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Research Article

Agrobacterium tumefaciens–Mediated Transformation of Sesame (*Sesamum indicum* L.)

Kemal Melih TAŞKIN
Akdeniz University, Graduate School of Natural and Applied Sciences, Department of Field Crops, Antalya–TURKEY
A. Gülhan ERCAN
Akdeniz University, Graduate School of Natural and Applied Sciences, Department of Field Crops, Antalya–TURKEY
Kenan TURGUT
Akdeniz University, Faculty of Agriculture, Department of Field Crops, Antalya–TURKEY

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Abstract: *Sesamum indicum* L. cv. Özberk was infected with various *Agrobacterium tumefaciens* strains to screen susceptibility to infection by Agrobacteria. Following infection, tumorigenesis was efficient with wild type *A. tumefaciens* strains, A281 and A136 NC, and transgenic cells were obtained with disarmed *A. tumefaciens* strain LBA4404/pBI121, harbouring a reporter gene. At the wound sites, tumorigenesis induced by the succinamopine strain A281 was more extensive than by the octopine strain A136 NC.

Key Words: *Sesamum indicum, Agrobacterium tumefaciens,* virulence, transformation.

Susam (*Sesamum indicum* L.) Bitkinin *Agrobacterium tumefaciens* Aracılığı ile Transformasyonu

Özet: *Sesamum indicum* L. cv. Özberk *Agrobacterium*'a duyarlılığının belirlenmesi amacıyla çeşitli *Agrobacterium tumefaciens* şuşları ile enfekte edilmiştir. Enfeksiyonu takiben, yabanıl tip *A. tumefaciens* suşları A281 ve A136 NC tümör indüklerken, reporter gen taşıyan disarmed *A. tumefaciens* suşu LBA4404/pBI121, transgenik hücreler oluşturmuştur. Yaralı bölgelerde succinamopin suşu A281 tarafından indüklenen tümörler, octopin suşu A136 NC tarafından indüklenen tümörlerden daha büyük olmuştur.

Anahtar Sözcükler: *Sesamum indicum, Agrobacterium tumefaciens,* virülentlik, transformasyon.

Introduction

Sesamum indicum L. *(Pedaliaceae)* is grown as an oil seed crop because sesame oil contains high percentages of linoleic and oleic fatty acids, but mostly used as food in Turkey. However, there are some problems limiting sesame production in Turkey. These are plant diseases, indeterminate flowering and dehiscent capsules. Plant diseases such as *Alternaria sesemicola* Kaw., *Oxysporum* Schlectend.: Fr., and *Phytophora parasitica* Dastur cause considerable damage and reduce the yield. Indeterminate flowering and dehiscent capsules are the most important factors limiting sesame production. Conventional plant breeding methods have produced some improvement but not enough. Therefore, new technologies such as biotechnology should be introduced to develop new varieties. Gene transfer via *Agrobacterium tumefaciens* is one of the useful technique to overcome such problems. Wild–type, oncogenic strains of *A. tumefaciens* harbour large plasmids called Ti (Tumor-inducing) plasmids, which contains several genes important for tumorigenicity (1). It is known that *A. tumefaciens* strains can be used to transfer individual genes of interest together with the Ti T-DNA, into the genome of plants. Successful foreign gene transfer to a plant was first reported by Zambryski et al. (2) using genetically manipulated strains of *A. tumefaciens.* According to host range studies (3) *Agrobacterium* can infect a wide spectrum of dicots and some monocots. Recently in vitro regeneration (4), in vitro propagation (5), shoot tip culture (6), protoplast culture (7) and naphthaquinone production (8) of sesame have been reported. However, the susceptibility of *S. indicum* to infection by *Agrobacteria* has not been reported. In this study, our aim was to determine the virulence of various wild type oncogenic *A. tumefaciens* strains against *S. indicum* and gene transfer to sesame using disarmed *A. tumefaciens* strains carrying a reporter gene.

