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Transfer and Integration of T-DNA without Cell Injury in the Host Plant

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Agrobacterium colonizes plant cells via a gene transfer mechanism that results in plant tumorigenesis. Virulence (*vir*) genes are transcriptionally activated in the bacteria by plant metabolites released from the wound site. Hence, it is believed that agrobacteria use injuries to facilitate their entrance into the host plant and that the wounded state is required for plant cell competence for Agrobacterium-mediated gene delivery. However, our experiments using *vir* gene–activated bacteria sprayed onto tobacco plantlets demonstrated that cells in unwounded plants could also be efficiently transformed. The condition of the plant cells was monitored using β-glucuronidase under the control of a wound-inducible promoter. Infection of leaf tissue is light dependent, and it is drastically reduced when abscisic acid is exogenously applied to the plant. Under these experimental conditions, stomatal opening seems to be used by Agrobacterium to circumvent the physical barrier of the cuticle. These results thus show that the proposed cellular responses evoked by wounding in higher plants are not essential for Agrobacterium-mediated transformation.

INTRODUCTION

The Gram-negative soil bacterium Agrobacterium is the causative agent of crown gall disease in dicotyledonous plants. A T-DNA fragment, which is part of a large plasmid (pTi, or tumor-inducing plasmid) found in the infectious bacterial strain, leads to plant transformation by integration into the genome. Expression of T-DNA–encoded genes in transformed plant cells gives rise to tumors because their encoded products synthesize the plant growth hormones auxin and cytokinin (reviewed in Winans, 1992; Zupan and Zambryski, 1995). Because this interkingdom interaction results in gene transfer and the growth of crown gall cells for the benefit of virulent bacteria, the process has been named genetic colonization (reviewed in Tempé and Schell, 1977).

Two additional genetic components, referred to as virulence (*vir*) genes, are important for plant cell transformation (see Hooykaas and Beijersbergen, 1994). They include eight operons (*virA* to *virH*), located on the Ti plasmid, that encode the *trans*-acting factors responsible for the excision and transfer of the T-DNA into the plant cell nucleus, and a number of chromosomal genes (*chv* genes) that have been described as affecting bacterial virulence, of which some are involved in the perception of plant signals, whereas others are related to bacterial attachment to the plant cell.

It is well known that infection by Agrobacterium requires injury to the plant, whereas certain other pathogenic bacteria do not (see Billing, 1982). Two hypotheses, not mutually exclusive, can be invoked to explain why Agrobacterium-mediated tumorigenesis requires wounding (see Kahl, 1982; Stachel et al., 1986; Binns and Thomashow, 1988; Cangelosi et al., 1990): wounding allows invasion by activating specific receptor sites on the plant cell that facilitate host–pathogen interactions (the portal of entry hypothesis); and wounding mediates T-DNA competence because of metabolic activation of the plant cell (the conditioning hypothesis).

One key process in Agrobacterium-mediated tumorigenesis concerns the activation of *vir* genes, because with the exception of the *virA* and *virG* genes, they are normally not transcribed in free-living bacteria (Stachel and Zambryski, 1986). It was found that agrobacteria perceive plant-released compounds that activate virulence, such as the phenolic compound acetosyringone (AS) and sugars, which are abundant elements in plant wounds. This finding led to the idea that wounded plant cells are especially susceptible to Agrobacterium and that the response in the wounded plant cell, with the concomitant cell divisions, is required for T-DNA integration into the plant genome (Citovsky et al., 1992). However, the natural competence of particular plant cells for T-DNA has yet to be determined.

Using nonmanipulated tobacco plantlets, we show that competence for Agrobacterium-mediated transformation is not necessarily linked to wounding. Induced bacteria provided as a fine aerosol on the surface of leaves were able to transfer T-DNA into apparently intact mesophyll cells and cause tumors. In addition, we suggest that agrobacteria can enter leaves via their stomata. Requirements of plant cells for Agrobacterium infection are also discussed.

