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Transfer of gene conferring herbicide bialaphos resistance into buckwheat plants

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Bar gene conferring resistance to herbicide bialaphos (phosphinothricin) was cloned from Streptomyces hygroscopicus. Bar gene under 35S promoter of cauliflower mosaic virus was introduced in binary pBin19 vector and constructed plasmid was transferred into Agrobacterium tumefaciens strain. Conditions of genetic transformation of cultivated buckwheat and interspecific hybrid Fagopirum esculentum × F. tataricum were worked out. Buckwheat explants were inoculated by the strain with a plasmid carrying bar gene nearby NPTII gene. Molecular analysis of 8 regenerated plants that were selected for kanamycin resistance was performed. 5 plants gave positive signal by dot- and Southern hybridization using as a probe DNA fragment with bar gene that is an evidence of integration of bar gene into plant genome. Transformed plants grow and rooted at Basta concentrations in the medium that totally inhibited nontransformed plants.

Introduction. The implementation of herbicides in modern agriculture is inevitable [1]. They control the weed growth and thus increase harvest [2]. Use of herbicides of a new generation that are very effective and as a rule inhibit single definite enzyme at very low concentration is very attractive. These herbicides are not toxic for human and animals. The ecological safety is connected to their quick degradation in soil and absence of target enzymes in vertebrates [1, 3].

Bialaphos is an example of a herbicide of this generation. It is a tripeptide and its acting substance is phosphinothricin (PPT) a toxic analog of glutamic acid [4]. But bialaphos as many other herbicides of this generation is not selective, inhibiting the growth of any plant and this character diminished the use of these herbicides in agriculture. Using gene engineering approach by introducing into plant genome of gene(s) conferring resistance to herbicides of this type it is possible to produce transgenic plants resistant to these herbicides [5].

The source of these genes is soil and other microorganisms. Bialaphos resistance gene *(bar)* was found in the genome of bialaphos producing streptomyces, conferring their resistance to produced tripeptide [4].

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Production of transgenic tobacco, potato, tomato, aspen plants resistant to herbicide PPT is reported [1, 3, 6, 7]. Earlier we described the introduction of modified *aroA* gene conferring resistance to herbicide glyphosate (Roundup) into potato, sugarbeet and soybean plants [8, 9]. In this paper we report about transfer of *bar* gene and its expression in buckwheat plants.

Materials and Methods. Cloning of *bar* gene was done from *Streptomyces hygroscopicus* using as vectors *pUC19* [10], *pCAMV* [11], *pBin19* [12] plasmids.

Alcali plasmid isolation, restriction, DNA ligation and electrophoresis, DNA elution and its cleaning, transformation of bacteria were done by the described methods [12, 13]. The binary vectors were transferred by tri-parental matings to the disarmed *Agrobacterium tumefaciens* strains 3850 [12] and LBA 4404.

All the enzymes used in this work were obtained from NPK «Biotekh» (Russia).

In all experiments we used mutant form of buckwheat Homostilnaya with high regeneration potential and hybrid of this form with tartari buckwheat (k-17). This hybrid was obtained via *in vitro* culture of immature interspecific embryos [14].

Internodes, petioles and leaves of these hybrids and Homostilnaya were inoculated by 3850 strain of

A. tumefaciens by two hours co-cultivation in overnight culture of agrobacteria in the presence of acetosyringone. Then explants were transferred onto Murashige-Skoog basal media with the addition of 1 mg/l BA and 0.5 mg/l NA for plant regeneration. To suppress agrobacteria regrowth and for kanamycin selection (*NPTII* gene is located on plasmids alongside *bar* gene) antibiotics kanamycin, carbenicillin and claforan were also added to the culture medium. Explants were cultivated first in the dark and after appearence of regenerants under 16:8-hr photoperiod with constant temperature of 26 °C. Regenerants that came through kanamycin selection were rooted and microclonally propagated. For evaluation of their herbicide resistance these regenerants were transferred onto MS medium with 3—30 μl/l concentrations of PPT (Basta).

Results and Discussion. It was shown that recombinant *pBA-1* plasmid conferring bialaphos resistance has additional 1.7 kb pair *Pst* fragment. To improve *bar* gene expression GTG translation initiation codon was changed for eukaryotic ATG codon.

35S promoter of cauliflower mosaic virus and 3′ region from nopalin synthase gene were introduced into *bar* gene expression cassette. Recombinant *pBA-3* plasmid was constructed for transfer of *bar* gene into buckwheat plants using agrobacteria transformation system. For this purpose *HindIII* fragment containing 35S promoter, *bar* gene and poly A region of nopalin synthase gene was cloned into corresponding site of *pBin19* binary vector.