Materials and Methods

Bacterial Strains

Six wild–type oncogenic (Table 1) and two disarmed (LBA4404/pBI121, pGV 2260/p35S-*GUSINT*) *A. tumefaciens* strains (obtained from Dr. Rod Scott, Bath University, UK) were used for transformation experiments. Octopine type disarmed strain LBA4404 (9) carries the binary vector pBI121 (10) and non-oncogenic vir helper plasmid. The plasmid pBI121 contains a nopaline synthase promoter sequence in front of a neomycin phosphotransferase II *(NPTII)* gene which confers resistance to kanamycin and a *GUS (β-glucuronidase)* gene driven by CaMV 35S promoter. Disarmed strain pGV2260 (11) carries plasmid p35S *GUS INT* (12). The plasmid p35S *GUS INT* contains the coding sequence of the *NPT II* gene under the control of nopaline synthase (NOS) promoter, and the *GUS* gene is under the control of the CaMV 35S promoter. In the p35S *GUS INT* construct, the *GUS* gene is interrupted by a plant intron and shows activity only in transformed plant cells. All the strains were grown in semi-solid Nutrient Agar (NA) or liquid Nutrient Broth (in a shaker at 150 rpm, Nuve SL350) media for 48 hours at 28 °C. NA medium contains 5 g/l peptone, 5 g/l sodium chloride, 1.5 g/l beef extract, 1.5 g/l yeast extract, 15 g/l agar and pH 7.4. NB medium contains 3 g/l bacto beef extract, 5 g/l bacto peptone and pH 6.8.

Plant Growth and Inoculation

Seeds were planted in 10 cm pots consisting of peat:perlite (1:1). Plants were grown in a growth chamber with 18 h photoperiod at 25 °C. Twelve weeks after germination, sesame plants (mature plants, during capsule growth) were inoculated with wild type *A. tumefaciens* strains. Bacterial inoculum was prepared by culturing each strain on semi-solid NA medium for 2 days at 28 °C. A syringe needle was used to scratch the stem about 2 mm deep and 0.5 mm wide for a length of 5 mm in 3 parallel lines at each internode (3 internodes per plant). A minimum of 3 plants per strain were used. The wound site was filled with bacteria (13, 14). Tumor formation was scored regularly for up to 2 months. Tumor frequency or the percentage of inoculated wounds that developed tumors as well as tumor size were monitored daily and scored.

Endogenous Level of Kanamycin Resistance of Plant Material

Kanamycin is usually used as a selection agent for transformation systems. For this reason, susceptibility of sesame to kanamycin was determined. The surface sterilized seeds (4) were aseptically placed in plates (9 cm petri dishes) containing MS medium (30 ml) supplemented with 0.8% agar (w/v), 3% sucrose (w/v) and 0, 50, 100 or 150 mg/l kanamycin for germination. The pH was adjusted to 5.7. Four repetitions and a minimum of 100 seeds were used per kanamycin concentration. Seedlings were grown at 25 °C with a 16 h photoperiod, under white fluorescent light.

Table 1. Tumors Induced by Wild–Type *A. tumefaciens* Strains After One Month Inoculation of Sasame Plants In Vivo.

Wild Type	*A. tumefaciens* Strains	Tumor induction Frequency (%)
A281	(Succinamopine)	54
A136NC	(Octopine)	50
T37	(Nopaline)	–
A6	(Octopine)	–
C58	(Nopaline)	–
ACH5	(Octopine)	–

