Genetic Transformation of Garlic (Allium sativum L.) by Particle Bombardment

Alejandra Robledo-Paz,1 José Luis Cabrera-Ponce,2 Víctor Manuel Villalobos-Árambula,3 Luis Herrera-Estrella,3 and Alba Estela Jofre-Garfias1

Departamento de Ingeniería Genética de Plantas, Centro de Investigación y de Estudios Avanzados del I.P.N. Km, 9.6 libramiento Norte Carretera Irapuato-León, Apdo. Postal 629, 36500 Irapuato, Gto, México

Additional index words. biotic, transgenic plants, somatic embryogenesis, genetic engineering, tissue culture

Abstract. Microprojectile bombardment was used to introduce DNA into embryogenic callus of garlic (Allium sativum L.) and produce stably transformed garlic plants. Embryogenic calluses, derived from garlic cultivar ’GT96-1’, were bombarded with plasmid DNA containing genes coding for hygromycin phosphotransferase and β-glucuronidase. Putatively transformed calluses were identified in the bombarded tissue after 4 months of selection on 20 mg·L–1 hygromycin B. The transgenic nature of the selected material was demonstrated by GUS histochemical assay and Southern blot hybridization analysis, and twenty transgenic plants were regenerated.

Genetic Transformation of Garlic (Allium sativum L.), family Alliaceae (Takhtahan, 1997), is a monocotyledonous crop used worldwide both as a condiment and for its medicinal properties. This crop is susceptible to numerous diseases caused by fungi, viruses, nematodes and insect pests. Garlic does not readily produce seeds, and is propagated vegetatively with a low multiplication rate. This mode of reproduction facilitates disease transfer (Novak, 1990) and prevents improvement of garlic by traditional breeding methods.

Both Agrobacterium and particle bombardment, have proven to be very useful techniques for the generation of transgenic plants of both dicotyledonous and monocotyledonous species (Bommireni et al., 1993; Bower and Birch, 1992; Cabrera-Ponce et al., 1995, 1997; Casas et al., 1993; Chee and Slightom, 1992; Christou, 1995; Eady, 2001; Eady et al., 2000; Fromm et al., 1993; Hiei et al., 1994; Ishida et al., 1996; Kihara et al., 1998; McCabe et al., 1988; Russell et al., 1993; Thomas et al., 1994; Vain et al., 1998). The use of genetic engineering techniques to obtain cultivars of garlic harbouring genes for pathogen resistance will surely have an impact on garlic production.

Received for publication 10 Feb. 2003. Accepted for publication 9 Dec. 2003. We want to thank Claudia Geraldine León Ramírez, Nélida Vázquez Sánchez and Rosa María Adame Alvarez for their technical assistance. This work was supported by Colegio de Postgraduados, Montecillo, México and The National Council for Science and Technology (CONACYT, México) by means of a Graduate Scholarship to ARP and a grant (28534N, awarded to AEJG).

1Former graduate student. Current address: Instituto de Recursos Genéticos y Productividad, Colegio de Postgraduados, Km. 35.5 Carretera México-Texcoco. Apdo. Postal 56230, Montecillo, Edo, México; e-mail aroblod@colpos.mx.

2Research assistant.

3Full professor.

4Associated professor. To whom correspondence should be addressed; ajofre@ira.cinvestav.mx.
for hybridizations with the hph gene. This enzyme liberates a 1.3-kbp fragment. Digested DNAs were separated on 1% agarose gels and transferred to nylon membranes (Hybond-N+, Amersham) using 2× SSC buffer (3 M NaCl and 0.3 M sodium citrate; pH 7.0). Membranes were prehybridized for 6 h at 65 °C in 2 m NaCl; 0.6 m NaH₂PO₄; 0.02 m EDTA•Na₂; 96 mg·mL⁻¹ dextran sulfate; 9.6 mg·mL⁻¹ sarkosyl; 0.52 mg·mL⁻¹ heparin; 1.9 mg·mL⁻¹ skimmed milk; 0.077 mg·mL⁻¹ sodium azide according to Sambrook et al. (1989). Hybridizations were carried out with a 1.6-kbp hph XbaI fragment from pWRG1515. This fragment was used in random priming reactions to generate the labeled probe (Feinberg and Vogelstein, 1983). Filters were washed in 2× SSC, 0.5% SDS at 65 °C for 1 h. Autoradiography was carried out using Kodak X-OMAT-AR film for 24 h at −70 °C.

