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MORPHOLOGICAL CHANGES IN ATYPICAL BIRD'S FOOT TREFOIL PLANTS OBTAINED DURING GENETIC TRANSFORMATION BY *AGROBACTERIUM*

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Atypical plants of bird's foot trefoil (*Lotus corniculatus* L., Bokor cv.) showing altered morphological characters that deviate from a normal phenotype were found after plant regeneration from transformed tissue. It had been obtained by genetic transformation of root sections of seedlings using *Agrobacterium tumefaciens* vector LBA4404/pBI121 on a medium supplemented with 0.2 mg l^{-1} BAP. The transformants 2b and 4a were found to have a greatly atypical habit, including shortened inter-nodes, elongated leaves, regular leaf arrangement along the stem and thicker leaves. Inheritance of altered characters was observed in the first progeny generation, and their genetic origin was considered.

Key words: *Lotus corniculatus*, leguminous plants, *in vitro* regeneration, genetic transformation, somaclonal variation, phenotypic variants

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INTRODUCTION

Successful genetic transformation requires stable inheritance and expression of transgenes in the progeny, as well as production of transformed plants with a normal phenotype equivalent to the characters of the transformed cultivar. However, both procedures, i.e. the genetic transformation and *in vitro* regeneration, may result in acquiring plants with atypical phenotype or somaclonal variation. Regarding leguminous plants, instances of somaclonal variation have been described mostly for soybean (BARWALE and WIDHOLM, 1987; 1990; RANCH and PALMER, 1987; SCHOEMAKER *et al*., 1991; GRIGA, 1999) and peas (STEJSKAL and GRIGA, 1992, GRIGA *et al*., 1995; GRIGA 2000). A number of phenotypic variations have been described in those plants, such as agglutinated seeds, multiple buds, dwarfish growth, abnormal morphology and leaf number, wrinkled leaves, leaf rolling, yellow-green plants, isoenzymatic variants, partial or complete sterility, etc. Somaclonal variants have also been detected for bird's foot trefoil (ORSHINSKY and TOMES, 1984; 1985; DAMIANI *et al*., 1985; PEZZOTI *et al*., 1985; WEBB and WATSON 1991). Bird's foot trefoil plants transformed by *Agrobacterium rhizogenes* (TABAEIZADEH, 1993) and *A. tumefaciens* (WEBB *et al*., 1996) displaying morphological deviation from the seed-grown control plant population have also been detected. This report gives a description of morphological alteration of bird's foot trefoil plants acquired by direct regeneration *in vitro* from *A. tumefaciens*-transformed plant roots.

MATERIALS AND METHODS

Transformed plants were regenerated from root sections (5mm) taken from bird's foot trefoil Bokor cv. seedlings aged 5-6 days that had been transformed by *A. tumefaciens* strain LBA4404 (HOEKEMA *et al*., 1983) carrying the plasmid pBI121 (JEFFERSON *et al*., 1987). The plasmid T-DNA carried the *nptII* selective marker gene for resistance to kanamycin and *uidA* reporter gene for β-glucuronidase. The *A. tumefaciens* strain LBA4404/pBI121 had grown on an agar solidified YEB medium (VAN LAREBEKE *et al*., 1977) supplemented with 50 mg l^{-1} of kanamycin antibiotic. Bacterial suspensions, made by subculturing a bacterial colony from a Petri dish into 5 ml of liquid YEB medium and leaving it to incubate overnight at 27°C, were used to infect the plant material. Isolated sections were immersed in the bacterial suspension for 5-10 minutes. Control root sections were immersed in a medium containing bacteria free of the plasmid (LBA4404/Ø). The explants were then transferred to filter paper to remove excessive bacteria. Ten root parts were transferred into each Petri dish plated with 20 ml MS (MURASHIGE and SKOOG, 1962) medium containing 0.2 mg l^{-1} BAP. After co-cultivation for 3 days, the parts were transferred onto the same medium supplemented with 300 mg l^{-1} cephotaxim to eliminate the bacteria. During the next several subcultures, cephotaxim concentration was reduced to 200 mg l^{-1}. After 15 days, the root parts with initial bud swell were transferred to a selective growth regulator-free MS medium supplemented with 100 mg l^{-1} of kanamycin.

The regenerated shoots were grown over two subcultures on a growth regulator-free MS medium supplemented with 100 mg l^{-1} kanamycin, followed by another two subcultures on a medium supplemented with kanamycin concentration reduced to 50 mg l^{-1}. The shoots that survived the selection treatment were rooted on a selective MS medium with 0.2 mg l^{-1} IAA or a kanamycin-free MS medium containing 0.5 mg l^{-1} IBA. Rooted plants were acclimatized in a greenhouse. Phenotypic variants were studied and compared with control seed-derived plants of identical genotype. (R_1 seeds from fertile plants were collected and stored for further evaluation.)

RESULTS

Kanamycin selection was survived by 18.3% of the shoots developing from the root sections transformed by LBA4404/pBI121, while no control shoots survived the selection treatment. Among the survivors were two rooted plants that, together with another two that failed to take root, belonged to the genotypes 2 and 4 showing altered morphological characters compared to normal control plants of the same genotypes. They also differed from the other shoots of other genotypes grown on kanamycin. Atypical plants of the genotype 4 (Fig. 1) had shorter internodes and elongated leaves evenly distributed along the stem. Atypical plants of

Fig. 1. Atypical plantlet of the genotype 4

the genotype 2 (Fig. 2) were short, with suppressed apical domination, shorter internodes at the stem top and broad and thick leaves. The morphological differences were so great that the plants' phenotype considerably deviated from normal shoots of bird's foot trefoil (Fig. 3).

