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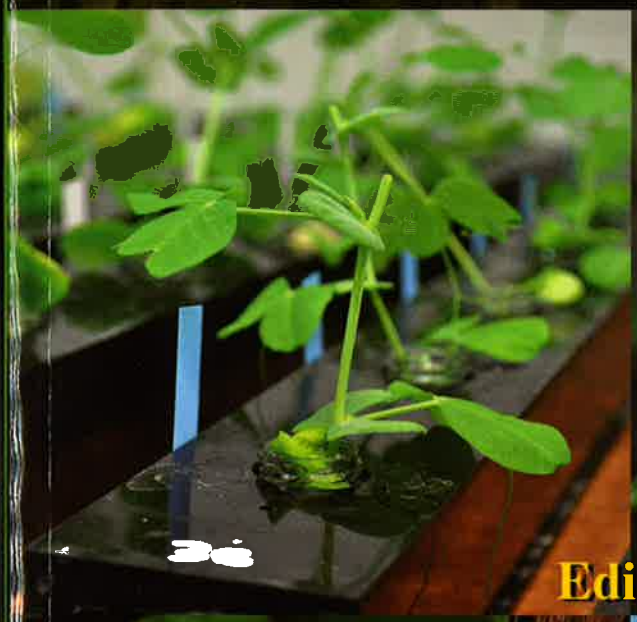

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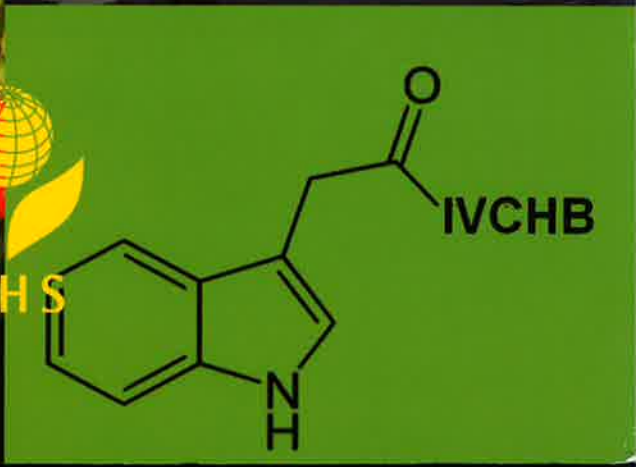

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An Improved Micro Propagation System for Successful Transformation of Cauliflower (*Brassica oleracea* var. *botrytis*)

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Keywords: cauliflower, micro propagation, *Agrobacterium tumefaciens*

Abstract

Cauliflower is described as a recalcitrant plant to the genetic transformation process from *Agrobacterium*. Cauliflower curd meristems were used in a fractionation and sieving protocol to produce microshoots in in vitro culture. This technique produced many thousands of small explants suitable for transfection studies. Explants were successfully transformed following standard transfection with *A. tumefaciens* carrying a construct with Kanamycin^R, GUS and the Ascorbate Peroxidase (APX) gene. Selection using Kanamycin produced normal plant phenotypic plantlets and these putative transformants were confirmed using GUS staining and PCR of the APX gene sequence. The transfection conditions were optimised during this study and 105 *Agrobacterium* cells/5 plants/pot successfully produced 1 transformed plant/pot.