Results and Discussion

Tissue culture of garlic. Garlic root-tip segments started to swell after 15 d of culture on induction medium B. Subsequently, cellular proliferation began and was limited to the cambial zone, resulting in the formation of distinct calluses by the end of the eighth week. Callus mass and size increased considerably upon transfer to medium C, and globular somatic embryos developed after 3 weeks. Embryos at the torpedo stage were easily discernible after 5 to 6 weeks of culture on medium C. These cultures were maintained in the same medium with a high capacity for the production of somatic embryos for at least 2 years. Embryos at the torpedo stage that were subcultured on medium B for 1 to 2 months proliferated by...
secondary embryogenesis. Only those embryos that grew from parts of the callus nearest to the culture medium achieved full development, while others stopped development at the globular stage. Mature somatic embryos developed complete plantlets 2 weeks after being transferred to medium D. These plantlets formed small bulbs (after 22 weeks from the start of the experiment) and were transferred to soil.

Selection of transgenic clones. Forty one percent of bombarded calluses expressed the gusA reporter gene after histochemical analysis (Fig. 1A). Barandiaran et al. (1998) reported that in order to get transient β-glucuronidase activity in garlic, it was necessary to include aurintricarboxylic acid to prevent degradation of exogenous DNA by nuclease activity. Under such conditions, they obtained transient expression in only 15% of bombarded calluses. Although they also reported stable transformation, it is not possible to compare these two transformation protocols with the results presented here, because the parameters of evaluation were different.

Regeneration of transgenic plants. Plant regeneration was achieved when embryogenic calluses were transferred to medium C and incubated at 24 °C under a 16-h photoperiod (50 µmol·m–2·s–1) for 7 weeks (Fig. 1D and 1E). Regenerated transgenic plants developed microbulbs (Fig. 1F) and were successfully established in soil conditions under a growth chamber environment (Fig. 1G). Bulbs were formed from these plants after 4 months. All regenerative transgenic plants showed leaves similar in shape and color to those of nontransformed plants.

DNA analysis. Five randomly chosen hygromycin B-resistant garlic clones were analyzed by Southern blot hybridization to confirm the presence of the hph gene in their genome. Four of the five clones gave strong signals for the expected 1.3-kbp EcoRI fragment of the pWRG1515 plasmid using the hph coding sequence as a probe (Fig. 2, lanes 5, 6, 8, and 9). Clone 3 (lane 7) was hygromycin B resistant and had β-glucuronidase activity, but showed a very weak hybridization signal. Nonbombarded calluses did not produce any signal (lane 4). Transgenic asparagus plants transformed with the plasmid pWRG1515 were used as a positive control (lanes 2 and 3). Comparison of DNA digested with EcoRI (lane 5) with undigested DNA (lane 10) indicated that the plasmid is integrated into the plant genome.

We propose, based on our results, that the key points for the establishment of a reliable protocol for genetic transformation of garlic are as follows:

1) The quality of the embryogenic calluses used. In this report, the calluses used were such that their rate of multiplication allowed them to increase their biomass three times, after 4 weeks in culture (data not shown). The importance of the quality of the tissues used as starting material has been also recognized by Birch (1997) and Myers and Simon (1998).

2) The methodology of bombardment used to achieve the genetic transformation. In a previous work Barandiaran et al. (1998), bombarded garlic calluses, but did not recover any transgenic clones. However, Myers and Simon (1998) as well as in this report showed success in regenerating transformed plants after using different protocols for genetic transformation than those reported by Barandiaran et al. (1998).

