THE INFLUENCE OF *VIR* GENES ON STABLE T-DNA INTEGRATION IN COTTON SHOOT APICES

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Abstract

Gossypium hirsutum cv. Sphinx is a commercially grown Texas cotton cultivar. A method to rapidly and efficiently transform cotton cultivars without modification of the genotype has been established in this laboratory. However, it is desirable to increase transformation efficiency. This report examines the effects of the additional virulent genes, *virG*, *virG/virE*, *virE*, and *virGN54D* on transient expression of the cotton shoot tip on selection medium with and without sonication at 60 watts, 47 KHz for 15 sec. The shoot establishment for 5 days between cocultivation and selection increased shoot tip survival from 8% to 18% on selection medium. pCH42 (*virE*) resulted in lower rates of survival, 13% and 17% without and with sonication treatment. The pCH32 (*virG/virE*) resulted in higher rates of survival, 23% and 27% without and with sonication treatment. The pCH30 (*virG*) and supervirulent plasmid, pAD1289 showed 18% survival without sonication, and 23% and 27% survival with sonication treatment. To investigate the effect of *vir* genes on stable T-DNA integration, primary plants will be tested.

Introduction

Agrobacterium-mediated transformation is the most commonly used method for plant genetic engineering. This vector can stably transfer foreign gene into crop genomic DNA. 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 (Binns, 1990). There are six *vir* complementation groups, *vir*A, *vir*B, *vir*C, *vir*D, *vir*E and *vir*G, which provide products required for plant cell recognition and T-DNA transfer (Engstrom et al., 1987). In response to chemical compounds (phenolic compounds, sugars, pH), v*irA* protein phosphorylates the v*irG* 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.

The *Agrobacterium*-mediated shoot apex transformation system has been established in this laboratory. 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. *Agrobacterium*-mediated transformation using shoot apex has been reported for cotton (Zapata et al., 1998), petunia (Ulian et al., 1988), pea (Hussey et al., 1989), sunflower (Bidney et al., 1992; Schrammeijer et al., 1990), corn (Gould et al., 1991), banana (May et al., 1995), tobacco (Zimmerman and Scorza, 1996), and rice (Park et al., 1996). A method to rapidly and efficiently transform cotton cultivars without modification of the genotype has been established. However, it is desirable to increase transformation efficiency. We have investigated the effects of the additional virulent genes, *virG*, *virG/virE*, *virE*, and *virGN54D* on transient expression of the cotton shoot tip on selection medium.

Materials and Methods

Plant Material

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 (Murashige and Skoog, 1962), and 2% of sucrose to germinate. Seeds were incubated at 28°C in the dark for approximately 3-5 days.

Bacterial Strains and Plasmids

Agrobacterium tumefaciens LBA 4404 (octopine strain) was used. The plasmid constructs used were *Ubi/Pat/orf25\Bt(syn trun)\(40cs)Mas* (6.7 kb). Additionally, the virulent plasmids, pCH30, pCH32, pCH42, and pAD1289, were used. The helper plasmid pCH30 carries *virG*; pCH32 carries *virG* and *virE* ; pCH42 carries *virE*. The supervirulent plasmid pAD1289 has *virGN54D*, mutation of *virG*, which causes constitutive expression of other *vir* genes independent of *virA*.

Shoot Apex Isolation, Co-cultivation, Selection and Regeneration

Three to five day-old seedlings grown *in vitro* were used to provide explants. Shoot apices 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. Acetosyringone (100 mM) was used in all experiments, and this was added at least 2 hr before cocultivation for induction of the *vir* genes. Shoot apices were inoculated by placing in bacteria suspension (OD_{600} 0.6-0.9) for 5 min 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°C for a period of approximately 72 hr. After cocultivation, shoots were established on MS and Clavamax medium for 5 days and then transferred to the selection medium containing 2.5 mg/l glufosinate-ammonium. The surviving shoot apices

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were transferred to a fresh medium every 2 weeks. The process was repeated until the controls were all dead. After 2 months, shoot apices that survived on selection were transferred to rooting medium containing 1/2 MS and 0.1 mg/l NAA. For sonication treatment, shoot tips were transferred to microcentrifuge tubes, containing 1 ml of bacteria suspension in YEB media and placed in the center of a bath sonicator (Bransonic 1210). Shoot tips were then sonicated for 15 sec. Shoot tips were removed from the tubes, placed on a sterile filter paper to blot off excess bacteria, and then transferred to wet filter paper for cocultivation.