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RESULTS

Induced Agrobacteria Sprayed onto the Plant Surface Result in T-DNA Transfer and Tumor Formation

Our study analyzed whether Agrobacterium could transform unwounded plant cells. To avoid injury, we assayed T-DNA transfer with the minimum number of manipulations and delivered the bacteria onto the surface of tobacco plantlets in a fine aerosol suspension. Agrobacterium-mediated DNA transfer into the plant cell nucleus was monitored using a binary plasmid (pCG5) containing a modified β-glucuronidase (*GUS*) gene (Schultze et al., 1990), as T-DNA marker, that is active only in eukaryotic cells. A coresident wild-type Ti plasmid provided a natural marker for tumor formation in the plant.

The bacterial strain A348 (pCG5) was cultured in the presence of the *vir* gene inducer AS and sprayed onto plantlets of two different tobacco lines, SR1 and Wisconsin 38 (W38). After histochemical staining, a large number of plant cells, normally in cell clusters (here referred to as GUS spots), showed GUS activity (Table 1 and Figure 1A), indicating that T-DNA transfer had occurred efficiently. Tumors were subsequently recovered, showing that stable transformation had occurred (Figure 1C). Interestingly, infection of root cells was also observed occasionally, indicating that some of the sprayed droplets reached the surface of the agar medium and that the agrobacteria then moved through it toward the roots (Figure 1B). In contrast, the same bacterial strain A348 (pCG5), cultured in the absence of AS before spraying, resulted in very few plant cells with GUS activity (i.e., poor T-DNA transfer) and no tumor formation (Table 1). This indicated that spraying the plants with bacteria did not lead to concentrations of inducing compounds that were sufficient for the activation of the *vir* genes. As expected, the use of a bacterial strain lacking the Ti plasmid (strain A136 [pCG5]) or a bacterial strain defective in plant cell attachment (strain A6.1h [pCG5]) resulted in neither detectable GUS activity nor tumor formation (Table 1).

Table 1. Plant Infection by Spraying Agrobacteria onto the Surface of Tobacco Plantlets[a]

Bacterial Strain[b]	Culture Conditions	Number of GUS Spots[c] SR1	W38	Tumor Formation[d]
A348	Induced	556	162	+
A348	Noninduced	3	0	−
A136	Induced	0	0	−
A6.1h	Induced	0	0	−

[a] Data from a typical experiment are shown.
[b] All bacterial strains listed harbor pCG5, a binary plasmid with a modified *GUS* gene active only in eukaryotic cells. Induced cultures were grown in the presence of 200 μm AS (see Methods).
[c] Scores represent the number of plant cells expressing the T-DNA marker gene *GUS* (GUS spots) in 10 plantlets after an X-gluc assay (see Methods).
[d] Twenty plantlets per treatment were included in the tumorigenesis assay. (−) indicates that no tumors were observed on the plantlets 4 weeks after spraying; (+), at least one tumor was detected on every sprayed plantlet.

Two other control experiments showed that there was no interference between the two T-DNA elements used in our assays (data not shown): the use of a bacterial strain carrying a disarmed Ti plasmid (wild-type *vir* genes but no T-DNA) together with the binary plasmid pCG5 led to GUS-positive plant cells at a frequency similar to that observed with the wild-type strain; and the number of tumors produced by strain A348 was independent of the presence of the binary plasmid pCG5 in the same bacterial cell.

AS-induced bacteria sprayed onto W38 plantlets gave rise to twice as many GUS spots if the plants were stabbed with needles (see Methods) before inoculation (Table 2). Efficient T-DNA transfer and transformation required the activation of the bacterial *vir* genes by induction with AS and/or injury of the plant cells before inoculation (Table 2). These two *vir* gene activation procedures resulted in a distinct pattern of plant cell infection, as deduced from the distribution of GUS spots (i.e., plant cell clusters showing GUS activity) over the sprayed leaf surface: AS-induced agrobacteria produced a fine array of numerous blue spots on unwounded plants, whereas bacteria sprayed onto injured plants (stabbed with needles) resulted in large patches of blue spots predominantly around the wounded areas (Figure 2). GUS quantification (determined by the 4-methylumbelliferyl β-D-glucuronide assay; see Methods) showed that reporter gene activity was superior if the plant tissue was wounded before infection (Table 2). Nevertheless, unwounded plantlets still showed a high level of competence for T-DNA when they were sprayed with AS-induced agrobacteria.

