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PYRAMIDING OF FOUR TRANSGENES IN ONE TOBACCO LINE BY SEXUAL CROSSES

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Abstract

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To combine four transgenes (*Np*, *CP*, *ttr* and *ahas 3R*) in one tobacco line a cascade of sexual crosses between four transgenic tobacco lines resistant to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean®, respectively, was performed. The first step of the experiment where genes for resistance were combined in pairs was previously described (Docheva et al., 2002). Here we report the second step of pyramiding of four transgenes and combining the resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® in one tobacco line (cross 2002). Complex resistance to all tree pathogens and the herbicide was analyzed in F1 and F2 generations and integration of all four transgenes in tobacco genome was proved by PCR analyses. Thirteen F2 tobacco lines with pyramided genes for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® were selected.

Key words: TSWV, PVY, *P. syringae* pv. *tabaci*, herbicide, cross pollination, resistance

Introduction

Pyramiding of transgenes aimed to combine more than two foreign genes in a plant genome and their coordinated expression. Different approaches have been developed for this purpose, like sexual crosses between transgenic lines, retransformation for consistent integration of genes and cotransformation with a construct containing multiple transgene cassettes.

The sexual crosses are easy and inexpensive approach to include the features encoded by different transgenes in one plant genome. This method was applied to combine genes for resistance to biotic and abiotic stress factors, as well as to join the transgenes that suppress the expression of target genes in plants. Pyramiding of different *Bt* genes by sexual crosses of transgenic lines was successfully used strategy for a pest management control of broccoli (Cao et al., 2002), rice (Yang et al., 2011) and cotton (Li et al., 2014). Complex resistance to bacterial blight and yellow stem borer, and tolerance to sheath blight has been developed by crossing of plants expressing the *Xa21* gene with plants expressing both a *Bt* fusion gene and a chitinase gene (Datta et al., 2002). Two genes for salt tolerance were stacked by cross breading of transgenic tobacco lines (Duan et al., 2009). Sexual crossing was successfully applied also in combination of transgenes encoding the synthesis of proteins, gene suppressors and for the incorporation of new biochemical pathways in plants that require the coordinated expression of several enzymes (Poirier et al., 1992; Nawrath et al., 1994; Ma et al., 1995; Slater et al., 1999; Poirier, 2000).

Despite of successive examples, sexual crossing has several disadvantages (Francois et al., 2002). It is time-consuming, particularly if the aim is to combine more than two transgenes. Two foreign genes that are integrated into one line are located in different chromosomal loci and make difficulties for further selection through traditional breeding techniques. Another disadvantage, that also concerns other techniques for pyramiding of transgenes, is accumulation of more copies of genes of interest or marker genes, leading to suppression of gene expression (Halpin, 2005; Eamens et al., 2008).

In a present study, combining of four transgenes for complex resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® in one tobacco line is presented. Pyramiding of the transgenes *Np*, *CP*, *ttr* and *ahas 3R* have been achieved

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by cascade of sexual crosses between four transgenic tobacco lines resistant to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean®, respectively. The complex resistance to all tree pathogens and the herbicide was analyzed in F1 and F2 generations and the presence of all four transgenes was proved by PCR analyses.

Materials and Methods

Plant material and sexual crosses

Transgenic tobacco plants resistant to TSWV and herbicide Glean® (cross I, F2) were cross pollinated with transgenic tobacco plants resistant to PVY and *P. syringae* pv. *tabaci* (cross II, F2) to combine four features in one tobacco line. Resulted F1 lines of the cross 2002 were tested for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® and presence of the transgenes *Np*, *CP*, *ttr* and *ahas 3R*. Selected plants with a complex resistance were self pollinated under insulator and seeds were collected individually for F2 generation of the cross 2002.

All initial transgenic tobacco lines involved in the crosses originated from the cultivar Nevrokop 1146. The combination of transgenes in pairs in a cross I and cross II was previously described (Docheva et al., 2002).

Test for resistance

Tests for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® of F1 and F2 lines of the cross 2002 were performed as previously described (Docheva et al., 2002).

DAS-ELISA test

Tobacco plants inoculated with TSWV and PVY were tested for virus infection using DAS-ELISA method (Clark and Adams, 1977). Presence of virus proteins in tobacco plants was analyzed by polyclonal rabbit antibodies using kit for plant virus detection, according to the manufacturer's instructions (Loewe Phytodiagnostica, GmbH).