Results and Discussion

The effect on shoot tip survival on selection medium (2.5 mg/l ppt) without and with sonication treatment after 2 months *in vitro* is shown in Table 1. The survival percentage with the *pat,* truncated *Bt* gene, and the supervirulent plasmid was 18%. This percentage is an increase in transient expression compared with our earlier report of 8% with same gene component (Lee et al., 1998). The only different factor between those experiments was shoot establishment for 5 days between cocultivation and selection. It appears that shoot establishment between cocultivation and selection has a positive effect on shoot tip survival.

The use of *virGN54D* improves the efficiency of *Agrobacterium*-mediated transformation, especially for recalcitrant plant species (Hansen et al., 1994). Recently, sonication was used to increase the level of wounding to improve transformation efficiency (Trick and Finer, 1997). Whereas pCH42 (*virE*) resulted in lower rates of survival, 13% and 17% without and with sonication treatment, the pCH32 (*virG/virE*) resulted in higher rates of survival, 23% and 27% without and with sonication treatment. The pCH30 (*virG*) and supervirulent plasmid, pAD1289 showed 18% of survival without sonication, and 23% and 27% of survival with sonication treatment. To investigate the effect of *vir* genes on stable T-DNA integration, primary plants will be tested.

References

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.

Gould, J., M. Devey, E.C. Ulian, O. Hasegawa, G. Peterson, and R.H. Smith. 1991. Transformation of *Zea mays* L. using *Agrobacterium tumefaciens* and the shoot apex. Plant Physiol. 95:426-434.

Hansen, G., A. Das, and M.D. Chilton. 1994. Constitutive expression of the virulence genes improves the efficiency of plant transformation by *Agrobacterium.* Proc. Natl. Acad. Sci. 91:7603-7607.

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.

Lee, B.-M., C. Zapata, S.H.Park, K.M. El-Zik, and R.H. Smith. 1998. Sonication effects and transient gene expression following Agrobacterium transformation. Proceedings Beltwide Cotton Conferences. p. 488-489.

May, G.D., R. Afza, H.S. 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.

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.

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.

Trick, H.N., and J.J. Finer. 1997. SAAT-Sonication-assisted *Agrobacterim*-mediated transformation. Transgenic Reseach 6:329-336.

Ulian, E.C., R.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.

Zapata, C., S.H. Park, K.M. El-Zik, and R.H. Smith. 1998. Transformation of a Texas cotton cultivar by using Agrobacterium and the shoot apex. Theor. & Appl. Gen. (In press)

Zimmerman, T.W., and R. Scorza. 1996. Genetic transformation through the use of hyperhydric tobacco meristems. Molecular breeding 20:73-80.

Table 1. Sphinx cotton shoot tip survival on selection medium (2.5 mg/l ppt) indicating transient expression of the *pat* gene. *Agrobacterium tumefaciens* LBA4404 was the vector.

	# shoot tips surviving (%)		
Gene construct	Without sonication	Sonication (15 sec)	Total
Pat/Bt (Trun) + *vir G*	11/60 (18)	15/65 (23)	26/125 (20)
Pat/Bt (Trun) + *vir G/E*	14/60 (23)	18/65 (27)	32/125 (25)
Pat/Bt (Trun) + *vir E*	8/60 (13)	11/65 (17)	19/125 (15)
Pat/Bt (Trun) + S	11/60 (18)	18/65 (27)	29/125 (23)

S = Supervirulence, pAD1289