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Use of *Agrobacterium* expressing green fluorescent protein to evaluate colonization of sonication-assisted *Agrobacterium*-mediated transformation-treated soybean cotyledons

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K.R. FINER AND J.J. FINER. 2000. Colonization and infection of soybean cotyledons by *Agrobacterium tumefaciens* and subsequent elimination of bacteria from cotyledons were monitored using bacteria expressing green fluorescent protein (GFP). GFP provided a quick, non-destructive method to evaluate, in real time, *Agrobacterium* colonization of cotyledon surfaces as well as infection of internal cells. GFP was first detected 7 h following inoculation of the cotyledon. By 36 h, GFP expression was very intense, and was limited to the adaxial surface of the cotyledon. Expression of GFP also served as a useful indicator of successful elimination of the bacterium from plant tissue following antibiotic treatment.

INTRODUCTION

Agrobacterium tumefaciens is a Gram-negative, soil-borne plant pathogen that has the ability to insert foreign genes into plant cells which then express the gene products. One major disadvantage of using *Agrobacterium* for plant transformation is the organism's host specificity, resulting in low levels of transformation in certain plant species. To increase transformation efficiencies in these species, much effort has been placed on understanding the molecular mechanisms of T-DNA transfer (Holford *et al*. 1992; Fullner *et al*. 1996) with the goal of manipulating and controlling the transfer process. Although these studies have been useful and have led to enhanced transformation rates in many plants, there have been few studies on the dynamics and optimization of agrobacterial colonization and infection, the initial events upon which gene transfer between plant and host are ultimately dependent.

The use of green fluorescent protein (GFP) from the jellyfish (*Aequorea victoria*) now provides us with a tool for monitoring pathogen infection in time and space without disturbing either the bacterium or the host tissue. As no manipulation of the tissue is required for GFP visualization, the integrity of the cell structure and the morphology of the target tissue are maintained. In addition, because visualization of GFP does not kill cells, the fluorescence can be used to study the timing of gene expression *in vivo* (Chalfie *et al*. 1994), or to monitor viral or bacterial infection events over time (Baulcombe *et al*. 1995; Gage *et al*. 1996; Oparka *et al*. 1997; Verver *et al*. 1998). By incorporating a plasmid into *Agrobacterium tumefaciens* that expresses GFP under the control of a constitutive bacterial promoter, we were able to monitor *Agrobacterium* growth, on the surface of and within soybean cotyledons, over time under different cultural conditions. Soybean cotyledons were selected as the target tissue in this study as they are capable of producing somatic embryos, which are preferred for soybean transformation (Finer and McMullen 1991).

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MATERIALS AND METHODS

Bacterial strains and growth conditions

The plasmid pTB93F (Gage *et al*. 1996; kindly provided by Sharon Long, Stanford University), encoding GFP was introduced into competent *Agrobacterium tumefaciens* EHA 105 (Hood *et al*. 1993; kindly provided by Beth Hood, Prodigene Inc., College Station, TX, USA) using a freeze/thaw method as previously described (Hofgen and Willmitzer 1988). Transformants which grew on modified Luria–Bertani (LB) agar (Trick and Finer 1997) supplemented with 300 μg ml^{-1} spectinomycin (Sigma, St Louis, MO, USA) were screened for GFP expression with a Leica M8 stereo dissecting microscope equipped with a fluorescence module consisting of a 100-W mercury lamp and GFP Plus™ excitation and emis-

sion filters (Leica, Heerbrugg, Switzerland). This system permits visualization of green fluorescence due to GFP following excitation with blue light. For plant tissue inoculation, *Agrobacterium tumefaciens* EHA 105, containing the plasmid pTB93F, was grown in LB/spectinomycin broth overnight at 27 °C on a rotary shaker (150 rev min^{-1}), harvested by centrifugation (4000 *g* for 15 min), washed with liquid D40 medium (Santarem *et al*. 1998), recentrifuged, and adjusted to an $O.D._{600}$ of 0·15 with liquid D40 medium. D40 medium contained Murashige and Skoog salts (Murashige and Skoog 1962), B5 vitamins (Gamborg *et al*. 1968), 3% sucrose, and 40 mg l^{-1} 2,4-dichlorophenoxyacetic acid (pH 7·0). All media were autoclaved at 121 °C at 103 kPa for 17 min.

