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GENETIC TRANSFORMATION AND HYBRIDIZATION

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Transformation and regeneration of garlic (*Allium sativum* L.) by *Agrobacterium*-mediated gene transfer

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Abstract By using highly regenerative calluses, we developed a stable transformation system in garlic (*Allium sativum* L.). The temperature and number of days of co-cultivation with *Agrobacterium tumefaciens* was shown to be an important factor in transient expression of the *uid* A gene. After a culture period of 5 months in selection medium containing hygromycin, 20 shoots were induced from ca. 1000 calluses, among which 15 plants expressed β-glucuronidase activity upon staining with X-Gluc. Shoots developed into transgenic garlic after 1 month. Integration of the *uid* A gene was confirmed by Southern blot analysis for genomic DNA of transgenic garlic plants.

Key words *Agrobacterium tumefaciens* · *Allium sativum* · Garlic · Genetic transformation · Transgenic plant

Abbreviations *BA*: 6-Benzylaminopurine · *CPM*: Callus propagation medium · *2,4-D*: 2,4-Dichlorophenoxyacetic acid · *GUS*: β-Glucuronidase · *LB*: Luria-Bertani medium · *LS*: Linsmaier and Skoog medium (1965) · *MS*: Murashige and Skoog medium (1962) · *NAA*: 1-Naphthaleneacetic acid · *PCR*: Polymerase chain reaction · *SE*: Standard error · *SIM*: Shoot inducing medium

Introduction

Garlic (*Allium sativum* L.) is an important and widely cultivated crop used both for food and for medicinal purposes because of its antithrombotic, lipid-lowering cardiovascular, and anticancer effects (Agarwal 1996). Garlic breeding has been limited to clonal selection of wild varieties or spontaneous mutants because this species does not form fertile flowers. Genetic transformation has proven to be a powerful tool for production of plants with desired traits in many kinds of crops. In monocotyledonous plants, many transgenic plants have been produced by *Agrobacterium*-mediated transformation following the successful transformation of rice with *Agrobacterium tumefaciens* by Hiei et al. (1994). In *Allium* species, Dommisse et al. (1990) reported that onion is a host for *Agrobacterium*; however, this species and other plants of the same family are recalcitrant to genetic transformation (Eady et al. 1996). Recently, preliminary results have been reported on the transformation of Welsh onion by *A. tumefaciens*(Dong et al. 1997; Ikeda et al. 1997) or particle bombardment (Peffley et al. 1995). In garlic, successful transformation has not been reported so far, although transient expression was shown with particle bombardment (Barandiaran et al. 1998). We investigated factors affecting the transformation of garlic by *A. tumefaciens* and produced stable transgenic plants expressing the *uid* A gene. To our knowledge, this is the first report on the production of transgenic garlic.

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Materials and methods

Plant materials

Shoot primordium-like tissues were initiated from apical meristems of garlic (*Allium sativum* L. cv. Fukuchi-howaito) in half-strength LS (Linsmaier and Skoog 1965)liquid medium supplemented with 0.02 mg/l NAA (1-naphthaleneacetic acid) and 0.2 mg/l BA (6-benzylaminopurine). Suspended cultures were incubated at 25 °C in a 12 h light/dark photoperiod with fluorescent illumination of 13.6 Wm^{-2}, shaken slowly at 2 cycles/min on a gyrated drum (1 m in diameter) placed obliquely, and were subcultured every 4 weeks. Calluses were induced from shoot primordium-like tissues on CPM from next page consisting of MS (Murashige and Skoog 1962) medium containing 1 mg/l

2,4-D (2,4-dichlorophenoxyacetic acid) and 0.75 % agar. These calluses were originally used for protoplast isolation and culture (Hasegawa et al. 1996). Calluses derived from protoplasts were maintained on CPM to former page and subcultured for propagation at intervals of 4 weeks. These calluses were used for transformation.