INTRODUCTION

Cauliflower (*Brassica oleracea* var. *botrytis* L.) is known as one of the most responsive species for tissue culture but one of the most recalcitrant *Brassica* species for genetic transformation (Passelgue and Kelam, 1996; Puddephat et al., 1996). Therefore the pre-requisite of the present investigation was to optimize an efficient protocol for tissue culture and the main goal was to establish a transformation protocol to integrate the Ascorbate Peroxidase gene (APX) into cauliflower. The most common method used for the transformation of other related *B. oleracea* species and sub-species is *Agrobacterium* and both *A. tumefaciens* and *A. rhizogenes* have been used to transform a number of other *B. oleracea* crops (Puddephat, 1996). *A. tumefaciens*-mediated transformation of cauliflower has been attempted with a variety of explants such as hypocotyl, leaf, seed and seedling stem with limited results. Oncogenic *Agrobacterium* was used with leaf discs by Srivastava (1988) and de Block (1989) but their procedure could not be reproduced in other laboratories (Kazan, 1997; Metz, 1995). Eimert (1992) experimented with the use of *Agrobacterium* with leaf disc and protoplasts by electroporation, and direct DNA uptake however, irrespective of the method used, stable transformation at very low frequency was reported. *A. tumefaciens* was used to infect seedling explants (cotyledon and hypocotyl) using a modified procedure of de Block (1989) and the following comments were made: "The present protocol (Clough and Bent, 1998; modified from Bechtold et al., 1993) is extremely simple. We have found that the MS salts, hormone, etc. make no difference, that OD of bacteria doesn't make much of a difference, that vacuum doesn't even make much of a difference as long as you have a decent amount of surfactant present. Plant health is still a major factor – healthy fecund plants make a big difference".

The use of antibiotic in culture media has recently become more widespread with the emergence of antibiotic resistance genes as selectable markers in transformation experiments and in transformation systems. In addition, cocultivation of *Agrobacterium tumefaciens* requires the use of an antibiotic to kill the bacteria. The antibiotic Kanamycin, Gentamycin and tetracycline have been found to be inhibitory to cell or tissue growth at comparatively low concentrations. Fiolaetal (1990) indicated that the addition of 10 mg L^{-1} or higher Kanamycin sulphate to *Rubus* cotyledon regeneration medium drastically

reduced the growth and organogenesis of explants.

MATERIALS AND METHODS

Plant Material

The January heading 'Dionis' F_1 hybrid cauliflower was grown in the field at Plymouth University, Devon, UK, following good commercial practice (Anon., 1982). The curds were harvested and taken to the laboratory where in vitro micro-shoots were produced in liquid culture according to the method of Kieffer et al. (1995, 2001) and Rihan et al. (2011). This method produced a high volume of single or double meristem explants in the size range 300-600 µm.

Agrobacterium tumefaciens

Agrobacterium tumefaciens (*A.t.*) strain APX was kindly supplied by the USDA, and has been used to investigate the biology of *A.t.* vectored transformation in other species. The *A.t.* APX strain used pRTL2 and pCGN1578 as vectors. The T-DNA of PCGN1578 has three 35S promoter elements and the construct has a dual CaMV 35S promoter as well as a TEV leader and CaMV terminator. It also contained the β-glucronidase (GUS) reporter gene under the transcription control of *Cauliflower mosaic virus* 35S promoter. The *A.t.* APX strain was grown in LB media supplemented with Kanamycin and Gentamycin.

EXPERIMENT PROTOCOL

Experiment 1

Large pieces of curd (1-5 cm) were surface sterilized by immersing for 15 min in 10% un-thickened commercial bleach (sodium hypochlorite 0.06% active chlorine), followed by 3 washings in sterile distilled water. Following surface sterilization, explants were produced manually. The first step eliminated the mass of non-responsive tissue (stem branches) by shaving off the upper meristematic layer using a scalpel under sterile conditions in a laminar flow hood. The second step was a mechanical partial homogenization of the selected meristematic tissue using a commercial blender (Waring model 800) at approximately 17000 rev min^{-1} for 30 s followed by the use of precision sieves (600, 300, 212 µm aperture size) to rank the explants into the size classes 212-300 and 300-600 µm. The culture density was controlled by using a constant volume of explants per container containing 20 ml of culture medium. Eighty to 100 culture containers were used per experiment and incubated on a shaker (<50 revs min^{-1}). Culture media were derived from Murashige and Skoog (1962), according to Anderson and Carstens (1977) and supplemented with Kinetin (0.2 mg L^{-1}) and IBA (indol-3-butyric acid (0.1 mg L^{-1}) for the shoot development medium, and IBA only (0.2 mg L^{-1}) and agar (7 g L^{-1}) for the rooting medium, Kieffer et al. (1995). Culturing was carried out in the laboratory at 23°C with adjacent lighting provided by white fluorescent tubes with a photoperiod of 16 h. The addition of the antibiotic Plant Preservation Mixture (PPM) (1 ml L^{-1}) was routinely added to reduce contamination risk (Fuller and Pizzey, 2001). All media, sieves, instruments and the Waring blender were sterilized by autoclaving at 121°C for 15 min.