PCR analyses

Gemonic DNA was isolated from tobacco leaf tissue as described by Delaporta et al. (1983).

PCR analyses were performed using Ready To Go PCR Beats (GE Healthcare) according to the manufacturer's instructions. PCR primers, amplification conditions and size of resulted products are presented on a Table 1.

Results and Discussion

Sexual crosses of four transgenic tobacco lines

Tobacco plants of F2 generation of the cross I, resistant to TSWV and herbicide Glean®, were cross pollinated with plants of F2 generation of the cross II, that were resistant to PVY and *P. syringae* pv. *tabaci*. Only plants with typical for the cultivar habit and presence of related transgenes proved by PCR analyses were selected for the cross breeding. Out of 86 reciprocal crosses were made and eight of them were chosen for future analyses.

Resistance to the pathogens and herbicide Glean® in F1 generation of the cross 2002

Tests for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® were subsequently performed using 40 plants of each of selected F1 lines of the cross 2002.

Resistance to TSWV in F1 generation

A mechanically inoculation of tobacco plants with TSWV was performed at the 2-3 leaf stage. Nontransgenic tobacco line Nevrokop 1146 and the initial parental line L1 (source of the *Np* gene for resistance to TSWV) were inoculated with the virus as negative and positive control, respectively.

Table 1
PCR primers, amplification conditions and size of resulted products used for PCR analyses of the cross 2002

Gene/PCR product	PCR primers	PCR program
Np/550 bp	5'-GGC AAA GAC CTT GAGT-3' 5'-CTT TGC TTT TCA GCAC-3'	94°C-5 min.; 33 cycles of 94°C-30 sec., 60°C-45 sec. and 72°C-1 min.; final elongation 72°C-5 min.
CP/550 bp	5'-TCG ATG CAG GAG GAA GCA CTA-3' 5'-TTC CGT CGC GCA GAT TAC GAA-3'	94°C-3 min.; 30 cycles of 94°C-30 sec.; 56°C-45 sec. and 72°C-1 min.; final elongation at 72°C-5 min.
ttr/340 bp	5'-CTT CGC CCA TTA TCG CCA TGG TC-3' 5'-CAG CCC GCG TTT GTG TTT TAC TG-3'	94°C-4 min.; 30 cycles of 94°C-45 sec., 64°C-45 sec. and 72°C-40 sec.; final elongation at 72°C-5 min.
ahas 3R/750 bp	5'-ACG ATG AGT TGT CCC TGC AG-3' 5'-AGA TCT CGT TCT CCC TTT CC-3'	94°C-3 min.; 30 cycles of 94°C-1 min., 62°C-1 min. and 72°C-1 min.; final elongation at 72°C-5 min.

The first symptoms of the virus infection were observed 10 days after inoculation with TSWV. Nontransgenic control plants were systemically infected with the virus, while all plants of the parental line L1 were completely resistant to TSWV. The most of tobacco plants of the cross 2002 showed no symptoms of viral infection. Some of plants were systemically infected with the virus, whereas other part demonstrated localized disease symptoms. A sign of virus infection was established more than a month after inoculation for a number of analyzed plants.

Tobacco plants without any symptoms of virus infection 45 day after inoculation were tested for absence of viral protein using DAS-ELISA method. Data confirmed lack of viral infection for all selected plants from the cross 2002.

The results of inoculation with TSWV and DAS-ELISA test showed that analyzed eight F1 tobacco lines demonstrated resistance to the virus varied from 38% to 100% (Table 2). Plants from a line 8 showed 100% resistance after inoculation with TSWV. A virus resistance rate of 85% demonstrated tobacco plants from lines 27 and 57. A lower resistance has lines 6, 13, 32 and 46 - 75%, 70%, 65% and 63%, respectively. Tobacco plants of the line 42 were the most sensitive to TSWV and only 38% of them were resistant to the virus.

Plants of the cross 2002 were tested for the inheritance of the *Np* gene by PCR analyses using specific for the transgene primers. Forty plants resistant to TSWV from all tested lines were selected and positive results have been achieved for 22 of them. No signal was detected for nontransgenic tobacco plants.

