the line L1 and the line L4 and the reverse ones were performed (cross I). More than 78 crosses were realized and seed samples were collected. Four of them were randomly chosen for examination - I 27 (L4 x L1), I 61 (L4 x L1), I 12 (L1 x L4) and I 57 (L1 x L4). The seeds from selected crosses germinated normally and all plants were typical in appearance for the cultivar. The plants were tested for resistance to TSWV and the herbicide Glean®.

Tests for resistance to TSWV and the herbicide Glean®

Tobacco plants were mechanically inoculated with TSWV at the 2-3 leaf stage. Three weeks after the treatment all F1 plants from the cross I showed no symptoms and were considered as resistant (Fig. 2). The control plants (nontransgenic tobacco Nevrokop 1146) were systemically infected. The results showed that resistance to TWSV was inherited in all plants of the cross I from the transgenic parental line L1 that had stable resistance to TSWV up to R9 generation.

The same plants from the cross I were sprayed with the herbicide Glean® (Fig. 3). The control plants demonstrated typical traits of sensitivity to the chlorsulfuron and ten days after treatment died. Different levels of resistance to Glean® were observed in the four different crosses, ranging from 100% to 43%. All tested plants from the crosses I 12 and I 57 were completely resistant to the herbicide. From the crosses I 27 and I 61 resistant were 32 (53%) and 26 (43%) plants respectively. Segregation in F1 progeny of the cross I was expected because the parental line L4 used for the crosses was not homozygous for the gene conferring resistance to the herbicide Glean®.

Fig. 2 Tobacco plants tree weeks after TSWV infection
1 - resistant plant from the cross I, F1 generation; 2 - control non transgenic plant

Fig. 3 Tobacco plants from the cross I (F1 generation) ten days after treatment with the herbicide Glean®
1 - resistant plant; 2 - susceptible plant; 3 - control plant

PCR analyses

The presence of genes conferring resistance to TSWV *(Np)* and Glean® *(ahas3R)* in plant genome was confirmed by PCR analyses. Five plants from each cross of I 27, I 57, I 61 and I 12 were randomly chosen for further experiments. PCR products for the specific genes proved the integration of both transgenes in the selected plants. PCR products with the expected size (550bp for the gene *Np* and 750bp for the ahas3R gene) were obtained in all selected plants from the cross I (data not shown). No signal was detected from the control DNA extracted from nontransgenic tobacco plants. The PCR analyses revealed that the *Np* gene and the *ahas3R* gene were inherited in the tested plants from the cross I and determined their resistance to TSWV and the herbicide Glean®.

Analyses of plants from F2 generation

Resistant plants from F1generation were self pollinated and seed samples were collected. The tests for resistance to TSWV and Glean® were done in F2 generation. Seven randomly selected numbers were chosen for further experiments - I 57/7 and I 57/11 (two plants that generated from the cross I 57 of F1 generation), I 12/10 and I 12/4 (from I 12), I 61/2 and I 61/19 (from I 61) and I 27/12 (from I 27).

Tests for resistance to TSWV and the herbicide Glean®

The plants from F2 generation were inoculated with TSWV and were sprayed with the herbicide Glean® as described for the plants from F1 generation. Only 4 plants (three were from the cross I 61 and one was from the cross I 27) were systemically infected with TSWV and were considered as susceptible to the virus. PCR analyses were performed to investigate whether the *Np* gene was present in the genome of the susceptible plants. No signal for the transgene was detected in the analyzed plants.

The plants from F2 generation of the cross I demonstrated different level of resistance to the herbicide Glean® varying from 93% to 50% (Table 1). The plants from cross I 57/11 demonstrated the highest level of resistance to the herbicide (93%). A lower resistance to sulfonylurea was shown by plants from the cross I 57/7, I 61/2, I 61/19 and I 12/4 - 71%, 77%, 70% and 67% respectively. The plants from the cross I 27/12 and I 12/10 demonstrated the lowest resistance to Glean® among all tested plants- 60% and 50% respectively.

The deviation from the Mendelian segregation is usually explained by different transgene copy number and/or integration site (5).