Resulting *A. tumefaciens* strains 3850/*pBA-3* and LBA 4404/*pBA-3* appeared to be resistant to 10 mg/l of bialaphos in nutrient medium.

Plants regenerated on kanamycin containing medium that was changed every 12—14 days were analyzed by dot- and Southern blot-hybridization. Out of 8 kanamycin resistant regenerants positive autoradiographic response gave 5 plants: 4 plants of hybrid *Homostilnaya* × k-17 and one plant of *Homostilnaya* that is a proof of *bar* gene integration into plant's genome (Fig. 1, 2).

These 5 plants were microclonally propagated and transferred into MS medium containing 3, 10, 15, 20 and 30 μl/l of herbicide Basta. On medium added with 3 and 10 μl/l of Basta all these plants formed roots and their growth did not differentiate from the growth of plants on MS medium without herbicide.

At 15 and 20 μl/l of Basta only 2 plants rooted and their development was normal. At 30 μl/l Basta concentration only one plant survived. The growth and root formation of nontransformed plants of *Homostilnaya* and *Homostilnaya* × k-17 hybrid were inhibited already at 3 μl/l of Basta.

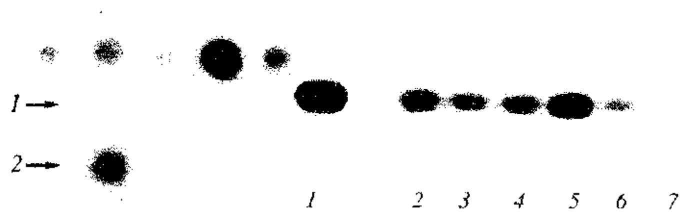

Fig. 1. DNA dot-hybridization of buckwheat plants Arrows show negative (*1*) and positive (*2*) controls. Intensive black spots correspond to DNA of plants with positive autoradiographic response

Fig. 2. DNA Southern hybridization of buckwheat plants: *1* — positive control (*HindIII* fragment with *bar*-gene); *2*—*6* — DNA of buckwheat plants after *HindIII* restriction with positive autoradiographic response; *7* — negative control (DNA of nontransgenic buckwheat plants)

That is a difference among transformed plants by the level of transferred *bar* gene expression was clearly seen.

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Перенесення гена стійкості до гербіциду біалафосу в рослини гречки

Резюме

Із Streptomyces hygroscopicus клонували bar-ген, який визначає стійкість до гербіциду біалафосу (фосфінотрицину). Ген під контролем 35S-промотора вірусу мозаїки цвітної капусти було введено у бінарний вектор pBin19 для трансформації рослин. Одержану рекомбінантну плазміду перенесено до штаму 3850 Agrobacterium tumefaciens. Підібрано умови трансформації культурної гречки і міжвидового гібрида Fagopyrum esculentum × F. tataricum. Експланти гречки інокулювали агробактеріальним штамом, у якому bar-ген знаходиться на T_i плазміді поруч з NPTII геном. Здійснено молекулярно-біологічний аналіз восьми регенерантів, які пройшли канаміциновий відбір. П'ять рослин дали позитивну радіоавтографічну відповідь при дот- і Саузерн-гібридизації з міченим зондом, який несе bar-ген, що свідчить про інтеграцію цього гена в геном рослин. Трансформовані рослини росли і утворювали коріння при концентрації гербіцида басти в середовищі, яке повністю пригнічувало нетрансформовані рослини.

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Перенос гена устойчивости к гербициду биалафосу в растения гречихи

Резюме

Из Streptomyces hygroscopicus клонировали bar-ген, определяющий устойчивость к гербициду биалафосу (фосфинотрицину). Ген под контролем 35S-промотора вируса мозаики цветной капусты был введен в бинарный вектор pBin19 для трансфор-

мации растений. Полученная рекомбинантная плазмида перенесена в штамм 3850 Agrobacterium tumefaciens. Подобраны условия трансформации культурной гречихи и межвидового гибрида Fagopyrum esculentum × F. tataricum. Экспланты гречихи инокулировали агробактериальным штаммом, в котором bar-ген находился на T_i плазмиде рядом с геном NPTII. Проведен молекулярно-биологический анализ восьми регенерантов, прошедших канамициновый отбор. Пять растений дали положительный радиоавтографический ответ при дот- и Саузерн-гибридизации с меченым зондом, несущим bar-ген, что свидетельствует об интеграции этого гена в геном растений. Трансформированные растения росли и образовывали корни при концентрации гербицида басты в среде, полностью ингибирующей нетрансформированные растения.

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