The number of tumors observed was in all cases high, except when uninduced bacteria were sprayed on unwounded plantlets (Table 2). Although tumor formation is not as quantitative as the histochemical GUS assay, because a few independent transformation events (detected as small separate GUS spots) close to each other could give rise to plant cell overgrowth that would be scored as a single tumor, it is a stable transformation assay and thus is a reliable measurement of genomic T-DNA integration into plants. These results clearly show that agrobacterial infection of tobacco is largely independent of plant cell wounding.

One week after defined tumors on the surface of the sprayed plant tissue had been scored, the hormone-independent growth of the transformed plant cells was tested by culturing the tumors on standard Murashige and Skoog (MS) agar medium. In vitro tumor growth, in the absence of plant hormones (Figure 1D), and GUS activity in most of the tumors (Figure 1E) indicated that tumorigenic cells expressed the T-DNA–encoded genes (i.e., the oncogenes present on the Ti plasmid and *GUS* on the binary plasmid). All of the tumors tested were

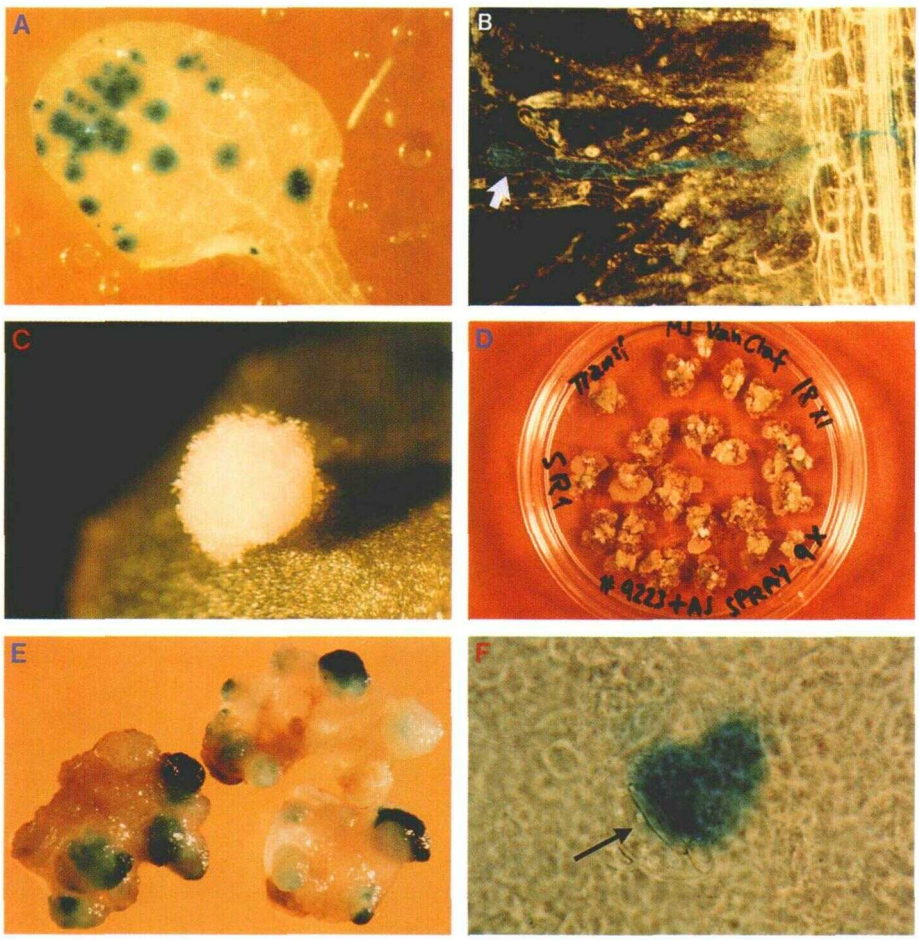

Figure 1. T-DNA Transfer and Integration in Tobacco Plantlets Sprayed with an Aerosol of Induced Agrobacterium Cells in Suspension.

(A) and **(F)** GUS activity in infected leaf cells. Note the stoma (arrow) above those mesophyll cells expressing *GUS* in **(F)**.
(B) GUS activity in a root hair cell (arrow) detected 3 days after spraying with bacteria.
(C) Appearance of tumors 3 weeks after spraying.
(D) Hormone-independent growth of tumors cultured on Murashige and Skoog (MS) medium.
(E) GUS activity observed in most of the tumors cultured on MS medium.

positive for octopine (data not shown). These results indicate that the galls observed on the plant leaves were due to the integration of the oncogenic T-DNA into the plant cell genome and its subsequent expression.

