Korean Journal of Life Science
Vol. 11. No. 4. 316~320, 2001

Effect of Sonication and *vir* Genes on Transient Gene Expression in *Agrobacterium*-Mediated Transformation

Byung-Moo Lee

Dept. of Plant Resources, Dongguk University, Seoul 100-715, Korea

Abstract

Sonication tremendously improves the efficiency of *Agrobacterium* infection by introducing small and uniform fissures and channels throughout the targeted tissue. Using shoot tips of cotton as explants, the effect of sonication treatment and virulence genes in *Agrobacterium tumefaciens* on transformation efficiency was investigated. The *pat* gene which encodes resistance to the herbicide, glufosinate, was used as a selectable marker. Transformation efficiency was evaluated on the basis of survival rates of cocultivated shoot tips on selection medium containing 2.5 mg/l glufosinate-ammonium (ppt) and 250 mg/l Clavamax. Sonication from 5 to 15 second has a positive effect on shoot tip survival. However, while *virE* as well as *virG* or *virGN54D* showed an enhancement in transformation efficiency, *virE*/*virG* resulted in the most significant enhancement. Overall, the combination of additional *virG*/*virE* gene and sonication treatment resulted in the most significant increase in transformation efficiency.

Key words – *Agrobacterium tumefaciens*, Sonication, Virulence genes, Shoot tip

Introduction

Agrobacterium-mediated transformation is the most commonly used method for plant genetic engineering. This system can stably transfer foreign gene into plant genome. The transformation process can be divided into four components: bacterial colonization and attachment to the plant cell, T-DNA processing and delivery, T-DNA integration and T-DNA expression[2]. There are six *vir* complementation groups, *virA*, *virB*, *virC*, *virD*, *virE* and *virG*, which provide products required for plant cell recognition and T-DNA transfer[3]. In response to chemical compounds (phenolic compounds, sugars, pH), *virA* protein phosphorylates the *virG* product, which in turn interacts with the promoters of other *vir* genes, causing induction. This interaction among *vir* genes should be especially important in transformation of recalcitrant plants such as cotton. In addition, Liu et al.[9] and Hansen et al.[5] demonstrated that additional copies of *virG* or *virGN54D* (mutation of *virG*, which caused constitutive expression of other *vir* genes independent of *virA*) in *A. tumefaciens* enhanced the transient transformation frequency using GUS expression assays.

Agrobacterium-mediated transformation using shoot apex has been reported for cotton[19], petunia[18], pea [7], sunflower[1,14], corn[4], banana[10], tobacco[20], and rice[13]. Theoretically, the advantage of the shoot apex explant over other regeneration systems is that plants may be obtained from any genotype rather than only those that regenerate from callus culture. However, it is desirable to increase transformation efficiency.

*To whom all correspondence should be addressed
Tel : (02) 2260-3307, Fax : (02) 2263-1227
E-mail : bmlee@dongguk.edu

The first publication using sonication for transformation was that of Joersbo and Brunstedt[8]. They demonstrated that mild sonication of sugar beet (*Beta vulgaris* L.) and tobacco (*Nicotiana tabacum* L.) protoplasts facilitated the uptake of plasmid DNA with some loss in viability of the protoplasts. Transient expression of the foreign DNA was increased by 7-15 fold over expression following electroporation. No long-term deleterious effects of sonication were observed. Zhang et al.[21] described using ultrasound for direct gene transfer into tobacco leaf segments with a frequency of 22% transgene expression, and transgene expression was observed in progeny. More recently, Trick and Finer[15,16,17] demonstrated that sonication resulted in small and uniform fissures and channels throughout the targeted tissue which may allow *Agrobacterium* easier access to the plant cell several layers within the tissue. They sonicated cotyledons of *Glycine max* Jack, embryonic cultures of *Aesculus glabra* and *Glycine max* Merrill, leaf tissue of *Vigna unguiculata*, seedlings of *Picea glauca* and *Triticum aestivum* and immature embryos of *Zea mays* and co-cultivated with *Agrobacterium*. The method is referred to as SAAT or sonication-assisted *Agrobacterium*-mediated transformation. They saw a 100 to 1,400-fold increase in transient expression of β-glucuronidase in these tissues. In this paper, the effects of sonication and additional copies of *vir* genes in *Agrobacterium tumefaciens* LBA4404 were evaluated using the shoot tip transformation systems.