Bacterial strain

A. tumefaciens EHA101 (Hood et al. 1986) was used for transformations. EHA101 harbored plasmid pIG121Hm, carrying a kanamycin-resistant gene driven by the *nos* promoter, a hygromycin-resistant gene driven by the CaMV 35S promoter, and a *uid* A gene with an intron of a catalase gene from the castor bean (Ohta et al. 1990) under the CaMV 35S promoter. Bacteria were cultured in Luria-Bertani (LB) medium (Sambrook et al. 1989) containing 50 mg/l kanamycin and 50 mg/l hygromycin at 28 °C until turbidity reached 0.6 OD_{600}. Prior to inoculation, bacteria were collected by centrifugation and then resuspended in MS liquid medium supplemented with 1 % glucose and 100 µM acetosyringone.

Transformation of garlic

Calluses were submerged in the suspension of *A. tumefaciens* EHA101 for 5 min. After blotting away the excess bacterial suspension, calluses were transferred to the MS medium (pH 5.2) solidified with 0.5 % Gelrite containing 1 % glucose, 1 mg/l 2,4-D and 100 µM acetosyringone, and co-cultivated in darkness for 3 days at 22 °C. Calluses were then washed five times with sterile water containing 500 mg/l cefotaxime (Claforan, Hoechst), blotted to remove excess liquid, and cultured on CPM solidified with 0.5 % Gelrite containing 500 mg/l cefotaxime and 10–40 mg/l hygromycin for 2 months. The concentration of hygromycin was initially at 10 mg/l, then was increased every 2 weeks to 20, 30, and 40 mg/l. After the calluses had proliferated, they were transferred to SIM (shoot inducing medium) consisting of MS medium containing 2 mg/l BA, 0.02 mg/l NAA, 500 mg/l cefotaxime, and 20 mg/l hygromycin. Calluses were transferred to new medium every 2 weeks. After 4–5 months, emerging shoots were transferred to MS medium containing 200 mg/l cefotaxime and 20 mg/l hygromycin without plant growth regulators for rooting. Rooted plantlets were transferred to vermiculite, slowly acclimated to ambient humidity, and then transferred to soil. Transgenic plants were maintained at 22 °C under constant fluorescent illumination in 9-cm pots.

β-Glucuronidase staining

Staining for β-glucuronidase (GUS) activity was performed according to Kosugi et al. (1990) 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 immediately after co-cultivation by counting the GUS-positive calluses appearing as blue zones (1 mm or more in diameter) on a callus after the staining procedure.

Southern blot analysis

DNA was isolated from leaves according to the manual of the PhytoPure plant DNA extraction kit (Nucleon Biosciences, UK). DNA (40 µg) from putative transformants and un-transformed plants was digested with *Eco* RI. Uncleaved and *Eco* RI-cleaved DNAs of each line were separated through a 1 % agarose gel and blotted onto Hybond N^+ membrane (Amersham). A 1755-bp fragment of the *uid* A gene used as a probe was amplified by PCR using pIG121Hm with primers 5′-TGGGCATTCAGTCTGGATCG-3′ and 5′-TCATTGTTTG CCTCCCTGCT-3′. Labelling, hybridization, and detection were carried out by the AlkPhos Direct System for chemiluminescence (Amersham Pharmacia Biotech).

Results and discussion

Influence of co-cultivation periods for transformation of garlic calluses

We had previously developed a highly efficient regeneration procedure for garlic protoplasts from calluses of the apical meristem (Hasegawa et al. 1996). With this regeneration system, we obtained an average regeneration frequency of 70 %. In studying various factors of garlic transformation, we observed that the culture periods and temperature of co-cultivation were important factors. The periods of co-cultivation differed according to plant species in the literature. Longer periods of co-cultivation seem effective for efficient transfer of the Ti plasmid to plant cells. In citrange explants, the numbers of spots stained with GUS increased sharply from days 1 to 5 (Cervera et al. 1998). Cao et al. (1998) suggested that low efficiency of transformation in earlier work on apples (James and Dandekar 1991) was due to an insufficient length of co-cultivation, since a prolonged period of co-cultivation from 2 to 4 days increased GUS expression by almost 100-fold (De Bondt et al. 1994).

