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Transformation of *Medicago* by *Agrobacterium* mediated gene transfer

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ABSTRACT

Shoot segments of Medicago varia genotype A2 were co-cultivated with Agrobacterium tumefaciens strain bo42 carrying pGA471, a plasmid coding for the kanamycin resistant determinant as transferable positive selection marker in plant cells (An et al., 1985). Resistant plants were regenerated at high frequency from green calli developed on inoculated stem cuttings under kanamycin selection. DNA–DNA hybridization analysis showed the presence of the structural gene of the kanamycin resistant determinant in total DNA isolated from several independent transformants. All data presented clearly demonstrate the transfer, stable maintenance and functional expression of the kanamycin resistance marker in Medicago varia cells which retain their morphogenic property.

Abbreviations: Km   = kanamycin
               KmR   = kanamycin resistant
               Cb   = carbenicillin
               2,4-D = 2,4 dichlorophenoxyacetic
                        acid
               BA   = 6-benzyladenine
               T-DNA = transferred DNA into plants

INTRODUCTION

Leguminous plants have been in the center of scientific interest for at least two main reasons. They are very important constituents of both human diet and animal feed, and as compared to other crop plants legumes are capable to supply their nitrogen demand from symbiotic associations with Rhizobia. The introduction of new genetic information into these plants would be of great impact on both crop yield improvement and the molecular understanding of symbiotic nitrogen fixation. Fortunately, recent scientific accomplishments of the natural gene transfer of Agrobacterium established the elaboration of the transformation system for alfalfa.

Virulent strains of Agrobacterium tumefaciens are capable to introduce a specific segment (T-DNA) of its tumor-inducing (Ti) plasmid into the cells of many dicotyledonous plants (DeCleene and DeLey, 1976; Chilton et al., 1977; Yadav et al., 1980; Nester et al., 1984) including Medicago species (Lariotti et al., 1984; M. Deak unpublished observations). The transferred T-DNA contains several genetic loci of which only the right border is required for integration into the plant genome (Lemmers et al., 1980; Thomashow et al., 1980; Zambryski et al., 1980; Leemans et al., 1982; Yadav et al., 1982; Ursic et al., 1983; Caplan et al., 1985). The other regions are dispensable in this respect and gives room for in vivo and in vitro genetic manipulations (Hernalsteens et al., 1980; Bevan et al., 1983). Thus, several structural modifications of the T-DNA led to the construction of versatile cloning vectors which can be used to transform different plant species (Bevan et al., 1983; Fraley et al., 1983; Herrera Estrella et al., 1983; Horsch et al., 1984).

In this paper we describe the transfer of the KmR determinant into alfalfa cells and the regeneration of whole plant from KmR tissues. The transformation procedure is based on the recently developed binary vector system of Agrobacterium (An et al., 1985) and the use of a Medicago genotype with high embryogenic potential.

MATERIALS AND METHODS

Plant material

Medicago varia genotype A2 exhibiting good plant regeneration capability in tissue cultures was kindly provided by Dr A. Atanassov, Tissue Culture Laboratory, Institute of Genetics, Bulgarian Academy of Sciences, Sofia, Bulgaria. Plants were propagated as sterile shoot cultures on hormone free UM medium (Uchimiya and Murashige, 1974). Young shoots of this material were used in transformation experiments.

Bacterial strains

Agrobacterium tumefaciens strain bo42 was kindly provided by Dr G. An, Institute of Biological Chemistry, Washington State University, Pullman, USA. This strain is A. tumefaciens A281 carrying plasmid pGA471 (An et al., 1985). Plasmid pGA471 contains a mini RK2 replicon, a transferable T-DNA carrying KmR determinant fused to the nopaline synthase promoter, ColE1 type origine of replication, lambda cohesive site and restriction endonuclease recognition sequences for cloning.

Media and Culture conditions

A. tumefaciens strain bo42 was grown at 28°C in LB medium containing tetracycline-HCl (SIGMA), 12.5 mg/l and kanamycin sulphate (SIGMA), 100 mg/l for selection of plasmid pGA471.

Callus tissues were induced on UM medium supplemented with 2,4-D (SIGMA), 0.5 mg/l , BA (SIGMA), 0.2 mg/l and Bacto Tryptone (DIFCO),250

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mg/1. For embryo induction a liquid B5N medium was used as modified B5 (Gamborg et al., 1968) supplemented with 3% sucrose, meso-inositol (SIGMA), 500 mg/1, adenine (SIGMA), 1 mg/1, glutathione (SIGMA), 10 mg/1, 2,4-D (SIGMA), 1 mg/1 and BA (SIGMA), 0.2 mg/1. All the other components of media were purchased from REANAL (BUDAPEST).