Materials and Methods

Plant Materials

Gossypium hirsutum cv. Sphinx was used in this study. Seeds were surface-sterilized in concentrated sulfuric acid (20 min), 50% Clorox (1 hr) in a rotary shaker at 50 rpm and rinsed at least three times in sterile, double-distilled water. The seeds were then placed on 0.2% (W/V) gelrite solidified medium, pH 5.7, containing the Murashige and Skoog (MS) inorganic salts[11], and 2% of sucrose to germinate. Seeds were incubated at 28℃ in the dark for approximately 3-4 days.

Bacterial Strain and Plasmids

An octopine strain of *A. tumefaciens*, LBA4404, was used. Two plasmid constructs, pAGM280 containing the *ubi/pat/orf25\bt(syn trun)\(4Ocs)⊿mas* (6.7 kb) expression vector, pAGM281 containing the *ubi/pat/orf25\bt(syn full)\(4Ocs)⊿mas* (8.4 kb) expression vector were used. The helper plasmids, pAD1289 carrying the mutant *virGN54D* (from the octopine Ti plasmid pTiA6), pCH30 carrying *virG* (from the L, L-succinamopine Ti plasmid pTiBo542), pCH32 carrying *virG* (pTiBo542)/*virE1virE2* (pTiA6), and pCH42 carrying *virE1virE2* (pTiA6), were mobilized into *A. tumefaciens* LBA4404 containing each of the expression vector by the freeze-thaw method[6].

Shoot Tip Isolation, Co-cultivation and Selection

Three to four day-old seedlings grown *in vitro* were used to provide explants. Shoot tips were isolated with the aid of a dissecting microscope. The shoot apex tissue used here was approximately 1 to 2 mm in height, and this size includes part of the cotyledon and radicle to supply nutrients (Fig. 1). Acetosyringone (100 μM) was used in all experiments, and added at least 2 hr before cocultivation for induction of the *vir* genes. For sonication treatment, shoot tips were transferred to microcentrifuge tubes, containing 0.5 ml of bacteria suspension in YEB media and placed in the center of a bath sonicator (Bransonic 1210). Shoot tips were then sonicated for various timed intervals (0, 5, 10, 15, 30 and 60 second at 60 watts, 47 KHz). Shoot tips were removed from the tubes, placed on a sterile filter paper to blot off excess bacteria, and then cocultivated on filter paper saturated with liquid MS medium (pH 5.7) at 19℃ for a period of approximately 3 days. After cocultivation, shoot tips were established on MS and 250 mg/l Clavamax medium for 5 days and then transferred to the selection medium

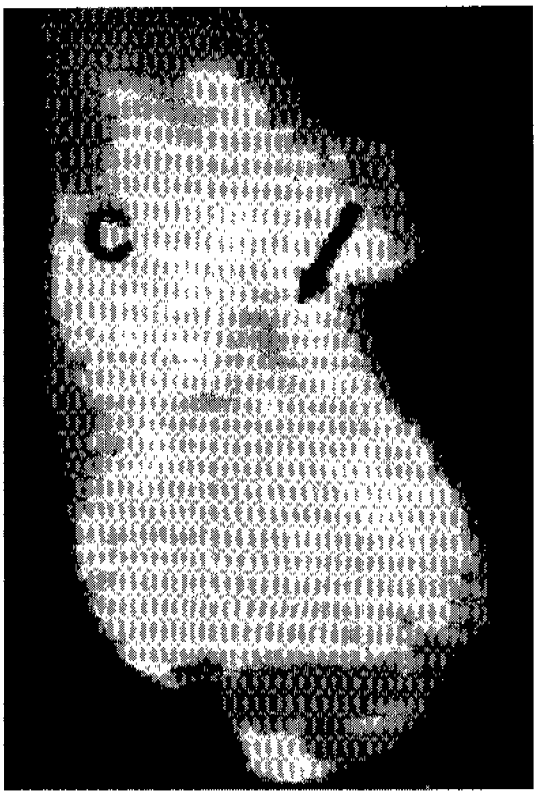

Fig. 1. Cotton shoot apex tissue including part of the cotyledon and radicle. Arrow indicates shoot tip and C indicates cotyledon.

containing 2.5 mg/l glufosinate-ammonium (ppt) and 250 mg/l Clavamax. This concentration of selective agent minimized the escapes which can cause cross-protection. The surviving shoot tips were transferred to a fresh medium every 2 weeks.