Experiment 2

For transformation experiments it is recommended that the bacterial cultures are in the log growth phase with an OD_{600}=0.6. The growth curve was obtained from cultures inoculated from actively growing liquid culture (1 ml culture in 100 ml LB media) supplemented with the appropriate combination of antibiotics. The presence of the APX gene construct in *A. t.s* was confirmed using PCR as follows:

1. Step 1. Bacterial colony template preparation; *A.t.* (strain APX) was grown in solid or liquid LB medium supplemented with 20 mg L^{-1} Kanamycin +6.0 mg L^{-1} Gentamycin.

The culture was incubated at 28°C in the dark for 24 to 48 hours.

2. Step 2. PCR Reaction; Plasmids were isolated using a Kit from sigma.com/oligos company. The primers 5'-CACGTCTTCAAAGCAAGTGG-3' (35SCaMV 5 frw), 5'-TT TCGGAACAATTAAGCACCAA-3' (APX 5) frw and 5'-AAGAGGGCGGAATACAG AGTCAGT-3' (APX 5 rev) were used for the PCR reaction. Each PCR reaction mixture was 50 µl PCR mixture, 2 µl DNA, 1 µl of each primer forward and 1 µl of reverse,1 µl DNTPS, 5 µl Taq buffer, 2 µl Taq and 38 µl deionized autoclaved water. Before starting the first PCR cycle, the thermo cycler (Gene Amp® PCR system 9700) was heated to 95°C for 30 min, and then the sample was heated to 94°C for 2 min. This was followed by 30 cycles of 55°C for 1 min, 75°C for 1 min. In the final cycle, the reaction period at 75°C was extended to 10 min. 1.5% agarose and 100 ml of 1x TAE buffer were thoroughly mixed and boiled in a microwave oven for 3-4 min, then 2.5 µl SYBR Safe DNA gel stain was used for detection.

Experiment 3

Firstly optimization of parameters enhancing transformation efficiency; the high number frequency of shoot regeneration from the curd micro propagation protocol appeared to be ideal for *Agrobacterium*-mediated gene transfer. Secondly selection for plant transformation; the explants co-incubated with *Agrobacterium* were washed for 30 s with 250 mg L^{-1} Cefotaxime to inhibit *Agrobacterium* growth, followed by three washes with sterilized water. After 5 days of growth in S23 medium with 250 mg L^{-1} Cefotaxime, transformation was assessed using the GUS assay. Explants were then transferred to S23 medium with 20 mg L^{-1} Kanamycin.

RESULTS

Effect of different blending durations on explant production; during explant development in agitated liquid medium, the majority of the good quality microshoots were floating at the surface of the culture medium, this is especially obvious when cultured at 22±1°C. For the two optimal size-classes the number of responding micro explants in culture for a constant volume of cultured micro explants decreased with blending duration (Kieffer, 1996). The optimal treatment duration to obtain the maximum of responding micro explants was 30 s for the two optimal size-classes (Fig. 1). The number of well-developed micro explants per container varied between 5-20±10 for the 212-300 µm and 40±20 for 300-600 µm size class. The standard culture medium (supplemented with Kinetin (0.2 mg L^{-1}) and IBA (0.1 mg L^{-1})) enabled regeneration of micro shoots which often displayed early signs of polarisation, root hair like structures close to the shoot apex.

Optimum Culture Conditions for *Agrobacterium tumefaciens* (APX)

The total mean of high concentration suspension CFU (colony forming unit/ml) was recorded for *Agrobacterium tumefaciens* strain (APX) growing on LB medium. Significant differences were recorded between the media at $P<0.001$. *A.t.* strain APX grew faster and gave a more homogeneous suspension in LB medium than YEB. The results showed that LB medium was more suitable for the growth of this strain. LB medium was used thereafter in all transformation experiments.

