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STABLE GENETIC TRANSFORMATION OF GARLIC PLANTS USING PARTICLE BOMBARDMENT

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Abstract: The improvement of garlic plants (*Allium sativum* L.) via biotechnological approaches is currently limited by the lack of an applicable direct gene transfer system. In this paper, we present the development of a genetic transformation system using particle bombardment for gene delivery and immature clove-derived callus as the gene target. Plasmid DNA (pBI221.23), containing the selectable “*hpt*” gene for hygromycin resistance and the reporter “*gus*” gene, was delivered into callus tissue that had been previously treated with aurintricarboxylic acid as an endogenous nuclease inhibitor. The transformed calli were selected using hygromycin B, regenerated, and analysed at the molecular level using DNA hybridization, transgenome rescue and histochemical β -glucuronidase assay. The results indicated that biolistic transformation can lead to the transfer, expression and stable integration of a DNA fragment into garlic chromosomal DNA. The relative simplicity of this system is a good recommendation for its future use in the production of genetically modified garlic plants.

Key Words: *Allium sativum*, Direct Gene Transfer, Particle Bombardment, Transgenome Rescue, Transgenic Plant, GUS Reporter Gene, Southern Blot Analysis

INTRODUCTION

Garlic (*Allium sativum* L.) is an important and widely cultivated plant with both culinary and medicinal uses stemming from its biological activities, which include antibiotic, anticancer, antithrombotic and lipid-lowering cardiovascular

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effects [1, 2]. It is cultivated vegetatively, and its breeding has been limited to clonal selection of wild varieties, the production of virus-free stocks [3, 4, 5, 6, 7, 8, 9, 10] or spontaneous mutants, because this species does not form fertile flowers, *i.e.* it is sexually sterile.

Genetic transformation technology has proven to be a powerful tool for the production of plants with desired traits in many kinds of crops [11, 12]. It promises to overcome some of the substantial agronomic and environmental problems that have not yet been solved using conventional plant breeding programmes [13].

Garlic, and other plants of the same family, like onion (*A. cepa*) and leek (*A. porrum*), have proved to be recalcitrant to genetic transformation [14]. A number of hypotheses have been proposed to explain the difficulties encountered in the transformation of onions, including genome size and the fact that it is a monocot [15]. Although endogenous nuclease activity has only been considered as an important factor in the transformation of specific cell types like pollen [16] or microspores [17], Barandiaran *et al.* [18] detected a strong endogenous nuclease activity in all kinds of garlic tissue, and showed that it is possible to obtain transient expression of foreign DNA only after blocking DNase activity.

Recently, however, preliminary results were reported on the transformation of the Welsh onion by *Agrobacterium tumefaciens* [19, 20] or particle bombardment [21], while a successful garlic stable transformation was only reported using *A. tumefaciens* [22]. Although transformation using *A. tumefaciens* is a suitable method for the introduction of cloned genes into the garlic genome, only a few clones have been transformed, and a high proportion of the regenerated plants exhibited somaclonal variation. Moreover, this transformation system requires the construction of a specific plasmid vector. In this paper, we present the first report on the production of transgenic garlic using direct gene transfer.

MATERIALS & METHODS

Plant material

Greenhouse grown garlic plants (*Allium sativum* L. cv. Giza 3) were used to obtain immature clove explants for the transformation experiments. The plants were cultivated at 24 °C under a 16-h photoperiod provided by cool white fluorescent tubes producing approximately 2000 lux.

Callus induction

Cloves were surface sterilized in 70% ethanol for 2 min, 20% Clorox (5.25% NaOCl) for 20 min, then rinsed four times in sterilized distilled water. Different explants were placed onto MS medium containing basal MS salts, 0.7% agar, 3% sucrose and B5 vitamins and supplemented with 2 mg/l 2,4-dichloro-

phenoxyacetic acid (2,4-D) + 0.5 mg/l kinetin (kin) [23]. Culture media were adjusted to pH 5.8 before autoclaving for 20 min at 121 °C (1.2 kg/cm²).

Calli were immersed for 24 h in liquid Gamborg's B5 medium [24] supplemented with 0.2 M sorbitol, 0.2 M mannitol and aurintricarboxylic acid (ATA) (Claforan, Hoechst) as an endogenous nuclease inhibitor. To increase the penetration of ATA, the tissues were subjected to vacuum infiltration for 60 min in the same medium. Before bombardment, circles (2 cm²) of the treated garlic tissues were centred in a Petri dish containing 20 ml of the medium described above, solidified with 0.7% agar.