In Vitro Transformation of Cotyledon Explants

Sesame seeds were surface sterilized (4) and then sown in 9 cm petri dishes containing 30 ml of Murashige & Skoog (MS) medium (15) supplemented with 0.8% (w/v) agar and 3% sucrose (w/v), pH 5.7. Seeds were germinated at 25 °C with a 16 h photoperiod, under white fluorescent light (3000 lux). Seven days after germination, cotyledons were excised and inoculated with *Agrobacterium* strains. Overnight bacterial cultures were grown in 25 ml NB medium at 28 °C in an orbital shaker (150 rpm). For disarmed strains, NB medium was supplemented with 50 µg/l kanamycin and rifampicin antibiotics. Cotyledon explants were immersed in overnight bacterial cultures diluted 1:100 (v/v) in 25 ml liquid MS medium containing 3% sucrose for 10 minutes then transferred to co-cultivation medium (semi-solid MS medium without antibiotics) for 2 days. After co-cultivation, cotyledon explants were transferred to MS medium supplemented with 0.8% (w/v) agar, 3% sucrose (w/v) 50 mg/l kanamycin as a selective agent and 400 mg/l augmentin to kill the bacteria. This medium also included 0.1 mg/l NAA + 8 mg/l BAP. Control explants were dipped into liquid MS medium without bacteria. Inoculations were carried out in a laminar flow hood. A minimum of 100 explants per strain were used. They were subcultured every 2 to 3 weeks.

Histochemical *GUS* Assay

Transgenic cells were confirmed by histochemical *GUS* assay (16). For analysis, 5 mg X-Gluc was dissolved in 100 µl dimethylformamide and total volume was

increased to 10 ml with 50 mM NaPO4 pH 7. Callus regenerated from co-cultivated explants were then treated with this solution at 37 °C for 72 hours. Finally, the reaction was stopped with 70% ethanol.

Results

Inoculations With Wild-type *A. tumefaciens* Strains

The response of sesame plants to strains containing wild-type Ti-plasmids are given in Table 1. Following inoculation, tumor formation was distinguishable within 2 weeks. Among the wild types, only two strains (A281 and A136 NC) induced tumors on sesame plants. The succinamopine strain A281 induced tumors at a frequency of 54% at inoculated sites on the 3 plants (Figure 1a). The octopine strain A136 NC induced tumors at a frequency of 50% at inoculation sites on the 3 plants. Furthermore, succinamopine tumors were larger than octopine tumors (average 0.2 mm and 0.1 mm respectively). However, the other wild type strains (ACH5, T37, A6, C58) were infective on sesame (Table 1) under the experimental conditions.

Determination of Kanamycin Resistance

Sesame seeds were germinated on MS media containing different concentrations of kanamycin and without kanamycin (control). After 10 days, in control plants (without kanamycin), all the seeds germinated and looked healthy. However, in the other MS media containing kanamycin, germination frequency was found to be 94% (50 mg/l kanamycin), 88% (100 mg/l kanamycin) and 80% (150 mg/l kanamycin). After 3 weeks of culture on MS medium supplemented with varying amounts of kanamycin, seedlings were bleached and dead regardless of kanamycin level. Thus, 50 mg/l kanamycin appeared to be convenient for selection.

Co-cultivation of Cotyledon Explants with *A. tumefacines* Strains

After exposure to wild-type *A. tumefaciens* strains and planting on hormone-free MS medium containing 400 mg/l augmentin, some cotyledons showed tumor formation. But only two strains (A281 and A136 NC) induced tumors (Figure 1b). These tumors covered the whole explants in two weeks of culture.

Following co-cultivation with disarmed *Agrobacterium* strains (LBA 4404/pBI121 and pGV 2260/ p35S *GUSINT*), cotyledon explants produced callus on regeneration medium (4) supplemented with kanamycin (50 mg/l) and augmentin (400 mg/l). However, for explants treated with liquid MS medium (control), callus formation did not occur on the same regeneration medium. Thus, histochemical *GUS* assay was performed with these calli to determine *GUS* activity. However, only the explants co-cultivated with LBA 4404/ pBI 121 strain revealed *GUS* gene expression (Figure 1c) and gave 4-5 blue spots on each cotyledon explant. However, transgenic shoots were not obtained in this study. In preliminary experiments, different dilutions of overnight bacterial cultures (1:10, 1:25, 1:50 and 1:100) were tested in order to improve transformation efficiency. However, generally more necrosis on cotyledon explants was seen at the highest concentrations of bacteria.