1. Determination of *Agrobacterium tumefaciens* Growth on LB Medium. The growth curve shows that, when quantifying bacteria growth via optical density, the maximum growth of *A.t.* APX strain (1.51) was obtained after 42 h. A classic sigmoid growth curve was obtained for APX strain and the log phase of growth (OD_{600}=0.6) was detected after 20 h. For transformation experiments it is preferable that the culture is still in the log phase.

2. Molecular Analysis of *Agrobacterium* Plasmid Using PCR. Since PCR amplification is very sensitive, it is imperative that sources of cross contaminating DNA, including extraneous microbes, be avoided. The PCR using the 35S5' foreword primer and APX3 reverse primer occasionally yielded 3 bands. The APX encoding sequences were detected

at 478 bp after PCR amplification.

The effect of high bacterial concentration was also reported by Orlikowska et al. (1995), Change et al. (2002) and Ismail et al. (2004). Diluted concentration (1:10 and 1:20 dilution) reduced necrosis to a great extent. In general the results confirmed that 1:10 (OD_{600}=0.6) bacterial density helped to improve transformation rate. These results agree with Srivestava et al. (1988) and Henzi et al. (2000). Whereas Chakrabarty et al. (2002) obtained maximum transformation efficiency with 1:20 dilution.

Healthy growing explants were transformed with the APX gene using a standard transfection protocol mediated by *Agrobacterium*. The explants that successfully grew under selection and presenting normal plant phenotypic features were considered putative transformants. The transfection condition optimised during this study successfully produced 1 transformed plant per culture vessel.

Confirmation of Transformation

1. Selection of Transgenic Plants. After co-cultivation the infected explants were washed for 30 seconds with 250 mg L^{-1} Cefotaxime to inhibit *Agrobacterium* growth, followed by three washes with sterilised distilled water and transferred to S23 medium with 250 mg L^{-1} Cefotaxime medium plus 25 mg L^{-1} Kanamycin with 2-4 weeks most of the untransformed shoots turned either pink or white and no further growth of shoot observed, while transformed shoots remained green on this medium and continued to grow (Fig. 2).

2. Histochemical and Fluorescence GUS Assay. Enzymatic analysis revealed GUS activity in explants used in this experiment. Samples taken from putative transformants showed GUS activity which indicated the presence of functional GUS enzymes in regenerated plants (Fig. 3).

3. DNA Analysis (PCR). Polymerase chain reaction was carried out (Fig. 4) to provide further evidence for the presence of the APX gene in the genomes of transformed plants. In addition, DNA extracted from the *A.t.* APX strain was prepared and used as a control. DNA from non-transformed cauliflower leaves was used as a negative control. PCR results confirmed that a fragment of approx. 478 bp appeared in transgenic plants and *A.t.* extracts whilst it was not present in non-transformed plants.

DISCUSSION

Different precision sieves and blending durations were used and the optimum conditions were 30 s blending and 300 µm sieving (Fig. 1). This produced a large amount of explants of uniform size suitable for transformation. Meristem destruction increased with increased blending duration, while less than 30 s did not disrupt the meristem clusters sufficiently.

Several factors are critical for successful production of transgenic cauliflower. One of these parameters is *Agrobacterium* density which plays an important role in the transformation process. The reduction in transformation rate caused by inoculation with high concentration of *Agrobacterium* (OD_{600}=0.6) appears to relate to an apparent hypersensitivity of explants to *Agrobacterium* eliciting a pathogenic response rather than a transformation event. Development of an efficient gene transfer system largely depends on a rapid and reliable in vitro regeneration system for the desired plant species and a competent transformation protocol. Even though in our lab a simple methodology allowing the production of ten thousands of micro shoots from curd meristems has been developed which would allow the necessary level of replication for transformation Kieffer et al. (1995) this technique was compromised largely because of bacterial overgrowth. In early experiments no meristems were able to survive the infection with *Agrobacterium*. Other workers have continued to favour using hypocotyls and cotyledons Chakraberty et al. (2002), Prem and Nicole (1998) and these were assessed in this investigation but found to be recalcitrant to transformation. This study showed that the most reliable results were obtained if small quantities of *Agrobacterium* cells from fresh bacterial colonies (one to two weeks after streaking) were used during transformation with microshoots produced

by fractionation and grading.