However, it was more difficult to eliminate *Agrobacterium* after longer periods of co-cultivation. Cervera et al. (1998) reported that the 5-day culture period resulted in overgrowth of *Agrobacterium* and a subsequent decrease in the regeneration frequency of transformed shoots, although 5-day co-cultivation was the most effective for increasing the frequency of transient GUS expression in citrange explants. Therefore, the period of co-cultivation should be optimized. We investigated the effect of varying the periods of co-cultivation with transient expression of the *uid* A gene. Co-cultivation for 3 days showed much higher GUS expression compared with 1- or 2-day co-cultivations (Fig. 1). However, transient GUS exprssion did not vary significantly over 3- to 6-day co-cultivation. *Agrobacterium* could be substantially eliminated from the co-culture medium of the 3-day co-cultivation in garlic calluses. Thus, we used a 3-day co-cultivation routinely.

Influence of temperature on the transformation of garlic calluses

Dillen et al. (1997) indicated that temperature plays an important role in transformation with *A. tumefaciens*. In their results, the best transformation efficiency was obtained at 22 °C in both *Phaseolus acutifolius* callus and tobacco leaves, irrespective of the type of helper plasmids. Although 25 °C is commonly

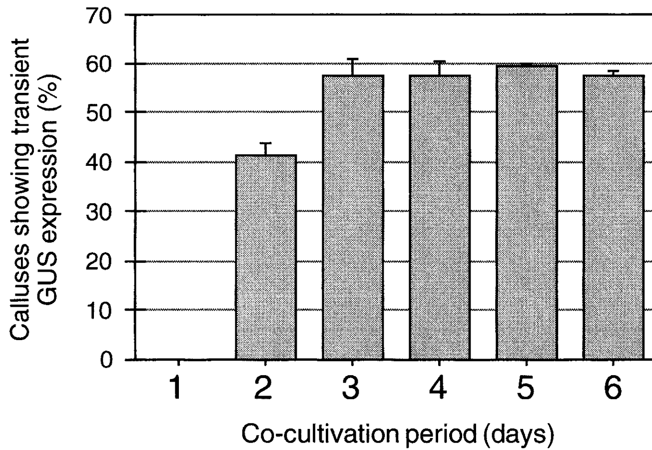

Fig. 1 Influence of co-cultivation period on transformation of garlic calluses. Transient GUS expression is represented as the percentage of GUS-positive calluses in 41–82 co-cultivated calluses. *Vertical bars* indicate the SE from two experiments

employed for in vitro co-cultivation experiments, they showed that a lower temperature (19–22 °C) is more optimal because *in planta* tumor formation occurs more frequently at 22 °C (Dillen et al. 1997). We investigated the effect of temperature in garlic transformation (Fig. 2). Among the temperatures tested ranging from 18 to 24 °C during co-cultivation, the highest transient GUS expression was observed at 22 °C, in which 64 % of total calluses showed GUS activity. The ratio of GUS-stained calluses to total calluses decreased by 85 % at 20 °C (54.2 % of total calluses) and by 69 % at 24 °C (44.2 % of total calluses) compared with that at 22 °C with EHA101. Suboptimal temperatures for transformation depend on the combination of *Agrobacterium* strains and plant materials. For example, when pEHA101 was used by Dillen et al. (1997), GUS activity was higher at 19 °C than at 25 °C, whereas the activity was higher at 25 °C than at 19 °C when pGV2260 and pMP90 were used. The results of our study, in which EHA101 was used as the *Agrobacterium* strain, showed that transient GUS expression was higher at 20 °C than at 24 °C; this is in good agreement with Dillen et al. (1997) using EHA101. Light and temperature are generally important factors affecting biological responses. Co-cultivation has been carried out in darkness for many kinds of plants. Ikeda et al. (1997) also co-cultivated Welsh onion with *Agrobacterium* in darkness at 22 °C. In pre-