Media were solidified by adding 9 g/1 of Bacto Agar. All media were autoclaved at $121^{\circ}C$ for 20 min. Calli were grown in sterile petri dishes at $25^{\circ}C$ under a 16 hrs photoperiod of fluorescent light.

Stem segments of M. varia genotype A2 produced dark green calli with several embryos on solid UM medium. Plantlets developed after transferring embryos onto UM medium with BA, while whole plants were recovered on the same medium without hormone.

The KmR phenotype of the transformed plants were tested as follow: small segments of the root were cut and placed on UM medium containing 100 mg/1 Km. KmR plant material produced calli with green embryos in three weeks, while control, sensitive tissues died.

DNA isolation, restriction digestion and hybridization

Total DNA was isolated from 200-600 mg of plant material according to Dellaporta et al., (1983). HindIII and EcoRI restriction digestion was carried out according to the supplyers instructions (Bethesda Research Laboratories). Restriction fragments were separated in 0.7 % agarose gel. Isolated BglII-SmaI fragment of plasmid pSUP1011 (Simon, 1984) covering the structural gene of the KmR determinant of Tn5 (Jørgensen et al., 1979) was labelled to $4.8-11.0 \times 10^{8}$ dpm/ug specific activity according to Feinberg and Vogelstein (1983). Hybridization was carried out according to Purello and Balazs (1983) and Singh and Jones (1984). Autoradiography was done at $-70^{\circ}C$ under Kodak XR5 X-ray film with Kodak X-Omatic intensifying screen.

Neomycin phosphotransferase assay

The activity of the enzyme neomycin phosphotransferase was measured according to Schreier et al.,1985.

RESULTS

Transformation of Medicago varia by Agrobacterium

The co-cultivation method originally described by Marton et al. (1979) for protoplasts was adapted to inoculate stem cuttings of alfalfa with Agrobacterium. 0.5-1.0 cm long shoot segments of steril plants of Medicago varia genotype A2 were placed into an Erlenmeyer flask (100 ml in size) containing 40 ml of liquid UM medium and 1 ml of appropriately deluted overnight culture of A. tumefaciens strain bo42 (5×10^{7} cells/ml). The tissues were incubated on a rotary shaker (150 rpm/min) at $25^{\circ}C$ for three days. The stem segments were then washed three times with sterile destilled water and transferred onto solid UM medium containing Cb, 300 mg/1 and Km, 100 mg/1. After three weeks of culture in average 3-4 green growing spots appeared on each of the treated stem segments in the presence of Km. The high efficiency in production of responsive growing tissues was repeatedly reproducable in large number of inoculation experiments. As control, shoot segments were treated as above without bacterial inoculation. In these cases the initial proliferation of the tissues was stoped by Km and the development of green calli was never detected.

Embryogenesis and plant regeneration from KmR calli

After several transfer on UM medium supplemented with Cb, 300 mg/1 and Km, 100 mg/1 somatic embryos developed from the green growing calli with low frequency. To improve the efficiency of plant redifferentiation several conditions were tested for embryo induction. Among others the following protocol was found to be the most successful:

First, the primary green KmR tissues were multiplied on fresh solid UM medium supplemented with Cb, 300 mg/1 and Km, 100 mg/1 for three weeks. Tissues with the proper size were then transferred into 40 ml B5N medium containing Km, 50 mg/1 in 100 ml Erlenmeyer flasks and incubated on rotary shaker (150 rpm/min) at $25^{\circ}C$. The suspensions were subcultured twice a week in the same medium. In the KmR suspension cultures, after several weeks large number of green globular and torpedo embryos appeared in addition to the unorganized cell aggregates. The suspension culture maintained their embryo-producing capability for more then three months. These embryos were transferred onto solid UM medium containing BA, 0.2 mg/1 and Km, 100 mg/ where they developed further and after 3-6 weeks plants started to regenerate.

Four developing plantlets designated P1, P2 P3 and P4 were randomly selected and characterise further (see below). The photos of six regenerate plants are shown in Figure 1. The stability of th KmR phenotype was tested on the roots of th transformants as follows: root segments were place on UM medium containing Km, 100 mg/1 and they forme green calli with embryos while control root segment never did so.
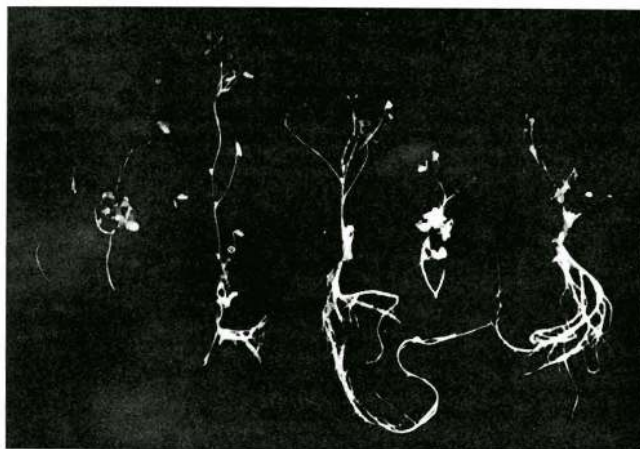

Figure 1. The picture of six randomly selected kanamycin resistant plants.