Inoculation and observation of soybean cotyledons

Freshly excised immature soybean cotyledons (*Glycine max* L. Merrill cv. Defiance) were isolated and prepared for sonication-assisted *Agrobacterium*-mediated transformation (SAAT) as previously described (Santarem *et al*. 1998). Ten cotyledons were placed in a 1·5-ml microcentrifuge tube containing 0·5 ml of the washed bacteria and sonicated 1·0 s in a bath sonicator (Model PC5, L & R Manufacturing Co., Kearny, NJ, USA). Cotyledons were then removed from the tube, blotted on sterile filter paper, and placed adaxial side (top surface) up on D40 medium, solidified with 0·2% Gelrite™ (Merck & Co., Rahway, NJ, USA) and supplemented with 100 μmol l^{-1} acetosyringone (Aldrich, St Louis, MO, USA). Cotyledons were incubated at 27 °C under a 23-h light photoperiod (30 $\mu Em^{-2} s^{-1}$). Control trials included cotyledons sonicated in liquid D40 only and cotyledons immersed in the bacterial culture but not sonicated. Cotyledons were observed and photographed at 3, 7, 18 and 36 h, after sonication. After 36 h of co-cultivation, cotyledons were washed with liquid D40 containing 400 μg ml^{-1} Timentin™ (SmithKline Beecham Pharmaceuticals, Philadelphia, PA, USA) and placed on D40/Gelrite medium supplemented with 400 μg ml^{-1} Timentin™ to eliminate the *Agrobacterium* from the plant tissue. Cotyledons were observed at 1, 2 and 3 d, and 1, 2, 4 and 6 weeks after the Timentin™ wash. Cross sections of 36 h post-SAAT cotyledons were prepared by slicing the tissue with a sterile scalpel from the abaxial to the adaxial edge (bottom to top). Cross sections were viewed using fluorescence microscopy immediately after cutting.

RESULTS

Bacterial GFP was initially detected, although faintly, 7 h after sonication (Fig. 1A). Eighteen hours following sonication, both small foci and large areas of bacteria were observed on the surface of the cotyledons (Fig. 1B). Although adaxial cotyledon surfaces were extensively colonized (as determined by intense GFP fluorescence) by 36 h (Fig. 1C), no GFP was detected on the surface of the cotyledons (abaxial) in contact with the plant tissue culture medium (Fig. 1D). In cross-sections prepared 36 h after inoculation, bacteria appeared to penetrate plant tissue four to five cell layers deep from the adaxial side of the tissue (Fig. 1E). SAAT-treated cotyledons were colonized sooner and more extensively than cotyledons which were not sonicated in the presence of the bacterium (Fig. 1F). Bacterial GFP could only minimally be detected in the cotyledons 3 d after transfer to the medium containing Timentin™ (Fig. 1G), with no GFP being detected by 5 d after transfer to the antibiotic-containing medium. In all experimental trials (six experiments, 100 cotyledons each), approximately 2·5% of *Agrobacterium*-treated cotyledons contained two to three small, localized foci of GFP on the surface 42 d after placement of the cotyledons on the Timentin™-containing medium (data not shown). No GFP was ever detected on the surface of control cotyledons sonicated in liquid D40 without bacteria. Using the Leica dissecting microscope equipped for GFP visualization, a lower limit of approximately 10 000 bacterial cells was required for detection (data not shown).