Spraying Induced Agrobacteria onto Plants Does Not by Itself Trigger a Wound Response

As shown in Tables 1 and 2, W38 tobacco plants were susceptible to T-DNA transfer from AS-induced, sprayed Agrobacterium strain A348 (pCG5). These results led us to suggest that agrobacteria are able to interact with undamaged plant cells and eventually to transform them. To test whether our experimental conditions resulted in wounding of plant cells, we used W38 transgenic tobacco plants in which *GUS* expression is controlled by a potato proteinase inhibitor II (*PIP*) promoter. These plants are referred to throughout as PIP–GUS. This *PIP* promoter has been shown to be active in both wounded and nonwounded leaves of plants that were wounded elsewhere (systemically induced) and stems of transgenic tobacco (Keil et al., 1989), making it a convenient indicator of wounding.

Neither the spray of aqueous solutions (10 mM $MgSO_4$ or M9 minimal medium, pH 5.5, and 0.2 mM AS) nor the spray of an AS-induced bacterial suspension of A348 cells (devoid in this case of pCG5) onto W38 carrying the *PIP–GUS* transgene activated the wound-inducible promoter (see Figure 3A). In contrast, injury of plant cells or the addition of methyl jasmonate, which is known to lead to rapid accumulation of

Table 2. Agrobacterium Transformation by Spraying Wounded and Unwounded W38 Tobacco Plantlets

Bacterial Culture[a]	Wounding[b]	GUS Assay[c] Number of GUS Spots	Enzymatic Activity	Number of Tumors
Induced	+	421	1000	134
Noninduced	+	317	150	102
Induced	−	188	75	127
Noninduced	−	2	1	1

[a] The wild-type A348 bacterial strain carrying the binary plasmid pCG5, which contains a *GUS* gene as T-DNA marker that is not expressed in bacteria, was used. Bacterial cultures were induced with 200 μM AS before spraying (see Methods).

[b] Wounding (+) was performed by stabbing the plantlets with needles (see Methods); (−) indicates intact plantlets.

[c] GUS spots represent scores after an X-gluc assay, as given in Table 1. GUS enzymatic activity was estimated by using the 4-methylumbelliferyl β-D-glucuronide assay (see Methods). The measured activity of AS-induced bacteria sprayed onto wounded plantlets was normalized to 1000. Scores correspond to 10 plantlets per treatment. Similar results were obtained from at least three independent experiments.

proteinase inhibitor proteins in leaves (Farmer and Ryan, 1990), to the in vitro plant culture medium resulted in GUS activity in cotyledons, leaves, and stems (data not shown). Besides cutting and stabbing the plant tissue, microprojectile bombardment with gold particles (~1.6 μm in diameter) was used to test the effects of microwounding on the PIP–GUS plantlets. This treatment resulted in the typical local as well as systemic induction of the wound response (Figure 3B). Even just a few gold particles, mainly reaching the first two cell layers of the leaf tissue, were sufficient to induce the *PIP–GUS* gene (Figure 3C). Apparently, a very subtle damage, affecting only a few cells in the plant, could trigger a wound response. This confirmed the suitability of these PIP–GUS plants for use in our tests. Mock bombardment of W38 PIP–GUS plantlets without gold particles did not induce marker gene activity (data not shown). From these results, we conclude that plant cell wounding, as detectable by the activation of a wound-inducible promoter, is not a requirement for either the transfer or integration of T-DNA into plant cells.

Sprayed Agrobacteria Infect the Leaf Tissue When Stomata Are Open

In the experiments described above, wounding was not the likely entry point for agrobacteria. An alternative entry route was stomata. These structures are known to link the otherwise impermeable plant surface with interior cell layers. To test whether stomata may be used by agrobacteria, we decided to spray plants that had been kept in either light or dark with bacterial suspensions. Light is one of the natural mechanisms directly controlling the opening of stomatal guard cells (reviewed in Sharkey and Ogawa, 1987). Conditions of either continuous white light or darkness were maintained for the 3 days of cocultivation before T-DNA transfer or tumorigenesis was assayed. Microscopic observation of leaf epidermal strips confirmed that the majority of stomata were open in our plants sprayed with agrobacteria in the light, whereas they were closed in the plants maintained in the dark (data not shown). Spraying of the A348 (pCG5) strain showed that successful infection with preinduced Agrobacterium, as determined by the number of GUS spots and galls, was dependent on maintaining the plants in light, whereas cocultivation in the dark for 3 days dramatically reduced the incidence of transformation (Table 3).