Detection of the KmR structural gene and its functional expression in the transformants

Three developing KmR plantlets P1, P2 and P3 (see above) and a KmS control plant were devided in two parts. One part was used to detect DNA homology to the structural gene of the KmR determinant while the other part was used to measure the activity of the neomycin phosphotransferase.

After total DNA isolation, Hind III digestion and agarose gel electrophoresis, DNA-DNA hybridization was performed using labelled probe specific to the structural gene of the KmR determinant (see Materials and methods). As seen on Figure 2. Panel B, hybridization signal could only be detected in the transformed plant material (lane

2 and 3). No hybridization could be observed with
λ from untreated calli (lane 4) and from whole
ant of M. varia, M. falcata, M. sativa, tobacco,
rrot and wheat (data not shown). The DNA of
antlet P1 contained multiple hybridization
agments from which one band is very intense.
antlet P2 had one hybridizing band. Though the
gestion of the DNA from plantlet P3 is partial the
bridization is obvious.

Similar experiments were done with eight
ndomly selected calli as well as from untreated
sensitive control material except that digestion
s with enzyme EcoR I. Specific hybridization was
tected to different EcoRI fragments of the DNA
om the eight KmR calli, while no hybridization was
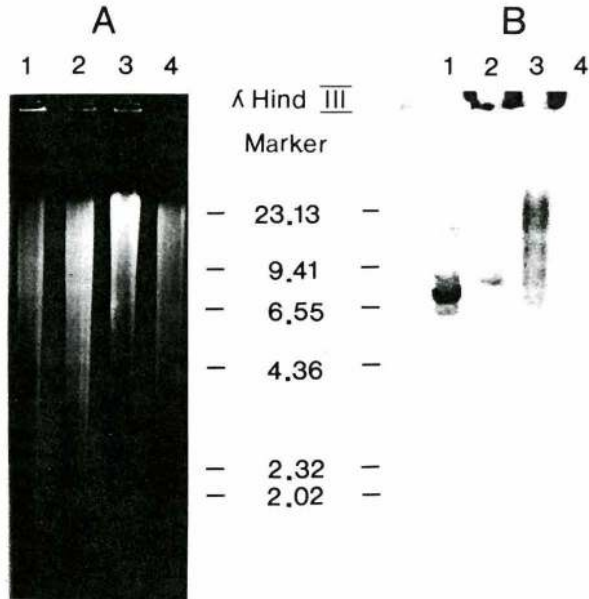und with DNA from Km sensitive callus (data not
own).

M. Deak (unpublished results) demonstrated that
transformed tissue of alfalfa could grow on hormone
free medium but no plant regeneration could be
achieved. In these experiments wild type Ti plasmids
were used where the integration of T-DNA resulted in
unbalanced phytohormone production (Akiyoshi et al.,
1983; Schroder et al., 1983; Nester et al., 1984).
As a consequence the highly morphogenic lines of
Medicago species also failed to produce plants from
hormone autotrophic tissues. Elegant series of
experiments showed that the phytohormone genes of
the T-DNA were dispensable for gene transfer, and
could be replaced by selectable markers (e.g. KmR
determinant) as well as other genes to be
transferred into plants (Herrera-Estrella et al.,
1983; Fraley et al., 1983; Herrera-Estrella et al.,
1984; Koncz et al., 1984). By creating "disarmed"
(hormone gene-deleted) T-DNA, the transferred
genetic material has no influence any longer on the
hormone metabolism of the recipient cell, therefore
it does not interfere with the morphogenic potential
(Bevan et al., 1983; Zambryski et al., 1983; De
Block et al., 1984).It has also been shown that the

Figure 2. HindIII restriction fragments of total
nt DNA was separated on 0.7 % agarose gel,
ined with ethidium bromide and photographed
nel A). The same gel was dried and hybridized
h a probe specific to the structural gene of the
determinant (Panel B). Autoradiography was done
-70 C for 9 days. Lane 1, 2 and 3 represents DNA
m three randomly selected regenerating plantlets
signated P1, P2 and P3 (see text). Lane 4 is DNA
m untransformed control tissue. HindIII digested
nbda DNA as indicated was used for molecular
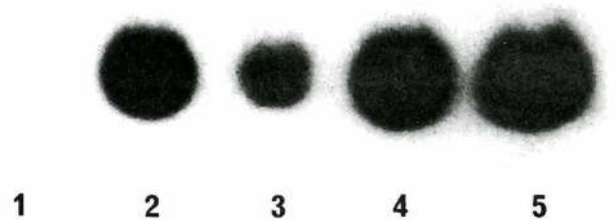ight marker (kb, kilobase pairs).