Number of the cross	Number of tested plants	Resistant plants (%)
I 12/4	30	67
I 12/10	8	50
I 57/11	30	93
I 57/7	17	71
I 61/2	30	77
I 61/19	20	70
I 27/12	30	60

Table 1 Resistance of plants from the cross I, F2 generation to the herbicide Glean®

Some plants delayed in the growth after treatment with the herbicide Glean®. To examine whether the *ahas3R* gene is present in the plant genome, PCR analyses were performed. The results showed that the *ahas3R* was present in some of the analyzed plants. In these cases susceptibility of the plants could be a result of transgene inactivation.

Combining of transgenes in one plant can result in the inactivation of one or more of introduced genes. The expression of transgenes varies and there are many examples where transgenic

trait is not expressed or disappears in subsequent generations, despite of the presence of the transgene. This loss of trait, but not of the transgene is known as gene silencing and can take two forms, transcriptional or post transcriptional (6). There are examples for gene silencing when homologous transgenes or a transgene with homology to an endogenous gene are present in the same genome (9, 11, 13).

DNA *hybridization analyses* Southern blot analyses on 14 randomly selected plants from F2 generation were performed with non radioactively labeled specific probes for the *Np* and the *ahas3R* gene (Fig. 4 A, B).

All tested plants from the cross I and the parental line L1 showed one copy of the *Np* gene. No signal was detected on control DNA extracted from non transgenic tobacco plants.

Positive signal for the *ahas3R* gene in all studied plants from the cross I and the parental line L4 was detected. Six of the plants were with one copy and eight plants had two copies of the *ahas3R* gene. No signal was detected on control DNA from non transgenic control plants. In all analyzed tobacco plants endogenous *ALS* gene was detected due to the high homology with the transgene *ahas3R*.

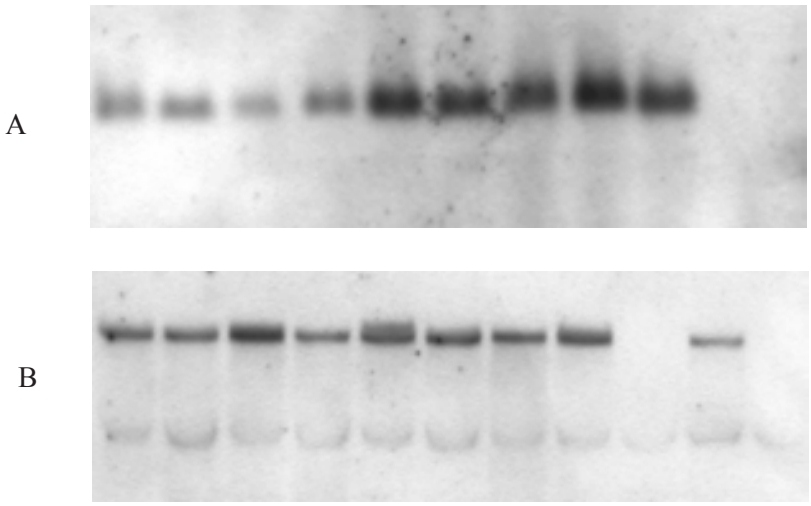

Fig. 4 DNA hybridization analyses of selected plants from the cross I, F2 generation. Specific probes for the Np gene (**A**) and the *ahas3R* gene (**B**) were non radioactively labeled. Lanes 1-8 - plants from the cross I; Lane 9 - parental line L1; Lane 10 parental line L4; Lane 11 - non transgenic cv. Nevrokop 1146

Cross II

Analyses of plants from F1 generation

To combine genes for resistance to PVY and *P. syringae* pv. *tabaci* sexual crosses between plants from the lines L2 and L3, and reverse crosses were performed. More than 57 crosses were done and seed samples were collected. Four of them were randomly chosen for examination - II 10 (L2 x L3), II 44 (L2 x L3), II 15 (L3 x L2) and II 19 (L3 x L2). The seeds from selected crosses germinated normally and all plants were typical in appearance for the cultivar. The plants were tested for resistance to PVY and *Ps. syringae* pv. *tabaci.*

Tests for resistance to PVY and Ps. syringae pv. tabaci.