Discussion

This study presents the first report on the susceptibility of a sesame cultivar to *A. tumefaciens.* Although there is one report on production of naphthaquinone by a hairy root culture induced by direct inoculation of *S. indicum* via *A. rhizogenes* ATCC 15834 (8) there is no report on the susceptibility of sesame to *A. tumefaciens* infection.

In this study, A281 resulted in larger tumor formation than A136 NC on sesame plants. The strain A281 is known as a broad host-range supervirulent strain (17) and has been very useful for transforming recalcitrant species. Davis et al. (18) reported that tumors induced by A281 strain were larger than other wild type strains on cotyledon explants of tomato. These reports support the results of the present study.

Recently in vitro regeneration (4), in vitro propagation (5) shoot tip culture (6), protoplast culture of sesame (7) have been reported. Taşkın and Turgut (4) obtained adventitious shoot regeneration which is important for *A. tumefaciens* mediated gene transfer systems. For this reason, this regeneration system was used for transformation studies. However, co-cultivation of cotyledon explants with disarmed *Agrobacterium* strains reduced regeneration efficiency over non-transformed controls. The reason for this reduction was not investigated further but may be related to the hypersensitive response of sesame explants to *A. tumefaciens* infections. This result is consistent with that of Orlikowska et al. (19). They described the characterization of factors affecting *A. tumefaciens*-mediated transformation of safflower seedlings explants and reported that hypersensitive response to bacterial infection may reduce organogenetic potential furthermore, negative effects of antibiotics (kanamycin) on regeneration were observed for safflower (19).

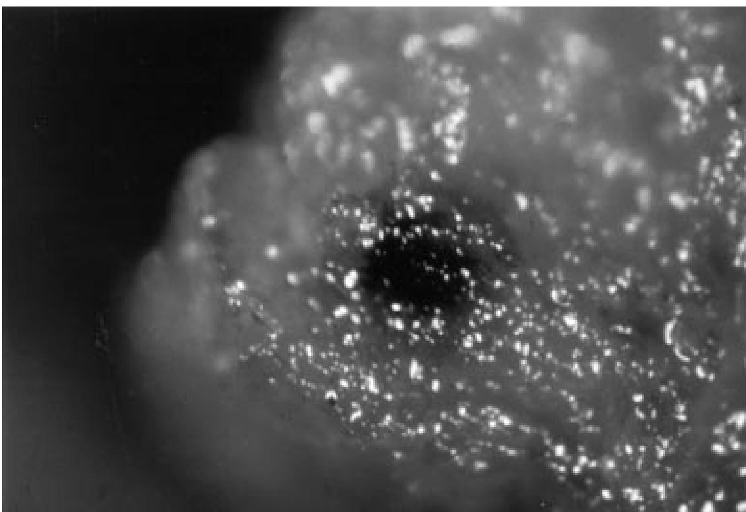

Figure 1 a) Tumors induced by wild type *A. tumefaciens* strain A281 after one month inoculation of sesame plants in vivo. b) Tumor induced by *A. tumefaciens* strain A136 NC on cotyledon explants of sesame after one week in vitro. c) Callus regenerated after co-cultivation with disarmed strain LBA 4404/pBI 121 in vitro showed stable *GUS* gene expression.

Therefore, it may be useful to try different antibiotic selection systems.

The results of this work demonstrate that sesame is susceptible to wild–type *Agrobacterium* strains A281 and A136 NC. Furthermore, it is possible to obtain stable T-DNA transfer by inoculating cotyledon explants with disarmed strain LBA 4404/ pBI121, although this occurs at a low frequency. However, no shoot regeneration was obtained in this study due to the low regeneration ability of sesame (4). Alternatively, it may be possible to obtain better regeneration systems from other genotypes. In conclusion, disarmed *Agrobacterium* strains based on wild-type oncogenic A281 or LBA4404/ pBI 121 could be used to obtain transgenic sesame plants.

