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Genetic transformation in the grain legume *Cicer arietinum* L. (chickpea)

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ABSTRACT. In the grain legume Cicer arietinum L. (chickpea), the seed-derived embryo axes deprived of the apical meristem were able to regenerate adventitious shoots on Murashige and Skoog (1962) medium supplemented with kinetin. This protocol was suitable for Agrobacterium-mediated gene transfer by the co-cultivation technique. Chickpea transgenic plants showed neomycin phosphotransferase II and ß-glucuronidase activities and the presence in their genome of integrated bacterial DNA.

Abbreviations: 6-BAP, 6-benzyl-aminopurine; CaMV, cauliflower mosaic virus; GUS, ß-glucuronidase; IAA, indole-3-acetic acid; Kn, kanamycin; MU, methyl umbelliferone; NAA, naphthaleneacetic acid; NPTII, neomycin phosphotransferase II.

INTRODUCTION

Grain legumes represent one of the most valuable sources of proteins for human and animal nutrition. These species are largely cultivated in the Mediterranean basin, Middle East, Asia and South America and, to date, they have been qualitatively and quantitatively improved by conventional breeding. However, the lack of resistance to several pest diseases still remains the major cause of significant loss of edible product. Recent advances in genetic engineering have clearly demonstrated the possibility of incorporating foreign genes for desired agronomic traits while preserving the existing characteristic of improved genotypes. In this context, the standardization of protocols of in vitro plant regeneration and Agrobacterium-mediated gene transfer is fundamental to future genetic manipulation of these crops.

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Legumes have been shown to be susceptible to Agrobacterium tumefaciens transformation (Mariotti et al.1984; Owens and Cress 1985; Jensen et al.1986; Bercetche et al.1987; Hussey et al.1989). Nevertheless, so far, few reports have been concerned with transformation of grain legumes (Vigna aconitifolia, Eapen et al.1987; Glycine max, Hinchee et al.1988; Phaseolus, Mariotti et al.1989; Pisum sativum, De Kathen and Jacobsen 1990).

It is well known that for practical purposes Agrobacterium-mediated gene transfer requires efficient plant regeneration methods, which, in spite of a considerable progress in developing tissue culture protocols in a variety of plant species, are difficult to obtain in grain legumes.

In this paper we describe a plant regeneration procedure from embryo axes deprived of the apex which can be successfully utilized in gene transfer experiments in the grain legume Cicer arietinum L. (chickpea).

MATERIALS AND METHODS

Plant materials and bacterial strains

Seeds of chickpea (Cicer arietinum L.), local ecotype (supplied by Consorzio Agrario Provinciale, Rome, Italy), were used as the source of plant material. Agrobacterium tumefaciens strain LBA 4404 containing pBI 121 (Jefferson et al.1987) was used. pBI 121 is a derivative of pBin 19 (Bevan 1984) containing the neomycin phosphotransferase gene, under control of the nopaline synthase promoter and terminator, and the E.coli ß-glucuronidase gene fused to the CaMV 35S promoter and nopaline synthase terminator.

Plant regeneration procedure

Seeds of chickpea were sterilized by 50% v/v commercial bleach

(5% sodium hypochlorite, 3% available chlorine, final concentration) for 30 min, washed six times with sterile water and left in water to soak overnight. Seeds were split open and the embryo axes removed. These were deprived of the vegetative apex and cultured on the following agar-solidified (1% w/v) media: hormone-free MS (Murashige and Skoog 1962); MS with 1.0 mg/L kinetin; MS with 1.0 mg/L kinetin and 0.1 mg/L NAA; MS with 1.0 mg/L 6-BAP; MS with 1.0 mg/L 6-BAP and 0.1 mg/L NAA. The pH of the media was adjusted at 5.6 before autoclaving (120°C, 15 min). The plates were placed at 23°C under cool fluorescent light (3000 lux) on a 12 h photoperiod. After three weeks, the newly formed shoots emerging from the residual epicotyl of the embryos were explanted and induced to form roots on rooting medium (MS with 0.5 mg/L IAA, 0.05 mg/L kinetin and 1.0 % w/v sucrose).