Myers and Simon (1998) report that only two out of five putatively transgenic lines subjected to molecular analysis (40%), showed integration of the transgenes (gusA and nptII). In contrast, in the present work we found that four of the five clones analyzed (80%), integrated in the genome contained the hph gene. Likewise, Kondo et al. (2000) reported the stable transformation and regeneration of garlic plants mediated by Agrobacterium tumefaciens but the efficiency was as low as fifteen transgenic plants from one thousand co-cultured calluses.

Using the methodology reported here we consider that the stable genetic transformation of garlic could be a viable technique for use in breeding programs of this crop.

Literature Cited

Barandiaran, X., A. Di Pietro, and J. Martin. 1998. Biolistic transfer and expression of a uidA reporter gene in different tissue of Allium sativum L. Plant Cell. Rpt. 17:737–741.

Birch, R.G. 1997. Plant transformation: problems and strategies for practical application. Annu. Rev. Plant Physiol. Plant. Mol. Biol. 48:297–326.

Birnboim, H.C. and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7:1513.

Bommireni, V.R., R.N. Chibbhar, R.S.S. Datla, and E.W.T. Tsang. 1993. Transformation of white spruce (Picea glauca) somatic embryos by microprojectile bombardment. Plant Cell Rep. 13:17–23.

Bower, R. and R.G. Birch. 1992. Transgenic sugarcane plants via microprojectile bombardment. Plant J. 2:409–416.

Cabrera-Ponce, J.L., L. López, N. Assad-García, C. Medina-Arevalo, A.M. Bailey, and L. Herrera-Estrella. 1997. An efficient particle bombardment system for the genetic transformation of asparagus (Asparagus officinalis L.). Plant Cell Rpt. 16:255–260.

Cabrera-Ponce, J.L., A. Vegas-García, and L. Herrera-Estrella. 1995. Herbicide resistant transgenic papaya plants produced by an efficient particle bombardment transformation method. Plant Cell Rpt. 15:1–7.