Particle bombardment process

Plasmid DNA (Fig. 1) was precipitated onto gold particles (Bio-Rad 1.6 µm) following a protocol modified from the original Bio-Rad procedure [18]. Bombardments were carried out using a PDS 1000/He microprojectile gun (Bio-Rad). Bombardment on garlic tissue was conducted at a distance of 3 cm (level 1) using a 1,155-psi rupture disk. Microcarrier travel distance was set by fixing the stopping screen support above both spacer rings and constant in all the experiments. Chamber vacuum was reduced to 0.006 atm (28 in Hg) before shooting.

Histochemical GUS assay

GUS activity was detected according to Kosugi *et al.* [25] using 5-bromo-4-chloro-3-indolylglucuronide (X-gluc) as a substrate. Tissues were cleared after staining by soaking in 100% methanol. Transient *GUS* expression was measured 24h after transformation by counting the *GUS*-positive spots appearing as blue zones (1mm or more in diameter) on a given callus after the staining procedure.

Regeneration and selection of transgenic lines

Calli inocula of 0.5 g fresh weight were transferred to regeneration media containing basal MS medium, 1 mg/l kin, and supplemented with 45 mg/l hygromycin B. The percentage of transformed calli regenerated plantlets was calculated after one month of cultivation.

Southern blot analysis

DNA was isolated from leaves according to the manual of the PhyloPure plant DNA extraction kit (Nucleon Biosciences, UK). 40 µg DNA from putative transformed and untransformed plants was digested with *HindIII*. Uncleaved and *HindIII*-cleaved DNAs of each line were separated through a 1% agarose gel and blotted onto Hybond N⁺ membrane (Amersham). A linearized plasmid pBI221.23 – supplied by Dr. M. Bevan (John Innes Center, Norwich, UK) – was used as a probe. Labelling, hybridization and detection were carried out by the AlkPhos Direct System for chemiluminescence (Amersham Pharmacia Biotech).

Transgenome rescue

Garlic genomic DNA from untransformed plants, transformed plants and plasmid DNA pBI221.23 were used to transform electro-competent *E. coli* DH10B cells with a Bio-Rad gene pulser [26]. The DNA treatments (*Hind III* or *BamHI* cut only or after religation) were used. The electroporated *E. coli* cells were plated on L-broth medium using a selection by ampicillin ($100 \mu\text{g}/\text{cm}^3$). The rescued transgenome was analysed by restriction analysis after digestion with *EcoRI* and *BamHI*.

RESULTS

The transformation treatments applied to garlic tissue were conducted using particle bombardment. Transient and stable transformations were evaluated by histochemical analysis of *gus* gene expression; gene rescue and Southern hybridization.

The garlic calli used in this study showed high levels of viability. Callus regeneration was vigorous, extensive root and shoot elongation occurred. These high levels of viability and vigour indicate that the tissue damage resulting from bombardment was not seriously detrimental to normal plant regeneration.

Transient transformation

Transient *gus* expression was measured 24 h after transformation. It manifested itself as small patches of blue coloration on the surface of the embryos, visible using a light microscope (Fig. 2). Our results show that the inhibition of nuclease activity in garlic tissue was a prerequisite for successful transient as well as stable expression of the *gus* gene. Conversely, direct treatment of microprojectiles or DNA with ATA was apparently not sufficient to prevent plasmid degradation after bombardment (Table 1).

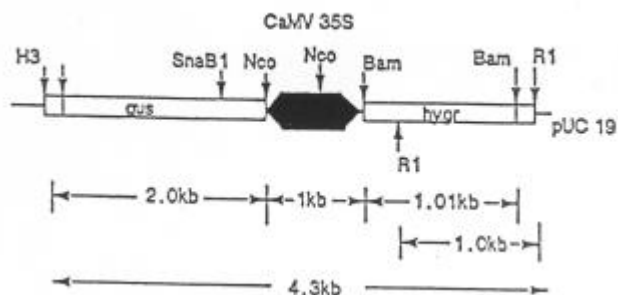


Fig. 1 Schematic representation of pBI221.23 plasmid

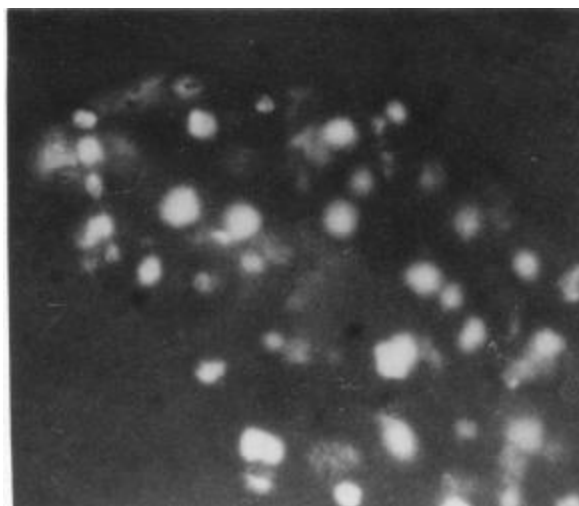


Fig. 2 Transgenic garlic tissue showing *gus* expression “blue spots”

Two types of *gus* expression were observed: mono- and multi-locations. Such multi-location expression may increase the possibility of a foreign gene being integrated into garlic chromosomal DNA.