To test whether the dark cocultivation conditions negatively influenced the efficiency of plant infection by Agrobacterium, infection of plant cells after wounding with needles was assayed (see Methods). T-DNA transfer and tumorigenesis were as efficient in the dark as they were in the light (data not shown), confirming that the bacterial spray renders the infection process light dependent.

Microscopic examination of sprayed plantlets revealed that plant cells expressing *GUS* were often located in the mesophyll layers and in all cases underneath a stoma (Figure 1F). Because the density of stomata in cotyledons and first leaves at this developmental stage in tobacco is high, and the distribution of sprayed droplets with bacterial suspension reaching the plant surface is uncontrolled, we could not analyze statistically whether the association between a particular GUS-positive patch of cells or their proximity to a particular stoma was fortuitous. Agrobacteria could be seen occasionally interacting with intact root cells (Figure 1B), obviously without the need for stomata but in the absence of the protecting cuticle.

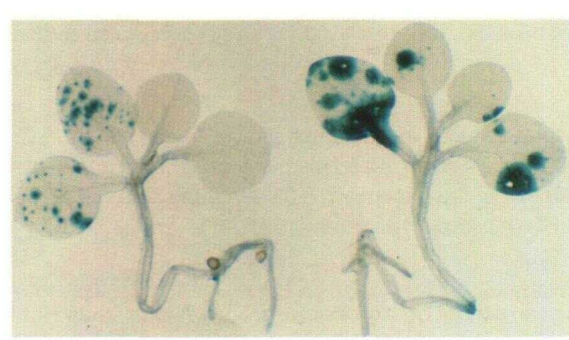

Figure 2. Pattern of Blue Spots after Histochemical Staining with X-Gluc in W38 Tobacco Plantlets That Were Sprayed with Agrobacterial Suspensions.

(Left) AS-induced bacteria sprayed onto intact plantlets.
(Right) Uninduced bacteria sprayed onto wounded (stabbed) plantlets. Note the different pattern of blue staining representing plant cells expressing the T-DNA reporter gene *GUS*.

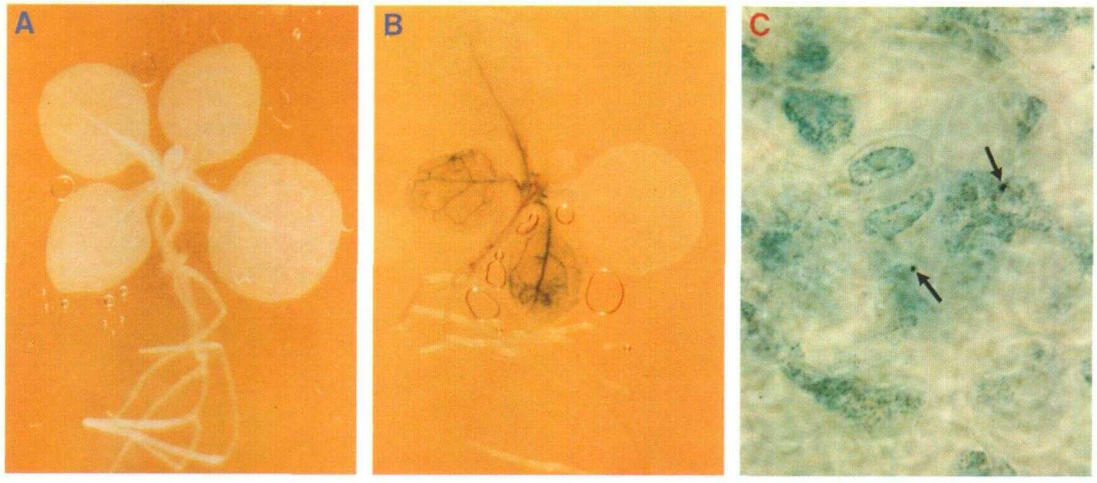

Figure 3. Test for Wounding in Transgenic (PIP–GUS) W38 Tobacco Plants.