Putatively transgenic plants (carrying APX) were identified by growing on the selective medium, and these were assayed for the GUS reporter gene. The substrate for GUS detection, X-Gluc, worked well but the quality of the histochemical localisation was affected by numerous variables such as tissue preparation and fixation. The putatively transformed plants all showed transient GUS staining and this confirmed this to be an easy reliable method of establishing optimal conditions of tansformation (Chakry et al., 2002). All the GUS positive plants in histochemical assay showed the APX band in subsequent PCR analysis.

These methods are routinely used to evaluate the presence of insert sequences constructed into vectors harboured in individual *Agrobacterium* strains and transformed plants (Ping et al., 1997; Jones et al., 1987). Although Perm and Nicole (1998) and proved the case with cauliflower too. PCR is a fast and sensitive method but it is relatively expensive and susceptible to cross-contamination. On the other hand the GUS assay used in this study have advantages as it was simple, required minimal use of chemicals and plant tissues and did not cause permanent damage to the plant. The present study provides a reliable transformation system for integration of some genes into the cauliflower genome. This provides an opportunity to introduce genes of agronomic interest such as abiotic resistance.

CONCLUSION

The fractionation and grading of cauliflower curd meristem tissues gave a reliable method to produce many thousands of clones suitable for a transformation protocol with *Agrobacterium*. The results of transformation with APX gene constructs indicated that the transient GUS assay approach is easy reliable method of establishing optimal conditions of transformation. The elimination of *Agrobacterium tumefaciens* and shoot regeneration by using cefotaxime and carbencillin confirmed that 250 mg^{-1} was the best concentration to enhance the regeneration frequency and efficiency and at the same time suppress the *Agrobacterium* growth. Molecular analysis of *Agrobacterium* plasmids using PCR can be considered as a routine method to evaluate insert sequence constructed into vectors harboured in individual bacterial colonies and this method substantially reduces time and effort required to evaluate the authenticity of inserts in agrobacterium binary vectors.

ACKNOWLEDGEMENTS

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Literature Cited

Anon. 1987. MAFF report on crops and supplies of fruit and vegetables in England and wales-position. MAFF report 707:25.

Anderson, J.A.W. 1977. Tissue culture propagation of broccoli, *Brassica oleracea* (Italic Group), for use in F_1 hybrid seed production. Hortic. Sci. 102:67 73.

Bhalla, P. and de Weerd, N. 1999. In vitro propagation of cauliflower, *Brassica oleracea* var. *botrytis* for hybrid seed production. Plant Cell, Tissue and Organ Culture 56(2):89-95.

Bechtold, N., Ellis, J. and Pelletier, G. 1993. In planta *Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C.R. Acad. Sci. Paris Life Sci. 316:1194-1199.

Clough, S.J. and Bent, A.F. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. The Plant Journal 16(6):735-743.

Chakrabarty, R., Viswakarma, N. et al. 2002. *Agrobacterium*-mediated transformation of cauliflower: optimization of protocol and development of Bt-transgenic cauliflower. Journal of Biosciences 27(5):495-502.