Tobacco plants were mechanically inoculated with PVY (Fig. 5). At the time of inoculation accidentally cross virus infection had occurred in the green house. Only plants that showed no typical symptoms of PVY infection, three weeks after inoculation, were chosen for ELISA test. From the cross II 10 32 plants were selected and 28 of them were ELISA negative. Thirty plants from the cross II 44 were selected and 25 of them had no coat protein. From the

cross II 19 12 plants were chosen and 11 of them demonstrated negative results. Only 10 plants from the cross II 15 were symptomless and 9 of them were ELISA negative. The presence of susceptible plants in F1 progeny of the cross II was due to the heterozygosity of the gene conferring resistance to PVY of the parental line L2 used for the crosses.

Plants from the crosses II 10 and II 44 showed higher percentage of resistance to PVY than the plants originating from the crosses II 15 and II 19. The crosses II 15 and II 19 were generated from the cross L2 x L3, where the gene conferring resistance to PVY came from the mother line, whereas the crosses II 10 and II 44 were originated from the reverse cross (L3 x L2).

Fig. 5 Resistance to PVY of tobacco plants from the cross II, F1 generation
1 - resistant plant; 2 - susceptible plant; 3 - control non transgenic plant

Plants from the cross II were tested for resistance to *Ps. syringae* pv. *tabaci* by detached leaf bioassay (Fig. 6). Typical infection symptoms were observed on control tobacco leaves three weeks after inoculation with the bacteria. All leaves from the plants of the cross II were not affected after bacterial inoculation. We considered these plants as resistant to *Ps. syringae* pv. *tabaci.* The results indicated that resistance was inherited in all tested plants of the cross II from the parental line L3 that had stable resistance to *Ps. syringae* pv. *tabaci* up to R9 generation.

Fig. 6 Detached leaf bioassay of tobacco plants for resistance to *Ps. syringae* pv. *tabaci*
1 - control non transgenic plant; 2 - resistant plant from the cross II, F1 generation

PCR analyses

Five plants resistant to both PVY and *Ps. syringae* pv. *tabaci* from each cross of II 10, II 44, II 19 and II15 were randomly chosen for the PCR analyses. Primers for 35S promoter and nos terminator were used for the amplification of the CP gene (conferring resistance to PVY) and the

ttr gene (coding resistance to *Ps. syringae* pv. *tabaci*). In all tested plans from the cross II two fragments with the expected sizes were obtained (data not shown). No signal was detected with DNA from the control tobacco plants. The PCR analyses of the selected plants from the cross II demonstrated that the CP gene and the ttr gene were inherited in the tested plants.

Analyses of plants from F2 generation

Resistant plants from F1 generation were self pollinated and seed samples were collected. Tests for resistance to PVY and *Ps. syringae* pv. *tabaci* were carried on in F2 generation. Eight randomly selected numbers were chosen for further experiments - II 10/26 and II 10/29 (two plants that were generated from the cross II 10 of F1 generation), II 44/15 and II 44/16 (from II 44), II 19/4 and II 19/19 16 (from II 19), II 15/20 and II 15/35 16 (from II 15).

Tests for resistance to PVY and Ps. syringae pv. tabaci

Tests for resistance to PVY and Ps. syringae pv. tabaci were performed as described for the plants of F1 generation.

Different levels of resistance to PVY, ranging from 90% to 40% were observed (Table 2). The highest resistance to the virus infection was found in plants of F2 generation from the crosses II 10/26 (90%) and II 10/29 (87 %). Intermediate level of resistance showed plants from the crosses II 44/15 (77%) and II 44/16 (70%). The rest of crosses of F2 generation were more susceptible to PVY infection. They demonstrated resistance to the virus varying as follows: II 19/4 (57%), II 19/19 (50%), II 15/20 (50%) and II 15/35 (40%).

Number of the cross	**Number of tested plants**	**Resistant plants (%)**
II 10/29	30	87
II 10/26	30	90
II 44/15	30	77
II 44/16	30	70
II 19/4	30	57
II 19/19	30	50
II 15/35	30	40
II 15/20	30	50