References

1. Watson, B., T. C., Currier, M. P., Gordon, M. D., Chilton, Nester, E. W., Plasmids required for virulence of *A. tumefaciens*, J. Bacteriol., 123, 255-264 (1975).
2. Zambryski, P., Joos, H., Genetello, C., Leemans, J., van Mantagu, Schell, J., Ti plasmid vector for the introducing of DNA into plant cells whithout alteration of their normal regeneration capacity. EMBO J., 2, 2143-2150 (1983).
3. Decleene, M., Deley, J., The host range of crown gall, Bot. Rev., 42, 389-466 (1976).
4. Taşkın, K. M., Turgut, K., *In vitro* regeneration of Sesame *(S. indicum L)*, Tr. J. Bot., 21, 15-18 (1997).
5. George, L., Bapat, V., Rao, P., In vitro multiplication of sesame (*S. indicum* L) through tissue culture, Ann. Bot. 60 (1) 17-21 (1987).
6. George, L., Bapat, V., Rao, P. S., Plant regeneration in vitro *in different* cultivars of sesame (*S. indicum* L), Proceedings of the Indian Academy of Sciences, Plant Sci., 99 (2), 135-137 (1989).
7. Bapat, V., George, L., Rao, P. S., Isolation, culture and callus formation of sesame (*S. indicum* L cv PT) protoplast, Indian J. Exp. Bio., 27 (2), 182-184 (1989).
8. Ogasawara, T., Chiba, Tada, K., Production of high yield of a naphtaquinone by a hairy root culture of *S. indicum*, Phytochem, 33, 1095-1098 (1993)
9. Hoekema, A., Hirsch, P. R., Hooykas, PJJ., Schilperoort, R. A., A binary plant vector strategy based on separation of vir and T-region of the *A. tumefaciens* Ti plasmid, Nature. 303, 179-180 (1983).
10. Bevan, M., Binary *Agrobacterium* vectors for plant transformation, Nucleic Acid Res., 12, 8711-8721 (1984).
11. Deblaere, R., Bytebier, B., De Greve, H., Deboek, F., Schell, J., Van Mantagu, M. and Leemans, J., Efficient octopine Ti-plasmid vectors for *Agrobacterium*-mediated gene transfer, Nucl. Acid. Res., 17, 8385 (1985).
12. Vancameyt, G., Schmidt, R., O'Conner-Sanches, A., Willmitzer. L., Rocha-Sosa M., Construction of an intron-containning marker gene: Splicing of the intron in transgenic plants and its use in monitoring early events in *Agrobacterium*-mediated plant transformation, Mol. Gen. Genet., 220, 245-250 (1990).
13. Hood, E. E., Fraley, R.T., Chilton, M. D., Virulence of *A. tumefaciens* strain A281 on Legumes, Plant Physiol., 83, 529-534 (1987).
14. Charest, P.J., Iyer, V.N., Brain, L.M., Virulence of *A. tumefaciens* strains with *Brassica napus* and *B. Juncea*, Plant Cell Rep., 8, 303-306 (1989).
15. Murashige, T., Skoog, A revised medium for rapid growth and bioassays with tobacco tissues culture, Physiol. Plant, 15, 473-497 (1962).
16. Draper, J., Scott, R., Armitage, P., Walden, R., Plant genetic transformation and gene expression, A Laboratory Manual, Blackwell Scientific. Publications, London (1988).
17. Hood, E. E., Helmer, G. L., Fraley, R. T., Chilton, M. D., The hypervirulence of *A. tumefaciens* A281 is encoded in a region of pTiBo542 outside of T DNA, J. Bacteriol., 168 (3), 1291-1301 (1986).
18. Davis, E. M., Lineberger, D. R., Miller, A. R., Effects of tomato cultivars leaf age, and bacterial strain on transformation by *Agrobacretium tumefaciens*, Plant Cell Tiss. Org. Cult., 24, 115-121 (1991).
19. Orlikowska, T. K., Cranston, H. J., Dyer, W. E., Factors influencing *A. tumefaciens*-mediated transformation and regeneration of safflower cultivars centennial, Plant, Cell Tiss. Org. Cult., 40, 85-91 (1995).