Changhe, Y., Huang, S. et al. 2002. Factors affecting *Agrobacterium*-mediated

transformation and regeneration of sweet orange and citrange. Plant Cell, Tissue and Organ Culture 71(2):147-155.
De Block M,d.B.D. and Tenning, P. 1989. Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacterium tumefaciens* and the expression of bar and neo genes in transgenic plants. Plant Physiol. 91:694-701.
Eimert, K.S.F. 1992. Transformation of cauliflower (*Brassica oleracea* L. var. *botrytis*): an experimental survey. Plant Mol. Biol. 19:485-490.
Fiola, J.A., Hassan, M.A. et al. 1990. Effect of thidiazuron, light fluence rates and kanamycin on in vitro shoot organogenesis from excised *Rubus* cotyledons and leaves. Plant Cell, Tissue and Organ Culture 20(3):223-228.
M.P. Fuller and Pizzey, T. 2001. Teaching fast and reliable plant tissue culture using ppm and Brassicas. Acta Hort. 560:567-570.
Kazan, K.C.M., Goulter, K.C. and Manners, J.M. 1997. *Agrobacterium tumefaciens*-mediated transformation of double haploid canola (*Brassica napus*) lines. Plant Physiol. 24:97-102.
Kieffer, M., Fuller, M.P. et al. 1995. Rapid mass production of cauliflower propagules from fractionated and graded curd. Plant Science107(2):229-235.
Kieffer, M. 2001. A cost effective protocol for in vitro mass propagation of cauliflower. Plant Science 160(5):1015-1024.
Metz, T.D., Dixit, R. et al. 1995. *Agrobacterium tumefaciens*-mediated transformation of brocoli (*Brassica oleracea* var. italica) and cabbage (*B. oleracea* var. *capitata*). Plant Cell Reports 15(3):287-292.
Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum 15(3):473-497.
Orlikowska, T.K., Cranston, H.J. et al. 1995. Factors influencing *Agrobacterium tumefaciens*-mediated transformation and regeneration of the safflower cultivar 'Centennial'. Plant Cell, Tissue and Organ Culture 40(1):85-91.
G. Ismail, H.S. and Zogluer, K. 2004. *Agrobacterium* mediated transformation of *Larix decidua*; an assessment of factors influencing the efficiency of Gus gene transfer. Applied Botany 78(2):83-90.
Henzi, M.X., Christey, M.C. et al. 2000. Factors that influence *Agrobacterium* rhizogenes-mediated transformation of broccoli (*Brassica oleracea* L. var. *italica*). Plant Cell Reports 19(10):994-999.
Jones, J.D.G., Gilbert, D.E. et al. (1987). T-DNA structure and gene expression in petunia plants transformed by *Agrobacterium tumefaciens* C58 derivatives. Molecular and General Genetics MGG 207(2):478-485.
Passelègue, E. and Kerlan, C. 1996. Transformation of cauliflower (*Brassica oleracea* var. *botrytis*) by transfer of cauliflower mosaic virus genes through combined cocultivation with virulent and avirulent strains of *Agrobacterium*. Plant Science 113(1):79-89.
Ping, X., Dae-Jin, Y., Paul, M. and Ray, A. 1997. An expedient and reliable method to identify gene constructs in *Agrobacterium* vector. Plant Tissue Culture and Biotechnology 3:37-40.
Prem, L.N.S. 1998. *Agrobacterium tumefaciens*-mediated transformation of cauliflower, *Brassica oleracea* var. *botrytis*. Molecular Breeding 4:531-541.
Puddephat, I.J., Riggs, T.J. et al. 1996. Transformation of *Brassica oleracea* L.: a critical review. Molecular Breeding 2(3):185-210.
Rihan, H.Z., Al-Issawi, M., Burchett, S. and Fuller, M.P. 2011. Encapsulation of cauliflower (*Brassica oleracea* var. *botrytis*) microshoots as artificial seeds and their conversion and growth in commercial substrates. Plant Cell, Tissue and Organ Culture 107(2):243-250.
Smirnoff, N. and Colombe, S.V 1988. Drought influences the activity of the enzymes of the chloroplast hydrogen peroxide scavenging system. .J. Exp. Bot. 39(8):1097-1108.
Srivastava, V., Reddy, A.S. et al. 1988. Transformation and regeneration of *Brassica oleracea* mediated by an oncogenic *Agrobacterium tumefaciens*. Plant Cell Reports 7(7):504-507.

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Figures

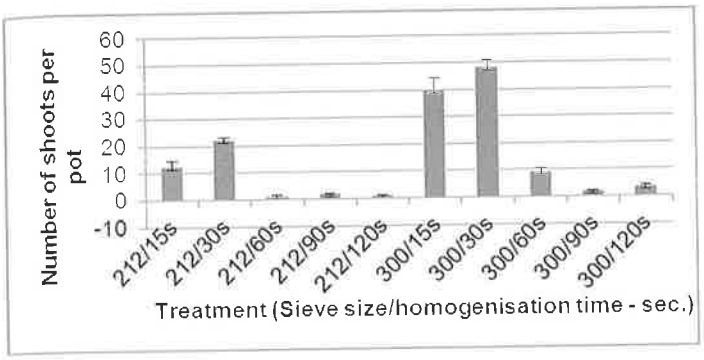

Fig. 1. The effect of different sieve sizes and duration of blending on number of explants produced.