Casas, M.A., A.K. Kononowicz, U.B. Zehr, D.T. Thomas, J.D. Axtell, L.G. Butter, R.A. Bressan, and P.M. Hasegawa. 1993. Transgenic sorghum...
plants via microprojectile bombardment. Proc. Natl. Acad. Sci. USA 90:11212–11216.
Chee, P.P. and J.L. Slightom. 1992. Transformation of cucumber tissues by microprojectile bombardment: Identification of plants containing functional and nonfunctional transferred genes. Gene 118:255–260.
Christou, P. 1995. Strategies for variety-independent genetic transformation of important cereals, legumes and woody species utilizing particle bombardment. Euphytica 85:13–27.
Christou, P., T.L. Ford, and M. Kofron. 1991. Production of transgenic rice (Oryza sativa L.) plants from agronomically important and japonica varieties via electric discharge particle acceleration of exogenous DNA into immature zygotic embryos. Bio/Technology 9:957–962.
Chu C.C., C.C. Wang, S.C. Sun, C. Hsü, K.C. Yin, C.Y. Chu, and F.Y. Bi. 1975 Establishment of an efficient medium for anther culture of rice through comparative experiments on the nitrogen sources. Sci. Sin. 18:659–688.
Eady, C.C. 2001. Allium transformation, p. 655–671. In: R. Learmonth, and G.G. Khachatourians (eds.). The handbook of transgenic plant. Marcel Deck
er, New York.
Eady, C.C., C.E. Lister, Y. Suo, and D. Schaper. 1996. Transient expression of uidA constructs in vitro onion (Allium cepa L.) cultures following particle bombardment and Agrobacterium-mediated DNA delivery. Plant Cell Rpt. 15:958–962.
Eady, C.C., R.J. Weld, and C.E. Lister. 2000. Agro-
bacterium tumefaciens-mediated transformation and transgenic plant regeneration of onion (Allium cepa L.). Plant Cell Rpt. 19:376–381.
Eriksson, T. 1965. Studies on the growth requirements and growth measurements of cell cultures of Hap-
lopappus gracilis. Physiol. Plant. 18:976–993.
Feinberg A.P. and B. Vogelstein. 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Ann. Biochem. 132:6–13.
Fromm, M., F. Morrish, C. Armstrong, R. Williams, J. Thomas, and T. Klein. 1990. Inheritance and expression of chimeric genes in the progeny of transgenic maize plants. Bio/Technology 8:833–839.
Hiei, Y., S. Ohta, T. Komari, and T. Kumashiro. 1994. Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium tumefaciens and sequence analysis of the boundaries of the T-DNA. Plant J. 6:271–282.
Ishida, Y., H. Saito, S. Ohta, Y. Hiei, T. Komari, T. Kumashiro. 1996. High efficiency transfor-
mation of maize (Zea mays L.) mediated by Agrobacterium tumefaciens. Nature Biotechnol. 14:745–750.
Kihara, M., K. Saeki, and K. Ito. 1998. Rapid production of fertile transgenic barley (Hordeum vulgare L.) by direct gene transfer to primary callus-de-
derived protoplasts. Plant Cell Rpt. 17:937–940.
Kondo, T., H. Hasegawa, and M. Suzuki. 2000. Transformation and regeneration of garlic (Allium sativum L.) by Agrobacterium tumefaciens gene transfer. Plant Cell Rpt. 19:989–993.
McCabe, D.E., B.J. Martinelli, and P. Christou. 1988. Stable transformation of soybean (Glycine max) by particle acceleration Bio/technology 6:923–926.
Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473–497.
Myers, J.M. and Simon, P.W. 1998. Microprojectile bombardment of garlic (Allium sativum L.). p. 121–126. Proc. 1998 Natl. Onion (and other Allium) Res. Conf. Sacramento, Calif., 10–12 Dec.
Novak, F.J. 1990. Allium tissue culture, p. 233–250. In: H.B. Rabinowitch and J.L. Brewster (eds.). Onions and allied crops. vol. 1. CRC Press, Boca Raton, Fla.
Russell, D.R., K.M. Wallace, J.H. Bathe, B.J. Marti-
nell, and D.E. McCabe. 1993. Stable transformation of Phaseolus vulgaris via electric-discharge mediated particle acceleration. Plant Cell Rpt. 12:165–169.
Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
Sanford, J.C., M.J. Devit, J.A. Russell, F.D. Smith, P.R. Harpending, M.K. Roy, and S.A. Johnston. 1991. An improved, helium-driven biolistic device. Technique 3:3–16.
Shure, M.S., S. Wessler, and N. Fedoroff. 1983. Molecular identification and isolation of waxy locus in maize. Cell 35:225–233.
Takhtajan, A. 1997. Diversity and classification of flowering plants, p. 49–69. Columbia Univ. Press, Cambridge.
Thomas, J.C., D. Adams, C. Nessler, H.J. Bohnert, and J.K. Brown. 1994. Reduced reproduction of whitefly (Bemisia tabaci) on transgenic tobacco expressing tryptophan decarboxylase. Proc. 4th Intl. Congr. Plant Mol. Biol., Amsterdam, 19–24 June. Intl. Soc. Plant Mol. Biol. Abstr.1924.
Tomes, D.T., M.C. Ross, and D.D. Songstad. 1995. Direct DNA transfer into intact plant cells via microprojectile bombardment, p. 197–213. In: O.L. Gamborg and G.C. Phillips (eds.). Plant cell, tissue and organ culture: Fundamental methods. Springer Lab Manual, Springer-Verlag, Berlin.
Vain, P., B. Worland, A. Kohli, J.W. Snape, and P. Christou. 1998. The green fluorescent protein (GFP) as vital screenable marker in rice transfor-
mation. Theor. Appl. Genet. 96:164–169.