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Creation of transgenic tobacco plants with two target genes by hybridization

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Motivation and aim: Creation of transgenic tobacco plants *Nicotiana tabacum* L., with constitutive expression of two transgenes using the hybridization method.
Materials and methods: The hybridization work used previously created transgenic tobacco plants with *ARGOS-LIKE*, glutathione-S-transferase *AtGSTF11*, expansins *NtEXPA1*, *NtEXPA5*, *PnEXPA3* and *AtEXPA10*, xyloglucan endotransglycosylases *PtrXTH1* and *NtEXGT*. Seeds of transgenic plants were germinated on Murashige and Skoog nutrient medium with the addition of the antibiotic hygromycin in «Binder» climate chambers (Germany) at a photoperiod of 16/8 hours, a temperature of +25 °C and an illumination of about 140 micromoles per square meter/sec. After 20 days, the seedlings were transferred to vegetation vessels with «Geolia» universal soil. Watering of plants was carried out 2 times a week. 2 weeks after transplanting into the ground, when the plants had acclimatized, DNA was isolated from them using CTAB to confirm their transgenicity by the marker genes hygromycin phosphotransferase (*HPT*) and β-glucuronidase (*GUS*), as well as the target transgene. A sample of 200 mg of each sample was ground in a micro-tube with 800 µl of 2×CTAB buffer, then incubated in a «Thermit» thermostat (DNA Technology, Russia) at 60 °C for 60 min. Then it was cooled in the refrigerator at 5 °C for 5 min, 600 µl of the chloroform:isoamyl alcohol (24:1) mixture was added and was shaken in an «Intelli Mixer RM-1 L» (SIA «ELMI», Latvia) for 20 min. Next, it was unscrewed in a 5430 R centrifuge («Eppendorf», Germany) at 12000 rpm for 2 min, 400 µl of the supernatant was taken, to which 400 µl of cooled isopropyl alcohol was added. The mixture was incubated for 20 min in a freezer at –20 °C, and then centrifuged for 15 min at 12000 rpm. Next, isopropyl alcohol was drained and the precipitate was washed with 80% ethyl alcohol, which was subsequently dissolved in 30-50 µl of sterile water. To test the transgenicity of plants, primers were used for the *HPT* gene: 5'-CTACACAGCCATCGGTCCAGA-3'/5'-ATCGTTATGTTTATCGGCACTTTG-3' and the *GUS* (*uidA*) gene: 5'-TGTGGAATTGATCAGCGTTGGTG-3'/5'-AAGCCGACAGCAGCAGTTTCATC-3'. The amplicon size for the *HPT* gene was 789 bp, and for the *GUS* gene it was 989 bp. The reaction took place in 30 µl of PCR solution with the addition of 0.1 µg of DNA and 1 unit of Taq DNA polymerase according to the scheme: initial denaturation for 3 min at 94 °C, 35 amplification cycles at a) denaturation – 94 °C, 40 sec, b) annealing – 59 °C, 40 sec, c) elongation – 72 °C, 60 sec. The final elongation was carried out for 5 min at 72 °C. The obtained amplicons were detected using agarose gel electrophoresis. 2 months after acclimatization to the soil conditions, the tobacco plants began to move to the flowering stage and were used for hybrid crossing. In order to cross the plants, the female specimens had flowers that had not had time to open and which had a protruding stigma of the pistil, while they had unripe pollen in the anthers, which were mechanically removed to prevent the possibility of self-pollination. Fully opened flowers with ripe pollen in the anthers were taken as males. The cross-pollination of transgenic plants among themselves took place according to the scheme: ripe pollen from the anthers from the male plant was applied using a synthetic brush to the stigma of the flower of the female plant and the pollinated flower was wrapped with food film to prevent other pollen from entering the stigma. A month later, fully matured seeds after artificial pollination were collected to produce tobacco plants of the T1 generation expressing two transgens simultaneously.
Results: During the experiments, 40 transgenic tobacco plants acclimatized to soil conditions were created with eight studied genes, from which DNA was isolated and the presence of *HPT* and *GUS* genes was confirmed. The presence of target *ARGOS-LIKE*, glutathione-S-transferase *AtGSTF11*, expansins *NtEXPA1*, *NtEXPA5*, *PnEXPA3* and *AtEXPA10*, xyloglucan endotransglycosylases *PtrXTH1* and *NtEXGT* genes was also confirmed in the same transgenic plants. Pairs of plants carrying the following target genes were selected for artificial crossing: *AtGSTF11* × *ARL*, *NtEXPA1* × *PtrXTH1*, *NtEXGT* × *NtEXPA5*, *PnEXPA3* × *AtEXPA10*. Artificial pollination of these transgenic plants was carried out and seeds of T1 generation descendants were obtained, which will be analyzed for the presence of both transgenes in one plant organism by PCR.
Conclusions: Transgenic tobacco plants overexpressing two target genes simultaneously can contribute to a greater degree of expression of a valuable trait: yield, resistance to abiotic factors, etc. The created tobacco plants with two transgens can also be used in basic research, since the functions of *ARGOS-LIKE*, glutathione-S-transferases, expansins and xyloglucan endotransglycosylases remain poorly understood.
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