Tab. 1. The effect of ATA on gene expression efficiency

ATA treatment	No. of colonies showing <i>gus</i> expression	Gene expression efficiency*
Control	0.0	0.0
Microprojectiles	19 ± 4	0.02
Plasmid DNA	7 ± 2	0.01
Callus tissue	151 ± 12	0.2

* Number of colonies showing *gus* expression per total number of colonies used.

Each value is an average of 3 independent transformation experiment ± S.E.

Stable transformation

The sensitivity of garlic tissue to hygromycin B was established prior to the transformation experiments in order to determine the effective concentration for selection. 45 µg/ml hygromycin B was found to be the optimal concentration for the selection of transgenic calli.

Immature clove-derived calli treated with ATA were bombarded with pBI221.23 plasmid DNA. The selection of transformants was initiated 3 days after transformation by adding hygromycin B to the culture media to a final concentration of 45 µg/ml. The regeneration of transgenic lines was recorded

after 4 weeks on the selective medium by counting the number of plantlets that survived the hygromycin selection. The control samples (bombarded without plasmid DNA, plasmid DNA without bombardment, neither plasmid DNA nor bombardment) did not show *gus* expression or grow under selective conditions. A summary of the results of 3 independent transformation experiments using particle bombardment is shown in Table 2.

Tab. 2. Summarized data on garlic transformation using particle bombardment.

Total colonies*	hyg ^r -colonies#	Regenerated transgenic lines	<i>gus</i> (+ve)	Southern (+ve)
910 ± 81	364 ± 66	254 ± 36	38 ± 7	8 ± 1

* The number of calli used per transformation.

The number of hygromycin B-resistant colonies obtained.

+ve: positive

Each value is an average of 3 independent transformation experiments ± S.E.

Table 2 shows a significant decrease in the number of hyg^r-colonies and *gus*-positive plants compared to the number of Southern-positive transgenic plants. This is because hyg^r-colonies did not mean that the cell line was transformed, *i.e.* false positive colonies might escape the selection pressure as a result of the density of cells and the nursing effect of transformants on untransformed cells. Thus, to exclude pseudotransformants, the calculation of the actual stable transformation frequency was based on the number of hyg^r-plants which tested positive for both the *gus* activity assay and the Southern blot analysis. The result was found to be 2×10^{-2} hyg^r-colonies. This is comparable to the relative transformation frequencies obtained by Zhu *et al.* [27] and Antonelli & Stadler [28].

Phenotypes of the transgenic plants

Plants regenerated from transgenic lines were similar to non-transformed control plants in that they were comparable in height and average thickness of the stem. In addition, the garlic bulbs produced were of comparable size and weight to those obtained by the usual clove cultivation method. Furthermore, there were no apparent abnormalities in the growth of the transgenic plants or in the resulting bulb morphology. However, several abnormal phenotypic characteristics were observed in some of the transgenic garlic plants, such as chlorophyll deficiencies (pale green & yellow green); necrotic leaves (necrotic leaf spots & lower leaf necrosis); and leaf morphology (wilted & adherent "epidermal cell fusion"). As morphological variations are expected to occur at a much lower frequency than cryptic variations (*e.g.* at the DNA level), the

absence of visible phenotypic somaclonal variations does not preclude the absence of all variation among tissue culture-regenerated transgenic plants [29].

Molecular confirmation of the transformation event

Plasmid rescue experiments indicated that the foreign transgenome can only be rescued in *E. coli* after *HindIII* or *BamHI* digestion followed by re-ligation (Table 3), *i.e.* the integrated plasmid molecule may be excised as linear fragments which would result in circular molecules that would be transformed at a much higher efficiency than linear molecules.