DISCUSSION

Although the plasmid used in this experiment, pTB93F, was initially designed for use in *Rhizobium*, transformation, replication and expression of pTB93F in *Ag. tumefaciens* was most efficient as GFP could be easily visualized both in bacterial colonies growing on solid media (Fig. 1H) as well as on plant tissue. Consistent elimination of *Agrobacterium* from transformed plant tissue has been problematic (Shackelford and Chlan 1996). Bacterial regrowth following antibiotic treatment poses particular problems when stably transformed tissues are evaluated for the gene of interest by either Southern analysis or PCR. In both cases, the analysis must be performed carefully to discriminate between DNA present in the residual bacteria or in the transformed plant tissues. Regrowth of *Agrobacterium* expressing GFP was easily detected as small, discrete, fluorescent foci in plant tissues as long as 6 weeks after transformation. GFP therefore provided a non-destructive tool to rapidly monitor cultures for the re-emergence of *Agrobacterium* under long-term plant cultural conditions.

When using SAAT to transform plant tissues, we were concerned that sonication of the bacterial culture might be detrimental to the bacterium, but in this study neither viability nor the ability to colonize and infect tissue appeared to be affected by the brief sonication treatment.

Electron microscopy studies can provide some information on the colonization and attachment of *Agrobacterium* to plant tissues (Matthysse *et al*. 1982; Graves *et al*. 1988; Starkbauerova and Srobarova 1993). Unfortunately, specimen preparation required by electron microscopy promotes tissue