Resistance to PVY in F1 generation

Tobacco plants of F1 generation of the cross 2002 were mechanically inoculated with PVY one week after treatment with TSWV. Plants of the parental line L2 (source of the *CP* transgene in the crosses) were used as a positive control and nontransgenic tobacco cultivar Nevrokop 1146 as a negative control.

A typical symptoms of PVY infection on some of tobacco plants of the cross 2002 appeared 5 days after inoculation with the virus. All nontransgenic control plants demonstrated susceptibility to PVY, whereas no symptoms of viral infection on the parental line L2 were observed.

The plants of the cross 2002 without symptoms of PVY infection were tested for viral proteins by DAS-ELIZA. A total number of 94 plants were analyzed and negative results for 89 of them were recorded.

The inoculation with PVY and DAS-ELIZA test showed that resistance of tobacco plants to the virus varied from 35% to 100% (Table 2). All transgenic plants of lines 6 and 27 demonstrated resistance to PVY (100%). A high level of susceptibility was estimated for the tobacco line 13 where only 35% of the plants were not infected by the virus. Other analyzed lines performed comparable rate of resistance, as follows - line 8 - 50% resistance, lines 32 and 46 - 58%, line 57 - 60% and line 42 - 68%.

PCR analyses with specific for the *CP* gene primers proved presence of the transgene in 28 of 29 tested F1 plants, that were resistant to PVY. No PCR signal was detected for nontransgenic tobacco plants.

***Resistance to P. syringae* pv. *tabaci* in F1 generation**

Tests for resistance to *P. syringae* pv. *tabaci* of F1 lines were performed using detached leaf bioassay. Plants demonstrated resistance to both viruses, TSWV and PVY, were selected for analyses. Nontransgenic control Nevrokop 1146 and the parental line L3 (source of the *ttr* gene for resistance to the bacteria) were also tested.

Development of chlorotic and necrotic symptoms of the bacterial infection was observed on nontransgenic tobacco leaves one week after application of *P. syringae* pv. *tabaci* suspension. Tested leaves from parental line L3 demonstrated complete resistance to *P. syringae* pv. *tabaci*.

Resistance to *P. syringae* pv. *tabaci* of analyzed F1 lines varied from 25% to 85% (Table 2). The highest level of resistance demonstrated lines 6 and 27, respectively 84% and 85%, a lower rate of resistance to the bacterial infection showed

Table 2
Resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® in F1 generation

Number of F1 tobacco line	Resistance to TSWV, %	Resistance to PVY, %	Resistance to *P. syringae* pv. *tabaci*, %	Resistance to herbicide Glean® , %
6	65	100	84	53
8	100	50	50	82
13	75	35	67	35
27	85	100	85	55
32	63	58	25	53
42	38	68	73	55
46	70	58	77	55

lines 46 and 42, respectively 77% and 73%. A similar level of resistance performed plants of three lines, as follows - line 13 - 67%, line 57 - 60% and line 8 - 50%. The most sensitive to *P. syringae* pv. *tabaci* were plants from line 13 and only 25% of them were resistant to the bacterial infection.

Resistant to *P. syringae* pv. *tabaci* plants of the cross 2002 were tested for inheritance of the *ttr* gene using PCR amplification with specific primers. Twenty plants of tested F1 lines were selected and PCR products with expected size were obtained for 10 of them. No signal was detected for nontransgenic tobacco plants.

Resistance to herbicide Glean® in F1 generation

All F1 plants of the cross 2002 were tested for resistance to herbicide Glean® by treatment with the herbicide at the stage of 4-5 leaf. A nontransgenic plants Nevrokop 1146 were used as a negative control and the parental line L4 (supplying the *ahas 3R* gene in the crosses) was a positive control for the experiment.

All tested nontransgenic tobacco plants developed typical symptoms of susceptibility to Glean® and died 10 days after treatment. Seven of eight tested plants of the parental line L4 demonstrated resistance to the herbicide.

Results of all analyzed lines of the cross 2002 showed that resistance to Glean® varied from 35% to 82% (Table 2). Highest resistance to the herbicide demonstrated line 8 - 82%. Plants of five lines showed similar reaction to the treatment with Glean®, as follow: lines 27, 42 and 46 - 55% resistance, lines 6 and 32 - 53% resistance. Lower rate of resistance to Glean® was observed for lines 57 and 13 - 40% and 35%, respectively.