Transformation procedure.

Bacteria were grown at 28°C on agar solidified (1% w/v) YMB medium (Hooykaas et al.1977) supplemented with 100 mg/L kanamycin. After 48 h bacteria were collected from the agar surface with a spatula, resuspended in hormone-free MS liquid medium and diluted to a concentration ranging from 1 to 5 x 10^8 cells/ml. Co-cultivation was carried out for 20 min at room temperature under gentle agitation (50 embryos/20 ml).
The explants were blotted on filter paper, cultured on agar solidified MS medium supplemented with kinetin (1.0 mg/L) and maintained under the previously described environmental conditions. After three days, the explants were transferred to plates containing the same medium supplemented with 300 mg/L carbenicillin (Geopen,Pfizer). Three weeks later, the shoots emerging from the residual epicotylary region were cultured on the above mentioned medium supplemented with 50 mg/L kanamycin. After three to four weeks the putative transformants were rooted in the rooting medium without kanamycin.

Enzyme assays

Before carrying out GUS and NPTII analysis, kanamycin resistant regenerants were tested for bacterial contamination by culturing small explants in YMB liquid medium at 28°C for three days. The neomycin phosphotransferase II (NPTII) assays were carried out following the simplified dot blot method described by McDonnel et al.(1987). ß-Glucuronidase (GUS) activity was determined by both the histochemical and fluorogenic assays according to Jefferson (1987).

DNA isolation and Southern hybridization

Genomic DNA was isolated using the method of Della Porta et al.(1983). The DNA samples were restricted with BamHI and EcoRI, separated in 0.8 w/v agarose gel, and blotted onto "Hybond" nylon membrane. A 2.1 kb BamHI-EcoRI fragment containing the GUS-Nos polyadenylation sequence was excised from pBI 221 for use as DNA probe. After hybridization with random primed 32-P labelled GUS gene fragment, the filter was washed and exposed to X-ray film at -80°C using an intensifying screen.

RESULTS

On MS medium without growth regulators the chickpea embryo axes deprived of the apex (Fig.1) developed only the root system and two lateral shoots, the latter deriving from axillary bud primordia on the cotyledonary node. Promotion of meristematic organogenesis from the remaining tissues of the epicotyl was obtained by supplying 1.0 mg/L kinetin (Fig.2). Other cytokinins, alone or in combination with low level of auxins (see Materials and Methods), failed to induce shoot formation. In general, one single shoot, occasionally more, derived from the tissue of the epicotyl. Taking into account the variability in size of the embryos, the regeneration capability was strongly related to the amount of the apex removed. The best results were obtained when 1.0 mm of the shoot apex was cut off, since larger sections removed inhibited regeneration from the remaining epicotyl tissues. In these conditions 40-50% of the manipulated embryo axes were able to produce adventitious shoots.
Regenerated shoots (Fig.3,4) were rooted in MS medium supplemented with 0.5 mg/L IAA, 0.05 mg/L kinetin and 1% w/v sucrose. On this medium, which proved to be the most efficient in promoting rhizogenesis, only 50% of the shoots rooted.
Prior to starting gene transfer experiments, chickpea embryos were tested in vitro for kanamycin sensitivity on MS medium supplemented with 1.0 mg/L kinetin. 50 mg/L of kanamycin totally inhibited embryo development. The same concentration was also effective in shoots explanted from in vitro grown plantlets. Lower concentrations of kanamycin gave unclear results with respect to the inhibition of plant growth.
Two to three weeks after the co-cultivation, the shoots which emerged from the remaining epicotylar region of embryo axes were explanted and submitted to kanamycin selection. The survival of these shoots was 10%. It is interesting to note that the lateral shoots derived from axillary bud primordia of the cotyledonary node did not survive on medium containing kanamycin. Moreover, experiments carried out by co-cultivating intact embryos or germinating seeds with the same bacterial strain did not produce kanamycin resistant plants (data not shown). Acetosyringone (50 µM), known to induce the VIR-genes of A. tumefaciens (Stachel et al.1985), did not improve the percentage of transgenic plants, nor cause transformation of intact seed-derived embryos or the above mentioned lateral shoots (data not shown).
Shoots surviving the kanamycin selection were induced to form roots in the rooting medium without kanamycin, since the selection agent (at 50 mg/L) strongly inhibited root morphogenesis. At the end of our procedure the percentage of transformation was