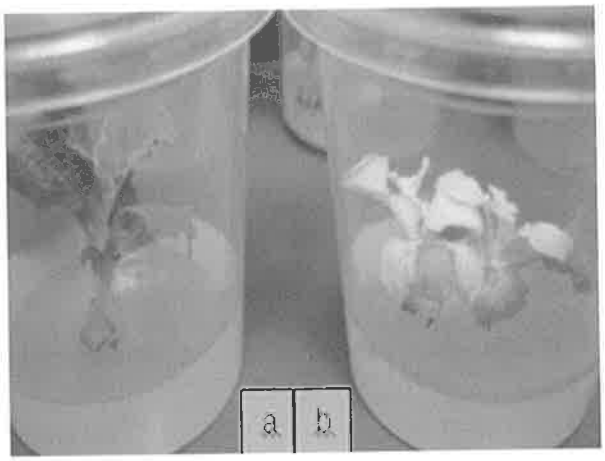

Fig. 2. Selection of transformed plants infected with APX strain: (a) transgenic plant; (b) non-transgenic plant.

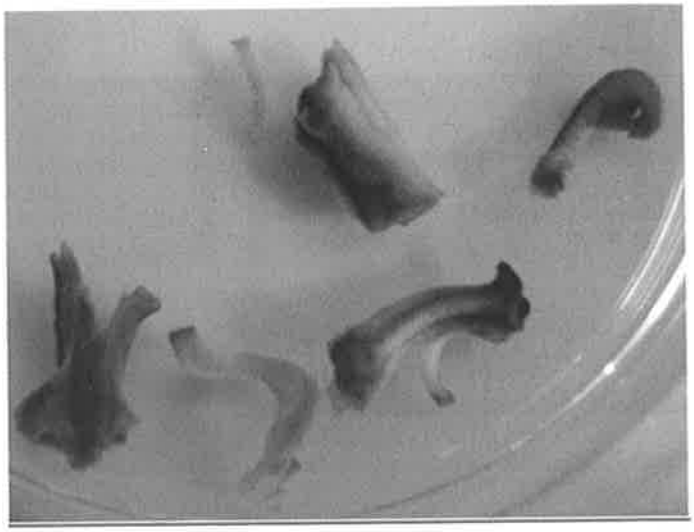

Fig. 3. Expression of GUS in cauliflower plants after transformation of cauliflower with *Agrobacterium*-carrying APX gene.

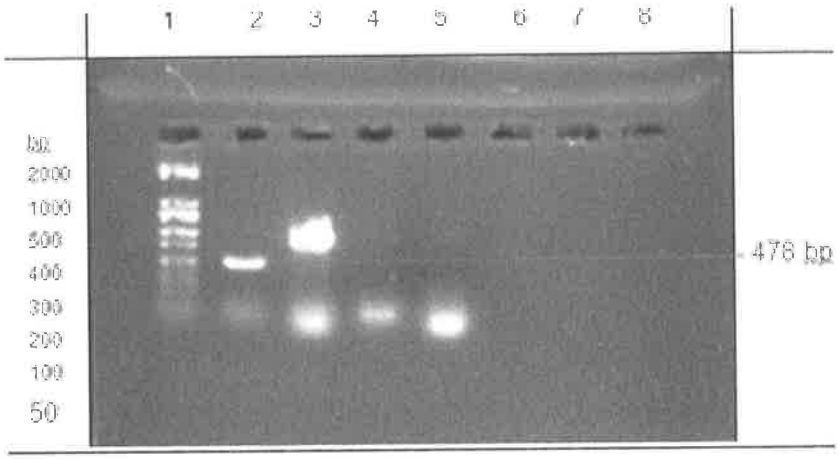

Fig. 4. Analysis of the presence of APX gene in putative transgenic plant. DNA molecular size marker (line 1), transformed plant carrying APX gene (lanes 2 and 3), negative control (non-transformed cauliflower leaves) (lanes 4 and 5) and water (lanes 6 and 7).