Results and Discussion

Table 1 summarizes the results of sonication effect on transient gene expression of the *pat* gene. Transformation efficiency was evaluated on the basis of survival rates of cocultivated shoot tips on selection medium containing 2.5 mg/l glufosinate-ammonium (ppt) and 250 mg/l Clavamax. Sonication resulted in 5, 10, 8, 8, 3 and 3% survival of shoot tips on selection medium at 0, 5, 10, 15, 30 and 60 second treatment, respectively. The addition of the supervirulent plasmid, pAD 1289, increased the survival rate of shoot tips on selection medium, respectively; 11, 20, 18, 19, 11 and 10% survival at 0, 5, 10, 15, 30 and 60 second treatment. It appears that sonication from 5 to 15 seconds has a positive effect on shoot tip survival. The sonication treatment for 5 second showed a twofold increase in the survival rate of shoot tips on selection medium compared with the control. Trick and Finer[15] suggested that sonication is advantageous when subsurface targets such as cotyledonary nodes and shoot meristems are the targets for transformation. And sonication did not affect *Agrobacterium* survival (data not shown).

Effect of sonication and *vir* genes on transient gene expression of the *pat* gene is shown in Table 2. Additional copy of pCH32 (*virG/virE1virE2*) resulted in higher rates of survival, 23% and 27% without and with sonication treatment, whereas pCH42 (*virE1virE2*) resulted in lower rates of survival, 13% and 17% without and with sonication treatment. The pCH30 (*virG*) and supervirulent plasmid, pAD1289 (*virGN54D*) showed 18% of survival without sonication, and 23% and 24% of survival with sonication treatment. While *virE* as well as *virG* or *virGN54D* showed an enhancement in transformation

Table 1. Sonication effect on transient gene expression of the *pat* gene

Vector+*vir* gene	Number of shoot tips in each treatment	Number of ppt-resistant shoot tips (%)					
		Sonication duration (second)					
		0	5	10	15	30	60
pAGM281	150	8(5.3)	15(10.0)	13(8.7)	13(8.7)	6(4.0)	5(3.3)
pAGM281+*virGN54D*	110	13(11.8)	23(20.9)	20(18.2)	21(19.1)	13(11.8)	12(10.9)

Table 2. Effect of sonication and *vir* genes on transient gene expression of the *pat* gene

Vector+*vir* gene	Number of ppt-resistant shoot tips / number of shoot tips inoculated (%)	
	Sonication 0 second	15 second
pAGM280	5/60 (8)	8/65 (12)
pAGM280+*virG*	11/60 (18)	15/65 (23)
pAGM280+*virG*/*virE*	14/60 (23)	18/65 (27)
pAGM280+*virE*	8/60 (13)	11/65 (17)
pAGM280+*virGN54D*	11/60 (18)	16/65 (24)

efficiency, *virG*/*virE* resulted in the most significant enhancement. Shorter T-DNA, pAGM280 (6.7 kb) in *A. tumefaciens* increased transformation efficiency compared with pAGM281 (8.4 kb) (Table 1, 2). Park et al.[12] demonstrated that shorter T-DNA or additional *vir* genes enhanced the transformation efficiency. Overall, the combination of additional *virE*/*virG* gene and sonication treatment resulted in the most significant increase in transformation efficiency.

Acknowledgment

This work is supported by the Dongguk University research fund (2000-0440-Z).