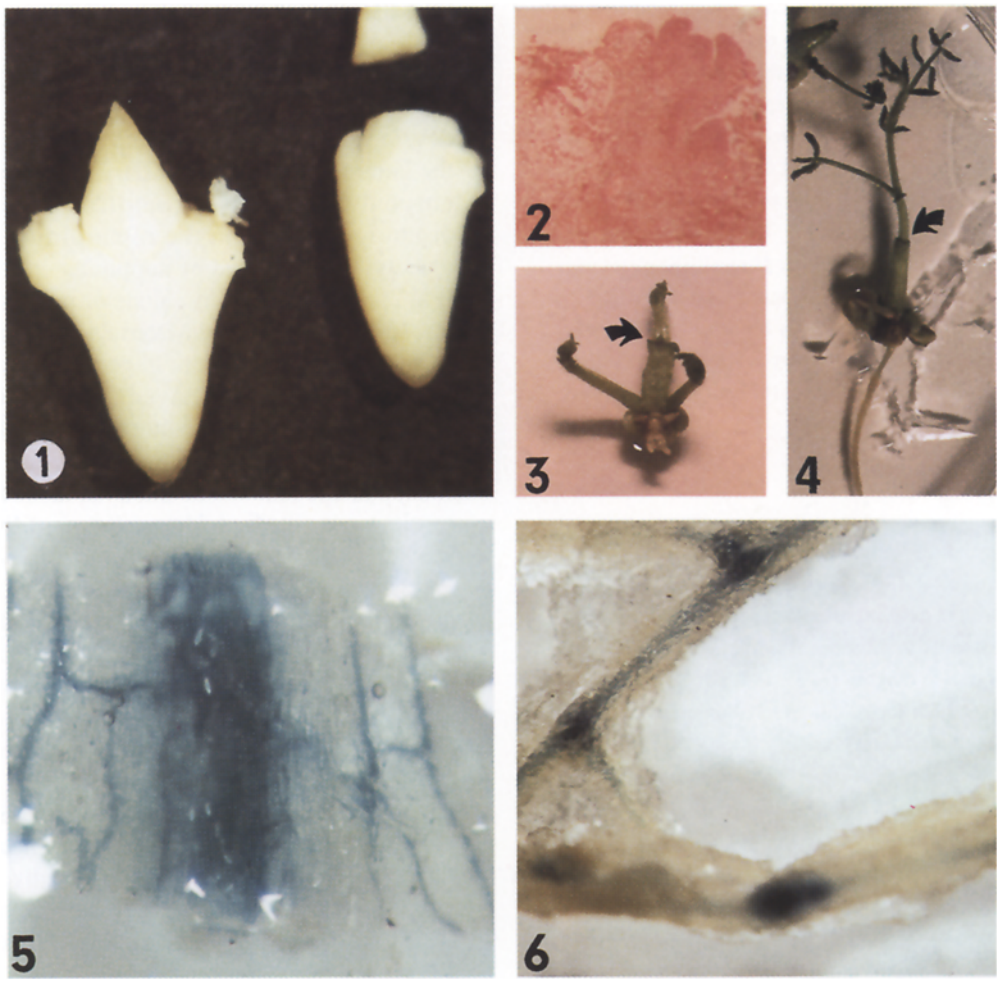

Fig.1-6: (1) Chickpea embryo axes deprived of the apical dome.(2) Longitudinal section of the residual epicotyl tissues showing shoot formation. (3,4) Well growing regenerated shoots; arrows indicate the site of shoot neoformation on the residual epicotyl.(5) GUS activity in transformed leaves and roots (6)

4% (number of whole plants transformed/initial number of embryos).