(A) Spraying of an Agrobacterium cell suspension does not induce the *PIP* wound-inducible promoter (no GUS activity detected).
(B) Effect of wounding by accelerated delivery of gold microparticles: expression of the *PIP–GUS* gene results in local as well as systemic GUS activity.
(C) Detail of **(B)** showing plant cells with GUS activity and 1.6-μm gold particles (arrows) responsible for the local activation of the *PIP* promoter on the leaf surface.

The plant growth regulator abscisic acid (ABA) is also known to mediate stomatal closure (see Mansfield and McAinsh, 1995). The exogenous addition of ABA to the tobacco plantlets was tested to determine whether it would influence the number of plant cells infected by sprayed agrobacteria. A 100 μM aqueous solution of ABA was sprayed directly onto the surface of tobacco plantlets 2 hr before the bacterial aerosol was administered. This resulted in closure of most of the stomata in the upper epidermis (data not shown). The ABA treatment was subsequently repeated every 12 hr during the first 2 days of bacteria–plant cocultivation. As a control, tobacco plantlets were sprayed with sterile water. Table 3 shows that plantlets sprayed with ABA, irrespective of their genetic backgrounds (SR1 or W38), had decreased Agrobacterium infection levels. However, plantlets sprayed with water did not show significant variation in the number of plant cells expressing the T-DNA reporter gene when compared with plantlets that had not been sprayed (data not shown). ABA was able to trigger stomatal closure in sprayed plants and thus limited the access of agrobacteria to their target cells. Altogether, these results show that under our experimental setup, Agrobacterium can use open stomata to infect plant cells in leaf tissue.

DISCUSSION

An important issue in the Agrobacterium–plant relationship is that the wounding of the plant is essential for successful infection (see Kahl, 1982). It has been hypothesized that the removal of physical barriers at the wound site allows exposure of specific receptor sites to the bacteria, thus facilitating the contact between virulent bacteria and the middle lamella of the host's cell wall (Lippincott et al., 1977). Most dicotyledonous plant species respond to injury by synthesizing

Table 3. Influence of Light and ABA on Infection after the Spraying of Agrobacteria onto the Surface of Tobacco Plantlets

Tobacco Line	Light Treatment[a]	ABA Treatment[b]	No. of GUS Spots[c]
SR1	−	−	0
	+	−	476
	+	+	8
W38	−	−	6
	+	−	143
	+	+	2

[a] Plantlets were kept in the dark (−) or under 2000 lux (+).
[b] A 100 μM aqueous solution of ABA (+) or sterile water (−) was repeatedly sprayed over the plantlets.
[c] GUS activity was scored as given in Table 1. Scores represent estimates of T-DNA transfer as the number of plant cells expressing *GUS*. Data correspond to 10 plantlets per treatment. The wild-type A348 bacterial strain, carrying a *GUS* gene as a T-DNA marker that was not expressed in bacteria, was used. Bacterial cultures were induced with 200 μM AS before spraying. Light and ABA treatments were maintained during cocultivation with agrobacteria. Similar results were obtained from three independent experiments.

phenolic compounds, such as AS, and by initiating adventitious cell divisions. Several reports (reviewed in Binns and Thomashow, 1988) have suggested that this wound healing in the plant triggers, at the cellular level, a sequence of reactions that is important for T-DNA–induced transformation. Hence, Agrobacterium could be described as a pathogen taking advantage of plant wounding and cell proliferation induced by it (i.e., DNA replication). It reacts to wound-excreted signals and may use enzymes needed for DNA replication for the integration of the T-DNA (discussed by Citovsky et al., 1992; Tinland and Hohn, 1995).