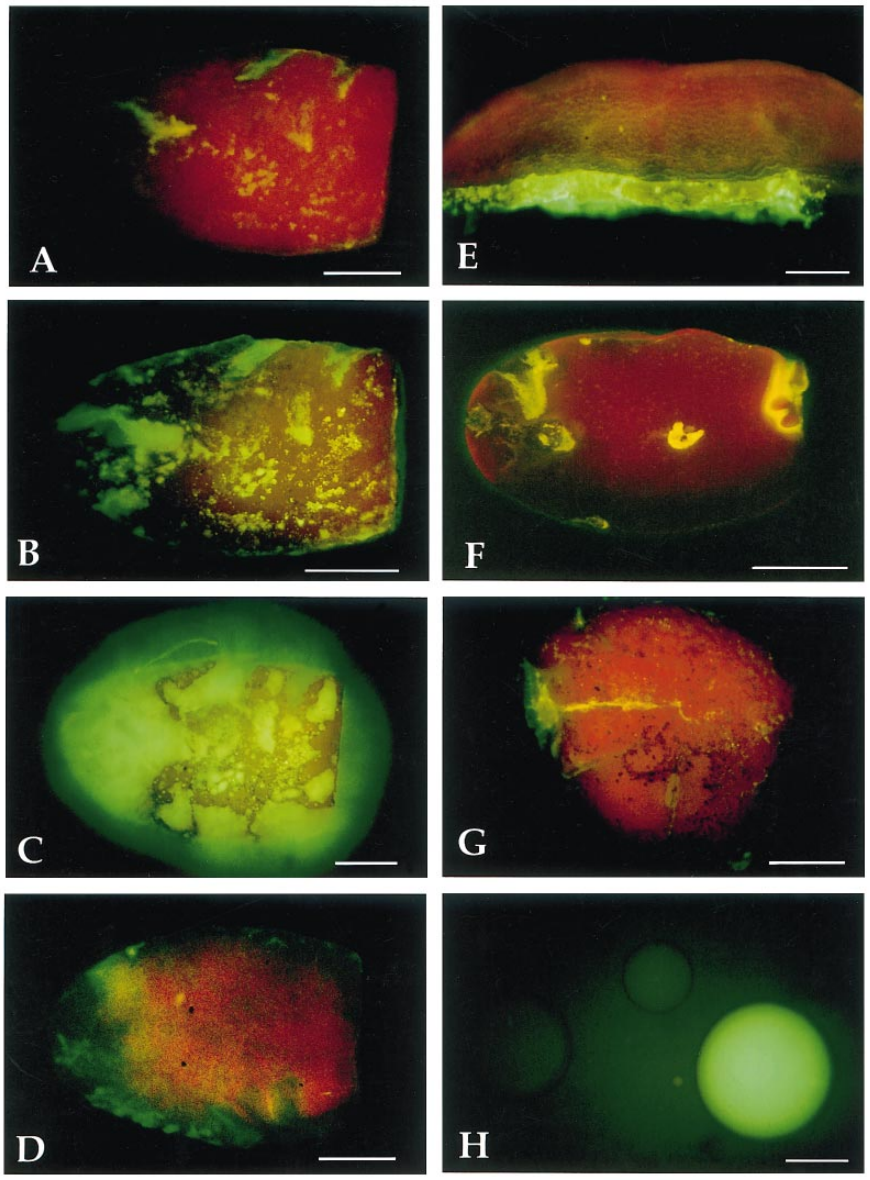

Fig. 1 (A) Soybean cotyledon 7 h after SAAT treatment, (B) 18 h post-SAAT, (C) 36 h post-SAAT. (D) Abaxial side of cotyledon, 18 h after treatment. (E) Cross-section of cotyledon, 36 h after SAAT treatment. (F) Unsonicated cotyledon, 18 h after exposure to the bacterium. (G) SAAT-treated cotyledon 3 d after Timentin™ wash. (H) colonies of *Agrobacterium tumefaciens* EHA 105 transformed with pTB93F. A–D, F, G, bar = 1 mm; E, bar = 500 μm; H, bar = 2·0 mm

disruption, produces specimen disorientation and prevents observation of the same sample over time. By monitoring bacterial colonization using GFP we observed that the organism did not colonize the abaxial (underside) surface of the tissue and would therefore not be sufficiently present to initiate *Agrobacterium*-mediated transformation of plant tissues. When using SAAT to introduce the plant expressible *GUS* gene in immature soybean cotyledons, Santarem *et al*. (1998) noted that the side of the tissue in contact with the medium, regardless of orientation, showed very limited GUS expression. Lack of transient gene expression in *Agrobacterium*-mediated transformation studies is often attributed to a fail-

ure to transfer T-DNA due to temperature influences (Holford *et al*. 1992; Fullner and Nester 1996; Dillen *et al*. 1997), pH effects inhibiting *vir* gene transfer (Vernade *et al*. 1988), or a lack of competent plant cells in a localized area (Villemont *et al*. 1997). Low oxygen availability on the underside of the cotyledon may inhibit growth of this aerobic bacterium leading to reduction or elimination of transformation. Although formation of the GFP chromophore is also oxygen dependent (Heim *et al*. 1994), we do not believe anaerobic conditions sufficient to eliminate GFP fluorescence existed underneath the cotyledons.

Increased transformation rates of soybean cotyledons using SAAT (Trick and Finer 1997; Santarem *et al*. 1998) are supported by our observations that sonicated cotyledons were more quickly and completely colonized than non-sonicated tissues. Sonication of cotyledons produces extensive microwounds in the surface of the plant tissue allowing increased access of the bacterium to plant cells. The bacterium is apparently able to extensively colonize infected plant cells (Trick and Finer 1997) resulting in high numbers of active bacteria that express GFP (Fig. 1C). The microwounded plant tissue may actually provide compounds that stimulate growth of the bacteria under aerobic conditions.

Although a lower limit of 10 000 bacteria was required for GFP detection, many fold higher bacteria are typically found in any given target tissue during a transformation experiment. GFP expression in a single bacterial cell can be observed using a different detection system (data not shown). However, the use of other detection systems with much higher magnification effectively eliminates our ability to observe the overall colonization of the cotyledon.

Successful genetic transformation of plants using *Agrobacterium* relies on optimization of a combination of several factors including plant tissue harvesting and manipulation, bacterial colonization and infection, T-DNA transfer, and post-transformation culture conditions. GFP can provide a simple, yet powerful tool for optimizing *Agrobacterium* colonization and infection of plant tissue, ultimately resulting in increased transformation frequency of plant tissues. In addition, bacterial GFP production can provide a visual means for monitoring elimination of the organism from transformed plant tissues.

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