Figure 3. Detection of the neomycin
phosphotransferase activity of the transformants.
The signal on the autoradiogram shows the presence
of phosphorylated kanamycin as the consequence of
the activity of neomycin phosphotransferase from
extracts of untransformed plant (lane 1), plantlet P1
(lane 2), P2 (lane 4),P3 (lane 5),and P4 (lane 3).
The assay was carried out as described in Materials
and Methods.

The activity of the enzyme neomycin
sophotransferase of the KmR plantlets P1, P2 P3
P4 as well as of the KmS control plant was meas-
d as described in the Materials and Methods. As
n from Figure 2, the transformed material showed
ivity (lane 2, 3, 4, and 5). No activity at all
ld be detected in the control plant (lane 1).

DISCUSSION

In this paper we demonstrate the transfer of
e KmR determinant into Medicago varia cells by
robacterium, and that the transformed tissues
tained their morphogenic property and regenerated
KmR plants at high frequency.

It was shown earlier that A. tumefaciens causes
own gall tumors on Medicago species (De Cleene and
Ley, 1976). Recently Mariotti et al. (1984) and

vir genes (necessary for transfer and integration
process) can act in trans making possible the
development of binary vector system, i.e. vir genes
and T-DNA are on different replicon.This allows, T-
DNA being on a plasmid of small size, to carry out
in vitro manipulations more easy (Leemans et al.,
1982; Hoekema et al., 1983).

As shown in this paper a recently developed
binary vector system (An et al., 1985) fulfilled
these expectations and made possible to transform
alfalfa cells which retained their morphogenic
potential. The co-cultivation procedure outlined in
the text resulted in KmR calli which in turn could
be regenerated to KmR plants. There are several
indications that the KmR phenotype is the
consequence of the functional expression of the
transferred and stably maintained neomycin
phosphotransferase gene:

I. Only the co-cultivated shoot segments could
grow, form green embryos and regenerate plants in

the presence of Km. Untransformed control calli never did so.

II. Root segments of the transformed plants grown under non-selective conditions retained the KmR phenotype, produced calli and developed embryos in the presence of Km. In contrast, root segments from untransformed plant were killed under these conditions.

III. The presence of the structural gene of the KmR determinant could be demonstrated by DNA-DNA hybridization in all KmR transformants tested. No homology has been detected in Km sensitive, untransformed tissues or plants.

IV. The structural gene of the KmR determinant could be detected on different restriction fragmets of the transformed plant DNA which might indicate integration of the KmR determinant at different location of the plant genome.

V. All KmR embryos and plantlets tested including P1, P2, P3 and P4 revealed high level of neomycin phosphotransferase activity. No activity at all could be detected in untransformed plants .

In our transformation experiments shoot segments of Medicago varia were treated with Agrobacterium to introduce new genetic informations. Under these circumtances, a number of independent transformation events could occur on one stem segment and the growing KmR calli could be considered as a chimera. Since embryogenesis can be induced efficiently in liquid culture the KmR tissues are dissected into independent sub-calli. These sub-populations might still contain cells originated from independent transformation events. However the embryogenesis originates from the progeny of a single transformed cell, the regenerated plants should be homogenous. To get more information of the stability and location of the transformed DNA generative propagation cycle of the transformed plants is necessary. These experiments are recently in progress in our laboratory.

The Agrobacterium strain used in these transformation experiments contains a wild type T-DNA which can also be transferred. Plant cells transformed by this T-DNA are probably not able to regenerate because of the unbalanced hormone production (see An et al., 1985). Therefore morphogenesis is expected only in those cases where the disarmed T-DNA is transferred alone and the insertion do not destroy gene(s) necessary for regeneration.

The transformation system described here is simple, reliable, efficient and may have general application. In fact we have successfully transformed other species of Medicago, M. coerulea and M. sativa with the same efficiency. We hope the procedure described here can be applied as an alternative method in Medicago breeding programs for crop yield improvement or introducing new resistant markers (i.e. against herbicides or pesticides) and other beneficial traits. This technique may also accelerate the study of symbiotic nitrogen fixation genes coded in Medicago.

ACKNOWLEDGEMENT

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