Histochemical analysis of GUS activity in axenic plants provided the first evidence of chickpea transformation. The highest activity was mainly localized in the meristematic regions of shoot apices. Both leaves and roots showed activity (Fig.5,6), but the number of roots expressing GUS was around 10%. The presence of GUS in the leaves was mainly confined to the veins, while roots showed GUS activity in the central cylinder with more intense staining where lateral roots branched. Fluorogenic assays carried out on tissue extracts from various organs of transgenic plantlets confirmed the histochemical analysis: roots had lower activity with respect to leaves and young shoots, the latter showing the highest level of GUS (Fig.7). NPTII assays proved the presence of enzymatic activity in all kanamycin resistant samples tested (Fig.8).

Southern blot analysis was performed on kanamycin-resistant, NPTII and GUS positive transgenic chickpea plants. A BamHI-EcoRI fragment from pBI 221 was used to probe both undigested and BamHI-EcoRI digested genomic DNA. Total genomic DNA of the putatively transformed T1 and T2 plants was blotted and hybridized with the above described probe: a characteristic 2.1 kb band GUS-Nos polyA sequence was shown to hybridize. A second Nos-polyA sequence was expected to hybridize and appears at a second location, due to the Nos-polyA site from NPTII gene in Bin 19. Assuming random insertion of pBI 121, the resulting hybridization pattern should indicate

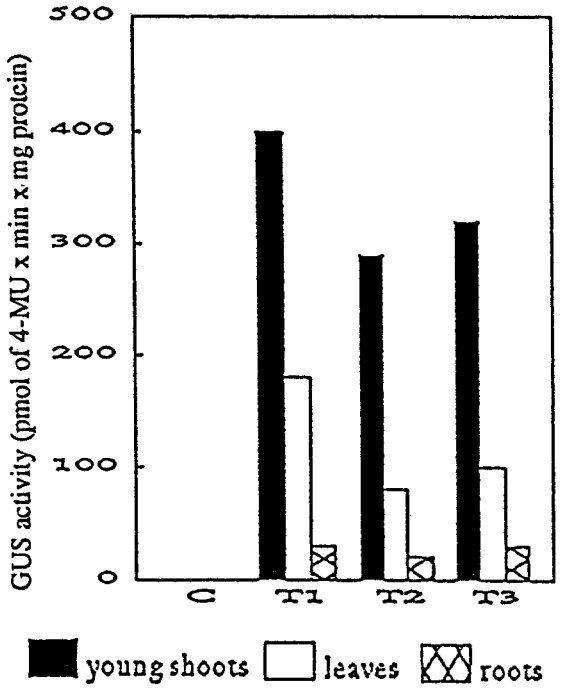

Fig.7: Fluorogenic GUS assay in various organs of transgenic chickpea plants (each bar is an average of 5 replicates). C.control; T1,T2,T3. transgenic plants.

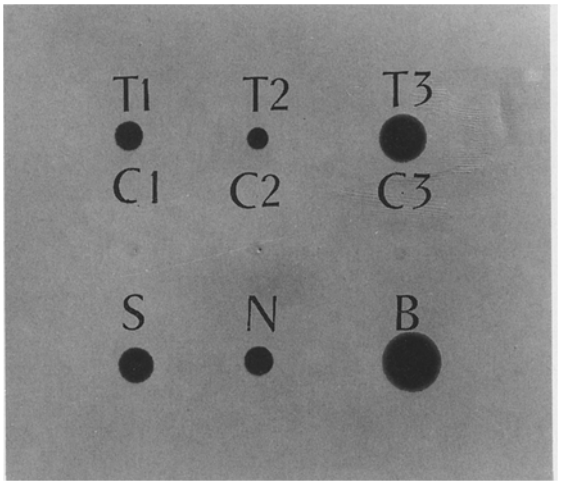

Fig.8: Dot blot analysis showing NPTII activity in transgenic chickpea shoots. T1,T2,T3. transgenic plants; C1,C2,C3. negative controls; S,N,B. positive transgenic potato, positive transgenic tobacco and positive bacterial control, respectively.

other bands which correspond to the number of NPTII genes (i.e. single or multiple copies) in the chickpea genome. The hybridization pattern in the plants T1 and T2 suggests a single pBI 121 insertion. Signals were not observed in the control (Fig.9).