References

1. Bidney, D., C. Scelonge, J. Martich, M. Burrus, L. Sims and G. Huffman. 1992. Microprojectile bombardment of plant tissues increases transformation frequency by *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **18**, 301-313.
2. Binns, A. N. 1990. *Agrobacterium*-mediated gene delivery and the biology of host range limitations. *Physiol. Plant* **79**, 35-139.
3. Engstorm, P., P. Zambryski, M. Van Montagu, and S. Stachel. 1987. Characterization of *Agrobacterium tumefaciens* virulence proteins induced by the plant factor acetosyringone. *J. Mol. Biol.* **197**, 635-645.
4. Gould J., M. Devey, O. Hasegawa, E. C. Ulian, G. Peterson and R. H. Smith. 1991. Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. *Plant Physiol.* **95**, 426-434.
5. Hansen, G., A. Das and M.-D. Chilton. 1994. Constitutive expression of the virulence genes improves the efficiency of the plant transformation by *Agrobacterium*. *Proc. Natl. Acad. Sci. USA* **91**, 7603-7607.
6. Holsters, M., D. De Waele, A. Depicker, E. Messens, M. Van Montagu and J. Schell. 1978. Transfection and transformation of *A. tumefaciens*. *Mol. Gen. Genet.* **163**, 181-187.
7. Hussey, G., R. D. Johnson and S. Warren. 1989. Transformation of meristematic cells in the shoot apex of cultured pea shoots by *Agrobacterium tumefaciens* and *A. rhizogenes*. *Protoplasma* **148**, 101-105.
8. Joersbo, M. and J. Brunstedt. 1990. Direct gene transfer to plant protoplasts by mild sonication. *Plant Cell Rep.* **9**, 207-210.
9. Liu, C.-N., X.-Q. Li and S. B. Gelvin. 1992. Multiple copies of *virG* enhance the transient transformation of celery, carrot and rice tissues by *Agrobacterium tumefaciens*. *Plant Mol. Biol.* **20**, 1071-1087.
10. May, G. D., R. Afza, H. H. Mason, A. Wiecko, F. J. Novak and C. Arntzen. 1995. Generation of transgenic banana (*Musa acuminata*) plants via *Agrobacterium*-mediated transformation. *Bio/Technology* **13**, 486-492.
11. Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plant* **15**, 473-497.
12. Park, S. H., B.-M. Lee, M. G. Salas, M. Srivatanakul and R. H. Smith. 2000. Shorter T-DNA or additional virulence genes improve *Agrobacterium*-mediated transformation. *Theor. & Appl. Genet.* **101**, 1015-1020.
13. Park, S. H., S. R. M. Pinson and R. H. Smith. 1996. T-DNA integration into genomic DNA of rice following *Agrobacterium* inoculation of isolated shoot apices. *Plant Mol. Biol.* **32**, 1135-1148.
14. Schrammeijer, B., P. C. Sijmons, P. J. M. Van den Elzen and A. Hoekema. 1990. Meristem transformation of sunflower via *Agrobacterium*. *Plant Cell Rep.* **9**, 55-60.
15. Trick, H. N. and J. J. Finer. 1997. SAAT: sonication-assisted *Agrobacterium*-mediated transformation. *Transgenic Research* **6**, 329-336.
16. Trick, H. N. and J. J. Finer. 1998. Sonication-assisted *Agrobacterium*-mediated transformation of soybean [*Glycine max* (L.) Merrill] embryonic suspension

culture tissue. *Plant Cell Rep.* **17**, 482-488.
17. Trick, H. N. and J. J. Finer. 1999. Induction of somatic embryogenesis and genetic transformation of Ohio buckeye (*Aesculus glabra* Willd.). *In Vitro Cell Dev. Biol.* **35**, 57-60.
18. Ulian, E. C., G. H. Smith, J. H. Gould and T. D. McKnight. 1988. Transformation of plants via the shoot apex. *In Vitro Cell Dev. Biol.* **24**, 951-954.
19. Zapata, C., S. H. Park, K. M. El-Zik and R. H. Smith. 1999. Transformation of a Texas cotton cultivar by using *Agrobacterium* and the shoot apex. *Theor. & Appl. Genet.* **98**, 252-256.
20. Zimmerman, T. W. and R. Scorza. 1996. Genetic transformation through the use of hyperhydric tobacco meristems. *Molecular breeding* **20**, 73-80.
21. Zhang, L.-J., L.-M. Cheng, N. Xu, N.-M. Zhao, C.-G. Li, J. Yuan and S.-R. Jia. 1991. Efficient transformation of tobacco by ultrasonication. *Bio/Technology* **9**, 996-997.

(Received May 16, 2001; Accepted June 25, 2001)

초록 : *Agrobacterium*을 이용한 형질전환에서 sonication과 *vir* 유전자들의 효과

이 병 무
(동국대학교 식물자원학과)

Sonication은 식물조직에 깊고 작은 상처를 만들어 줌으로서 *Agrobacterium*이 원하는 조직에 침투할 수 있는 능력을 향상시켜준다. 이 실험은 면화의 생장점을 이용하여 sonication과 virulence 유전자들이 형질전환효율에 미치는 결과를 조사하였다. 형질전환의 효율성은 2.5 mg/l ppt를 포함하는 selection 배지에서 생장점의 생존율을 기초로 하여 평가하였다. 5-15초간 sonication을 처리한 생장점에서 생존율이 높게 보였다. *virE*, *virG*, *virGN54D* 유전자들을 *Agrobacterium*에 첨가시켰을 때 생존율이 증가되었지만, *virE*/*virG* 유전자를 첨가시켰을 때, 보다 높은 효율을 보였다. 전체적으로 *virE*/*virG* 유전자의 첨가와 sonication 처리의 조합이 형질전환효율을 가장 많이 증가시키는 것으로 나타났다.