PCR analyses for presence of the *ahas 3R* in resistant to herbicide Glean® plants confirmed presence of transgene in 24 out of 29 tested samples. A negative result was observed for nontransgenic tobacco plants.

Selection of F1 plants carrying all four transgenes (*Np*, *CP*, *ttr* and *ahas 3R*) and segregation analyses

After screening for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean®, and PCR analyses performed, pyramiding of *Np*, *CP*, *ttr* and *ahas 3R* genes was proved in 12 plants of the cross 2002. Selected plants with combined all four transgenes for resistance belongs to five of analyzed F1 lines (lines 6, 8, 13, 27 and 42), whereas plants with all genes of interest were not detected in lines 32, 46 and 57.

A complete resistance of line 8 to TSWV and of lines 6 and 27 to PVY means that parental lines source of the *Np* gene and the *CP* gene, respectively, were homozygous to the transgenes. When resistance to any of tested diseases or the herbicide is close to 100%, we can suppose that the parental line source of corresponding transgene is also homozygous, but a part of genes are inactivated. Analyzed lines that demonstrated resistance about 50% probably have a hemizygous parental lines source of the corresponding transgene. Line 8 demonstrated 50% resistance to both PVY and *P. syringae* pv. *tabaci* meaning that parental lines were hemizygous on *CP* and *ttr* genes. A resistance rage of 53-55% to herbicide Glean® was accounted for most of analyzed F1 lines (6, 27, 32, 42, 46) probably due to hemizygous *ahas 3R* gene in a parental lines. Non Mendelian segregation of the genes for resistance to tested diseases and the herbicide in a part of the F1 lines could be based on gene silencing, presence of more than one copy of transgenes or lower expression level (Matzke and Matzke, 1995; Yin et al., 2004).

Resistance to the pathogens and herbicide Glean® in F2 generation of the cross 2002

To asses inheritance of resistance and stability of pyramided transgenes analyses were continued in the F2 generation. F1 plants that demonstrated resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean®, and with positive results for all four transgenes were self-pollinate under insulator and seeds were collected individually. Six F2 lines were chosen for tests for resistance to the diseases and the herbicide (6/14, 6/35, 8/27, 8/30 27/14 and 42/18). Fifty plants of each F2 line were subsequently analyzed for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean®.

Resistance to TSWV in F2 generation

Plants of the F2 generation of the cross 2002 and nontransgenic control plants were mechanically inoculated with TSWV at a stage of 2-3 leaves. F2 lines demonstrated resistance to the virus varied from 66% to 100% (Table 3). All analyzed plants of the line 8/30 showed resistance to TSWV. Lower resistant rate demonstrated the line 27/14 (82%). Other tested lines presented similar reaction to TSWV with a following resistance level: lines 6/35 and 42/18 - 70%, line 8/27 - 68% and line 6/14 - 66%. The systemic infection with the virus was observed on control nontransgenic plants.

Resistance to PVY in F2 generation

Plants of the F2 generation of the cross 2002 and nontransgenic control plant were mechanically inoculated with PVY 10 days after TSWV application. Results of the tested tobacco lines demonstrated that resistance to the virus varied in a range of 68% to 94% (Table 3). A resistance level of 94% was accounted for lines 27/14 and 42/18. A lower resistant rate to PVY showed lines 6/35 (84%), 6/14 (76%), 8/27 (72%) and 8/30 (68%). Control nontransgenic plants developed typical symptoms of viral infection.

***Resistance to P. syringae* pv. *tabaci* in F2 generation**

Resistance of F2 lines to *P. syringae* pv. *tabaci* was assessed using detached leaf bioassay. Plants with no symptoms of the viral infection were choosen for analyses, as well as control nontransgenic tobacco plants. Results demonstrated a range of resistance to *P. syringae* pv. *tabaci* from 44% to 67% for assessed lines (Table 3). The highest level of resistance to the bacterial disease demonstrated line 27/14 (67%). Similar resistant rate showed lines 42/18 (57%), 6/14 (55%) and 8/27 (55%). The lowest resistance to *P. syringae* pv. *tabaci* was observed for lines 6/35 and 8/30 - 45% and 44%, respectively. Leaves of nontransgenic tobacco lines demonstrated typical symptoms of the bacterial infection one week after inoculation with *P. syringae* pv. *tabaci*.