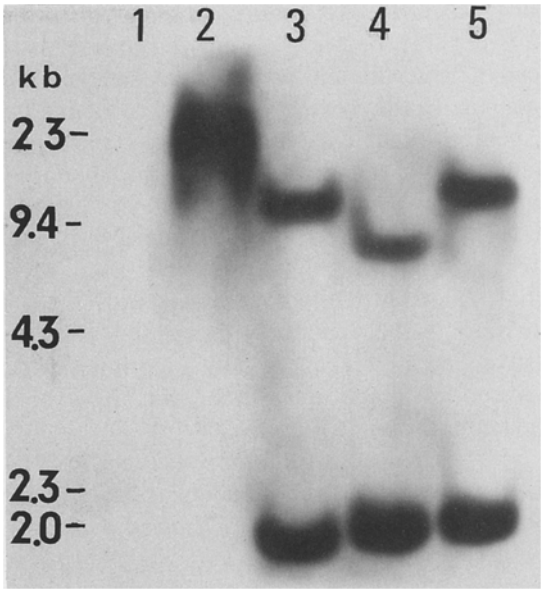

Fig.9: Southern blot analysis of DNA isolated from transformed plants of chickpea. Lane 1: control plant ; Lane 2: undigested total DNA from T1 plant. Lane 3:digested total DNA from T1 plant; Lane 4: digested total DNA from T2 plant; Lane 5 : digested plasmid pBI 121. The DNAs were restricted with BamHI and EcoRI and probed with the radioactively labeled 2.1 kb BamHI-EcoRI GUS fragment from pBI221.

DISCUSSION

The production of transgenic plants requires an efficient shoot regeneration system. The in vitro "plant-to-plant" process usually includes the induction of the callus from which morphogenesis must be achieved. As is well known, the length of the callus phase is negatively correlated with the regenerative ability; moreover, somaclonal variation can influence the phenotype of the regenerated plants. Currently, various approaches are under study to avoid the in vitro cell manipulation step (e.g. biolistic methods). In several species, the leaf disc transformation technique has shown a strong reduction of callus phase correlated to an improvement of plant regeneration. Chickpea embryo axes deprived of the apical dome satisfy this requirement since the cells of the wounded area are able to re-constitute the meristematic apex with no evident callus formation under cytokinin stimulus. This gives rise to the conditions (wound response, neoformed shoots) necessary for a successful application of the Agrobacterium-mediated transformation system.

In our procedure, it was particularly important to apply kanamycin selection to well grown rootless shoots instead of "regenerating" embryo axes, and the lack of kanamycin was also essential in root production from transgenic explanted shoots. The use of an effective kanamycin-selection timing and the absence of kanamycin in the root development phase have also been shown to be crucial in other species (e.g. apple, James et al.1989). The multicellular origin (transformed and untransformed cells) of the regenerating shoots and/or the initial low number of transformed cells unable to withstand the selection pressure, could account for the inhibition of the regeneration.

The NPTII activity and the presence of GUS DNA sequence proved the simultaneous transfer of both marker genes, even though they showed quantitative differences among the clones. These diversities could be ascribed either to the copy number of the inserted genes, or to "position effects" arising from the integration of the marker genes on different chromosomal locations.

Histochemical as well as fluorogenic assays showed distinct GUS activity patterns among the different tissues of transgenic plants. Although the CaMV 35S promoter is considered constitutive, its level of expression may change with respect to the cell cycle or the various tissues (Jefferson et al.1987; Nagata et al.1987; Terada and Shimamoto 1990).

Finally, similar results obtained in other agronomically important species with the same protocol (e.g. pea, D.Mariotti unpublished) are consistent with the possible extension of this approach to other grain legumes.

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