Table 2 Resistance of plants from the cross II, F2 generation to PVY

Plants from F2 generation of the cross II were tested for resistance to *Ps. syringae* pv. *tabaci* by detached leaf bioassay. Most of the infected with the bacteria tobacco leaves showed no symptoms of infection and the plants were considered as resistant to *Ps. syringae* pv. *tabaci.* However, on some of the inoculated leaves from different crosses, chlorotic halos caused by the production of bacterial tabtoxin appeared. No further spread of chlorosis was observed. This might result from hypersensitive response. Typical symptoms of *Pseudomonas* infection, that are necrotic lesions surrounded by yellow halo, were observed on a few tobacco leaves. To examine whether the ttr gene was present in the plant genome PCR analyses were performed. Plants with different level of chlorosis were chosen. The results showed that only plants with typical symptoms of *Pseudomonas* infection had lost the *ttr* gene. The transgene was lost in the susceptible plants in F2 generation most probably as a result of recombination events. Positive signal for the *ttr* gene was observed of the plants with the localized chlorotic spot on the infected leaves. However, further studies are needed to investigate whether the chlorotic symptoms were a result of transgene silencing.

DNA hybridization analyses

Southern blot analyses on 16 randomly selected plants from F2 generation were performed. Specific probes for the *CP* gene and the *ttr* gene were non radioactively labeled.

All tested plants from F2 generation of the cross II and the parental line L2 showed more than two copies of the *CP* gene (Fig.7). High copy number of the transgene could lead to the lower gene expression than the single copy due to methylation. This might be the reason for the lack of Mendelian inheritance for the resistance to PVY in F2 generation (7, 10).

Positive signal for the transgene *ttr* in all analyzed plants from F2 generation of the cross II and the parental line L3 was detected (data not shown). No hybridization signal was detected from the control DNA.

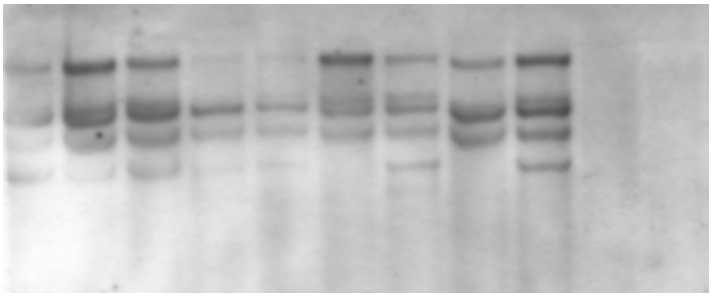

Fig. 7 DNA hybridization analyses of selected plants from the cross II, F2 generation
Non radioactively labeled specific probes for the CP gene were used.
Lanes 1-8 - plants from the cross II; Lane 9 - parental line L2;
Lane 10 - parental line L3; Lane 11 - non transgenic tobacco

CONCLUSION

Promising results for combining genes for resistance to TSWV, PVY, *Pseudomonas syringae* pv. *tabaci* and the herbicide Glean® in pairs by sexual crosses of four transgenic tobacco lines were obtain. Further crosses will be performed in order to combine four traits in one tobacco line.

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ТРАНСГЕНИЧЕН ТУТУН СО КОМПЛЕКСНА ОТПОРНОСТ НА БАКТЕРИСКИ И ВИРУСНИ БОЛЕСТИ И НА ХЕРБИЦИДОТ Glean®

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РЕЗИМЕ

Создавањето тутунски растенија отпорни на различни патогени има теоретско и практично значење. Целта на о вој труд е да се добие линија тутун со комплексна отпорност на бактериски и вирусни болести и на хербицидот **Glean®.** Како почетен растителен материјал се земени четири различни трансгенични линии со потекло од сортата Неврокоп 1146 отпорни на *TSWV, PVY, Pseudomonas syringaepv. tabaci* и на **Glean®**. Овие тутунски линии се вкрстувани за комбинирање на својствата во парови. Во крстоската I комбинирана е отпорноста на TSWV и **Glean®**. На растенијата од потомството Ф1 испитувана им е отпорноста по пат на заразување со TSWV и прскање со **Glean®**. Во крстоската II комбинирана е отпорноста спремаPVY и *P. syringae pv. tabaci* и во F1 генерацијата се проверува отпорноста и на двата патогена по извршената вештачка инокулација. Отпорните тутунски растенија од потомството на секоја комбинација се селектирани врз база наELISA тестот и PCR анализата. Во оваа фаза на истражувањата резултатите ветуваат успешно комбинирање на отпорноста спрема сите три патогени и хербицидот **Glean®** кај секоја линија тутун.

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