Resistance to herbicide Glean® in F2 generation

Plants of the F2 generation were tested for resistance to the herbicide Glean® at stage 4-5 leaves. Nontransgenic tobacco plants Nevrokop 1146 were used as a control.

Typical symptoms of susceptibility to Glean® were observed on all tested nontransgenic plants and 2 weeks after treatment died. Resistance to the herbicide of F2 plants varied from 44% to 70% (Table 3). The highest resistance level demonstrated the line 8/27 (70%), a lower resistance rate have lines 42/18 (64%), 6/35 (62%), 6/14 (60%) and 27/14 (56%), and the line 8/30 was the most susceptible (44% resistance).

Selection of F2 plants carrying all four transgenes (*Np*, *CP*, *ttr* and *ahas 3R*)

After testing for resistance to the pathogens and the herbicide selected F2 plants were analyzed for the presence of *Np*, *CP*, *ttr* and *ahas 3R* genes using PCR amplification with specific for each transgene primers. Results of analyses confirmed inheritance of all four transgenes in 13 tobacco plants that is 4.3% of all tested plants. A similar percentage of selected F2 plants with combined four transgenes have been reported by Kalunke et al. (2013). Pyramiding of *Pvpgin2*, *Acpmei* and *Taxi - III* was achieved by crossing of durum wheat plants containing *Pvpgin2* and *Acpmei* transgenes with transgenic lines carrying *Taxi - III* (Kalunke et al., 2013). Segregation analyses in F2 generation showed that only 4.5% of the progeny inherited all four transgenes, including the marker *bar* gene.

The stable inheritance and expression of more than 2 transgenes in one plant genome by sexual crosses is difficult and requires cultivation of 4 to 6 generations (Halpin et al., 2001). Datta et al. (2002) get together resistance to bacterial blight and yellow stem borer, and tolerance to sheath blight by crossing plants expressing the *Xa21* gene with plants expressing both a *Bt* fusion gene and a chitinase gene. They confirmed homozygosity and stability in expression and function of all three transgenes using molecular methods and bioassay to F4 generation. Enhanced resistance against major sap-sucking pests have been achieved by sexual crosses of rice lines harbouring *Allium sativum* (*asal*) and *Galanthus nivalis* (*gna*) lectin genes (Bharathi et al., 2011). Segregation analysis of F2 progenies showed digenic inheritance (9:3:3:1) of the transgenes. Homozygous F3 plants carrying *asal* and *gna* genes were selected based on genetic analyses, molecular methods and insect bioassays. Stable and consistent resistance against tested insects of the pyramided lines was tracked up to F6 generation.

A multiple virus resistance including the *Np* gene of TSWV and the *CP* gene of PVY in a combination with other viruses like TuMV, ToLCTWV and WMV have been achieved using different transformation techniques (Jan et al., 2000; Lin et al., 2011, 2012). Our experiments represent opportunity to use sexual crosses of transgenic lines for pyramiding of genes for resistance to two viral diseases, one bacterial pathogen and one herbicide.

Conclusion

A lot of single genes for resistance to biotic stress factors have been integrated in a different plant species using

Table 3
Resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® in F2 generation

Number of F2 tobacco line	Resistance to TSWV, %	Resistance to PVY, %	Resistance to *P. syringae* pv. *tabaci,* %	Resistance to herbicide Glean®, %
6/14/	66	76	55	60
6/35/	70	84	45	62
8/27/	68	72	55	70
8/30/	100	68	44	44
27/14/	82	94	67	56
42/18/	70	94	57	64

genetic engineering approaches. A further enhancement of stress tolerance can be achieved by combining in one genome of transgenes for resistance to viral and bacterial diseases, fungal pathogens, parasitic plants and herbicides.

Here we describe the possibility for a successful pyramiding of four transgene for resistance to TSWV, PVY, *P. syringae* pv. *tabaci* and herbicide Glean® in one tobacco line. A cascade of sexual crosses between four transgenic lines was performed to combine four genes (*Np*, *CP*, *ttr* and *ahas 3R*). Although pyramiding of transgenes by sexual crosses is a slow process with low efficiency a complex resistance to all tree pathogens and the herbicide has been achieved in 13 F2 tobacco lines. Selected plants demonstrated combined resistance to some of the most widespread and economically important tobacco diseases.

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