Fig. 2. Atypical plantlet of the genotype 2

Two atypical rooted plants, i.e. 4a and 2b, were successfully acclimated in the greenhouse and transferred into experimental field to continue growth. Over the winter period, the plant 2b froze to death. The surviving plant 4a continued to grow

Fig. 3. Plantlet with normal fenotype

and develop until springtime (Figs. 4 and 5), showing the same altered morphological characters that had been previously observed during its growth *in vitro* compared to control plant. In conditions of natural growth, it was observed to have good tillering capacity, and stems with shortened internodes fairly abundant with leaves of dark green color. The plant was singled out as a specific form of bird's foot trefoil compared to the normal type. The plant's morphological characters

Fig. 4. Plants after 50 d of acclimation (A-atypical plant 4a, B-normal fenotype)

compared to normal control plants are shown in Table 1. The plant flowered in spatial isolation of an experimental field but the number of seeds at an early stage of organogenesis was low. The seeds were collected and made to germinate in laboratory. The seedlings were then planted into pots and grown in the greenhouse.

Fig. 5. Plant 4a in experimental field

Alterations of morphological characters found in the five filial plants were observed to be identical as those observed in the mother plant. Atypical genotypes have been cultivated for the past two years in the required distance to prevent cross pollination and multiplied by seeds.

Table 1. Morphological characters of atypical plants of the genotype 4

Character	Transformed plant	Control
Average stem length, cm	32	28
Number of stems	48	40
Longest stem length, cm	35	31
Longest stem number of nodes	17	13
Longest stem length of nodes, cm	2.0	2.4
Sepal length, cm	2.7	1.5
Sepal width, cm	0.82	0.96
Upper leaves length, cm	1.54	1.42
Upper leaves width, cm	0.54	0.60
Flowering date	22	22
Number of flowers	410	360
Color of flowers	Dark yellow	Yellow
Color of leaves	Dark green	Green
Habit	Horizontal	Upright
Stem shape	Round	Round

DISCUSSION

The main question the results presented here raise is what provokes the occurrence of atypical plants of bird's foot trefoil following *Agrobacterium* transformation and *in vitro* regeneration. The atypical plants 4a and 2b survived the kanamycin selection. As all other survivors of the selection treatment had normal phenotype and did not differ significantly from control plants, the occurrence of atypical plants cannot be attributed to kanamycin activity.

There are two other possible explanations of the phenomenon. Firstly, the transformation itself may have brought about specific characters in the transformants. Transformed plants with specific characters (T phenotype) different from the corresponding characters in control plants had already been reported for *A. rhizogenes*-transformed bird's foot trefoil plants (TEPFER and CASSE-DELBRAT, 1989). Studying the morphological characters of *L. corniculatus* plants transformed by *A. tumefaciens*, WEBB *et al*. (1996) had observed plants with abnormal flowers. Those plants had reduced petals, while stigma and anthers protruded from the corolla. The next year, the plants produced flowers of similar abnormal morphology.

Another explanation relates to somaclonal variation. A variation of morphological and agricultural characters of bird's foot trefoil regenerants, compared to corresponding characters in control seed-derived plants, had been observed earlier (DAMIANI *et al*., 1985; 1990; WEBB *et al*., 1986) and investigated in field conditions (PEZZOTTI *et al*., 1985). WEBB and WATSON (1991) studied regenerants of the bird's foot trefoil cv. Leo derived from three different types of explants, and identified some morphological variants showing morphological (or even cytological) difference from control seed-derived plants.

The occurrence of atypical variants is hardly favorable regarding clonal propagation, i.e. multiplication of specific genotypes, or production of transgenic plants with an improved character but needing to have all other characters of the cultivar with normal phenotype unchanged. The occurrence of phenotype variants, on the other hand, enables selection of genotypes with some superior characters that may, as sources of genetic variability or carriers of desirable traits, be included in breeding procedures. Transformed plants of altered phenotype may therefore be used in studies of the functioning and expression of transgenes and other genes in the genome of transformed plants.

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MORFOLOŠKE PROMENE KOD ATIPIČNIH BILJAKA ŽUTOG ZVEZDANA DOBIJENIH U TOKU GENETIČKE TRANSFORMACIJE SA *AGROBACTERIUM*

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Izvod

Atipične biljke žutog zvezdana (*Lotus corniculatus* L. kultivar Bokor) sa izmenjenim morfološkim karakteristikama koje su uslovile odstupanje od normalnog fenotipa dobijene su regeneracijom iz transformisanog tkiva. Ono je dobijeno genetičkom transformacijom odsečaka korenova klijanaca sa *Agrobacterium tumefaciens* vektorom LBA4404/pBI121 na medijumu koji je sadržao 0.2 mg l^{-1} BAP. Transformanti 2b i 4a su imali veoma izmenjen habitus uključujući skraćene internodije, izduženo lišće, pravilan raspored listova na stablu, zadebljale listove. Zapažena je nasledljivost ovako izmenjenih karakteristika u prvoj generaciji potomaka i razmatrana njihova genetička uslovljenost.

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