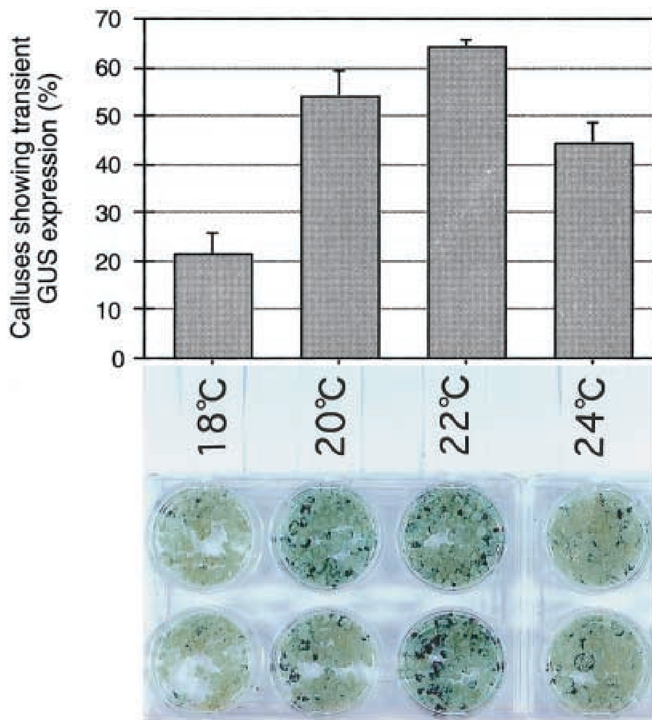

Fig. 2 Influence of co-cultivation temperature on transformation of garlic calluses. Transient GUS expression is represented as the percentage of GUS-positive calluses (*above*) in 80–96 co-cultivated calluses (*below*). *Vertical bars* indicate the SE from two experiments

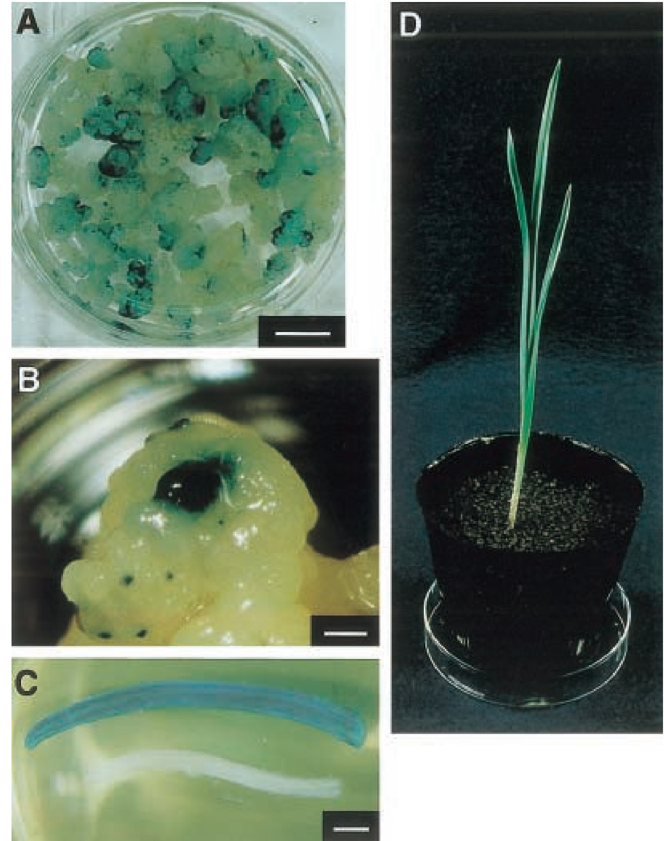

Fig. 3A–D Regeneration of transgenic garlic. **A** Histologically stained garlic calluses after 4 days of co-cultivation (*inner bar* represents 6 mm). **B** Strongly stained leaf-primordia candidate in garlic calluses after 2 months of culture (*inner bar* represents 1 mm). **C** Histological GUS staining with shoots of a transgenic plantlet (*above*, stained shoot) and non-stained shoot of non-transformed garlic (*below*, white shoot; *inner bar* represents 1 mm). Shoots were dissected from regenerating plants after 5 months of culture. **D** Transgenic garlic plant transferred to soil