Tab. 3. Summarized data of transgenome rescue experiments

Genomic DNA treatments	Transgenome rescue		
	Control*	Untransformed garlic	Transformed garlic
Undigested	+	-	-
HindIII-digested	+	-	-
BamHI-digested	+	-	-
HindIII-digested + religation	+	-	+
BamHI-digested + religation	+	-	+

* Plasmid DNA was used in the transformations

- No transgenome rescue

+ Transgenome rescue occurs

Digested and undigested genomic DNA from transformed and untransformed garlic plants produced no *E. coli* transformants. This excludes the possibility that genomic DNA was contaminated with plasmid DNA. Although the restriction pattern of one of the rescued plasmids and pBI221.23 which had not been passed through garlic was identical (data not shown), the possibility that some re-arrangements and linkage between plasmid DNA and garlic genomic sequences had occurred and that these plasmids were not rescued cannot be discounted. This is because, in plasmid rescue experiments, only those DNA molecules which retained a competent origin of replication and a functional antibiotic (*amp^r*) gene through passage in garlic cells would have been recovered, so the possibility exists that DNA re-arrangements and garlic flanking sequences

render a substantial proportion of the plasmid DNA incapable of transforming *E. coli*.

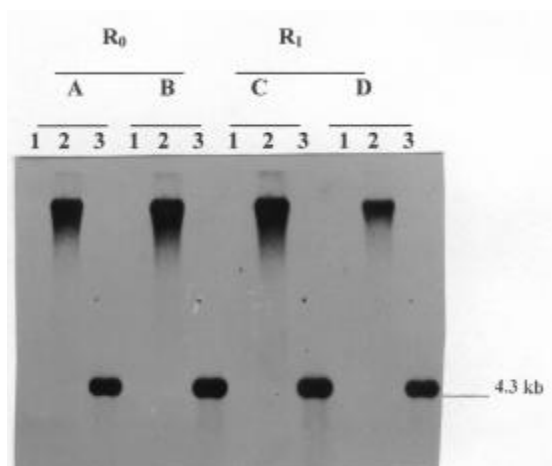


Fig. 3. Southern blot analysis of genomic DNA isolated from four independent transgenic lines of primary transformant plants R_0 and the next vegetatively obtained generation R_1 . Lanes (1): DNA isolated from untransformed controls. Lanes (2): Uncut DNA from transformed plants. Lanes (3): *HindIII*-digested DNA from transformed plants. A and B: Primary transformant plants R_0 . C and D: The next vegetatively obtained generation from the primary transformant plants R_1 .

To confirm the stable integration of the transgene into garlic genomic DNA and its transfer to the next generation, Southern blot analysis (Fig. 3) was carried out using genomic DNA isolated from four independent transgenic lines of the primary transformant plant R_0 and the next vegetatively obtained generation R_1 . These independent lines were hygromycin B-resistant and *gus*-positive. The linearized plasmid probe was hybridised to genomic DNA of all four plants, which had been digested with *HindIII*, and, for all four transgenic plants, bands of the expected size of about 4.3 kb were observed (Fig. 3, lanes 3). The probe was further hybridised to undigested DNA of all the transformants. Hybridisation only occurred with the uncut high molecular weight chromosomal plant DNA, and not with smaller pieces of DNA, indicating that the expressed transgene had integrated into the chromosomal DNA of the transgenic plants (Fig. 3, lanes 2). The profile of the genomic DNA digested with *HindIII* showed a single band in all the transgenic lines, suggesting that each transgenic line had a single integration site with one or more copies of the transgene. There was no hybridisation signal from the untransformed garlic DNA (Fig. 3, lanes 1).

DISCUSSION

This study enabled us to evaluate the potential use of particle bombardment as a tool for genetic transformation. There is no intrinsic feature of the particle transformation process that precludes stable integration and the expression of the foreign transgenome or its transfer to the next generation. Furthermore, the foreign transgenome can be rescued in *E. coli*, i.e. the mobility of the transgenome in and out of the host genome can be controlled.

Although traditional breeding programmes have produced steady improvements in garlic agronomical traits, the lack of useful economical character in commercial garlic cultivars still remains a major challenge. Despite the fact that commercial garlic entered the biotechnology arena rather recently [30, 31], advances in genetic transformation technology promise to meet this challenge by way of incorporating foreign genes for desired agronomic traits.

Although the percentage of *gus* expression is low, the procedure described here is simple and reproducible, and can serve as a means for the production of transgenic garlic with agronomically useful traits. Traditionally, garlic has been cultivated vegetatively because of its sexual sterility; consequently, viral diseases are a very serious problem. Almost all commercial garlic plants have been shown to be infected with a complex of viruses, such as leak yellow stripe virus, onion yellow dwarf virus, shallot latent virus and garlic common latent virus, as well as unclassified novel rod-shaped viruses [32, 33, 34, 35]. Although tissue culture is a useful technique for producing virus-free garlic seedlings [6, 7], the propagation rate of virus-free plantlets is very low, and the process is laborious and time-consuming. It is possible to use the genetic transformation system developed here for the production of transgenic garlic with the desired characteristics.

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