liminary experiments, we compared light and dark conditions with respect to transformation efficiency. No significant differences between the cultures in light and dark conditions were observed for transformation efficiency in garlic (data not shown).

Regeneration of transgenic plants

When calluses were transferred to SIM directly after co-cultivation with EHA101, no transgenic plants were obtained. Therefore, calluses were transferred to CPM after co-cultivation with EHA101 and elimination of the excess bacteria. After 4 days of co-cultivation, many blue spots appeared in the calluses (Fig. 3 A). The hygromycin concentration was increased gradually following subculture. At first, calluses were cultured for 2 weeks in CPM containing 10 mg/l hygromycin, then 20 mg/l for 2 weeks in the second subculture. Hygromycin was increased to 30 mg/l and then to 40 mg/l for each of the successive 2-week subculture periods. During this selection with an increasing concentration of hygromycin, numerous calluses became brown or grey; however, several calluses developed and formed leaf-primordia candidates (Fig. 3B). Five months after co-cultivation in selection medium, 20 shoots appeared from ca. 1000 of the inoculated calluses and, among them, 15 shoots showed positive GUS staining. Fourteen of these were stained completely blue (Fig. 3C), while the staining of one shoot was weak. Transgenic shoots were rooted in the presence of 20 mg/l hygromycin and developed whole, transgenic plants (Fig. 3D) after acclimatization. Among shoots stained positively with GUS, a few shoots showed vitrification that resulted in a failure to root. All regenerated transgenic garlics appeared normal morphologically.

Detection of *uid* A gene by Southern blot analysis

pIG121Hm could not express the *uid* A gene in *Agrobacterium*(Ohta et al. 1990) because of the presence of a stop codon at the 3′ splicing site of a catalase intron connected with *uid* A gene, and *uid* A gene was expressed only in higher plants after splicing.

To exclude the possibility of the expression of *uid* A gene by residual *Agrobacterium* and to confirm the mode of integration of *uid* A gene in transgenic garlic, transgenic garlic was assayed by Southern blot analysis (Fig. 4). In two putative transgenic lines, the undigested genomic DNA was hybridized to the probe; this means that the *uid* A gene was integrated into the plant genome. The hybridization profiles resulting from genomic DNA digested with *Eco* RI provide an estimate of the number of integration sites, since the plasmid used for transformation contained a unique *Eco* RI restriction site, and therefore the second *Eco* RI restriction site must reside in the plant genome.

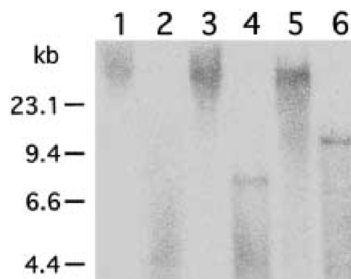

Fig. 4 Southern blot analysis of genomic DNA isolated from two GUS-positive transgenic garlic plants transformed with pIG121Hm. Un-transformed garlic DNA was represented in *lane 1* (undigested) and *lane 2* (digested with *Eco* RI). *Lanes 3–6* show undigested DNA (*lanes 3* and *5*) and DNA digested with *Eco* RI (*lanes 4* and *6*) in two transgenic garlics. *Hin*dIII-digested λ size markers are indicated

The profile of genomic DNA digested with *Eco* RI showed a single band in both transgenic lines, suggesting that each transgenic line had a single integration site. There was no hybridization signal from the untransformed garlic DNA.

The procedure described here is simple and reproducible, and can serve as a means for production of transgenic garlic with agronomically useful traits.

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