Results from this study, however, show that injury is not essential for the Agrobacterium-mediated transformation of plants. Experiments described here using induced bacteria sprayed onto tobacco show clearly that the transfer and genomic integration of T-DNA into undamaged plant cells do occur, as determined by the absence of activation of a wound-inducible promoter. Thereby, wounding had to be "replaced" by preinduction of bacteria and inoculation of the plant under conditions in which bacteria could enter intact leaf tissue. Apparently, under these circumstances, stomata can be used by agrobacteria to gain access to cells in the mesophyll layers underneath. However, this infection mechanism is probably rare for Agrobacterium as a rhizosphere inhabitant in the wild. Although similar observations have been reported for other bacterial species (for instance, in necrotic diseases, such as leaf spot, *Pseudomonas* spp invades the plant host tissue via stomata; Panopoulus and Schroth, 1974), Agrobacterium is special in being able to use this route for infection and transformation. Hence, the competence of plant cells for Agrobacterium-mediated DNA transfer is not necessarily linked to cell damage. T-DNA integration, therefore, does not absolutely need the triggering, by wounding, of specific DNA-metabolic activities in the plant cell. This indicates that the well-known requisite of a wound for transformation is probably a special sensory attraction that agrobacteria developed to recognize a natural niche: the presence of target plant cells.

During cocultivation experiments, exhaustive wounding of plant tissue has been observed to increase the number of plant cells expressing T-DNA–encoded *GUS*. This occurred in tobacco plantlets (Z. Koukolíková-Nicola and J. Escudero, unpublished data) as well as in maize plantlets (Shen et al., 1993), despite the fact that AS was used as a bacterial virulence inducer. In this study, wounding rendered between approximately two and 13 times as much bacterial infection as when an aerosol of induced agrobacteria was used in the absence of wounding. This phenomenon could be explained by the attraction of Agrobacterium to wounded sites in the plant, the very efficient activation of *vir* genes in bacteria precisely at the wounded infection site, and higher access of bacteria to plant cells in wounded plants compared with intact plants.

This report describes a significant finding on the competence of plant cells to Agrobacterium and a novel process in this bacterial colonization, which shows that agrobacteria can transform nontraumatized host cells. It has been suggested recently that a host cell cycle control mechanism of T-DNA transfer exists in petunia plants treated with phytohormones (Villemont et al., 1997). Because our results show that nondividing, intact mesophyll cells can take up and integrate T-DNA, plant cells might posses a mechanism for regulating DNA repair and/or recombination, which would be either constitutive or pathogen induced. It remains to be determined how infection of unwounded plants by Agrobacterium occurs at the cellular level.

METHODS

Bacterial Strains and Plasmids

Agrobacterium tumefaciens strains are listed in Table 4. Bacteria were generally maintained in YEB medium (Vervliet et al., 1975) with appropriate antibiotics (rifampicin, 5 to 20 μg/mL; kanamycin, 50 μg/mL; gentamycin, 20 μg/mL) at 28°C. Plasmid DNA constructions were maintained in *Escherichia coli* DH5α and manipulated as described previously (Sambrook et al., 1989). Plasmid transfer to Agrobacterium was done by electroporation (Cangelosi et al., 1991). The artificial T-DNA in plasmid pCG5 (Shen et al., 1993) contains a β-glucuronidase (*uidA* or *GUS*) gene (Jefferson et al., 1987) that is not expressed in bacteria because it contains a translational fusion between the *GUS* gene and open reading frame five from cauliflower mosaic virus (Schultze et al., 1990).

Plant Material

Tobacco (*Nicotiana tabacum*) cultivar Petit Havana SR1, cultivar Wisconsin 38 (W38), and W38 plantlets carrying a potato proteinase inhibitor II promoter (*PIP*)–*GUS* transgene (J. Sánchez-Serrano, CNB, Universidad Autonoma Madrid, Spain; unpublished line B1239-29) were used in this study. The seeds were germinated on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) under sterile conditions, and 10 to 20 days after germination, plantlets were assayed for infection with agrobacteria. Plants were maintained in vitro within plastic boxes in a growth chamber with a 16-hr-light (2000 lux) and 8-hr-dark regime at 25°C.

Plant Handling and Infection

Wounding of otherwise nonmanipulated whole plantlets was done either by stabbing the plantlets with a 9-cm disk equipped with multiple needles (such as those used for the replica plating of bacteria) or using a particle gun device activated with gas (Biolistic PDS-1000/He particle delivery system; Bio-Rad) and delivering of gold microparticles (~1.6 μm in diameter).

Initial bacterial cultures were grown in YEB liquid medium at 28°C at 250 rpm for ~16 hr and then washed and diluted to an OD_{600} of 0.5 in M9 minimal medium (Sambrook et al., 1989) for subsequent 10-hr culture (the bacterial titer normally doubled during this period). Bacteria were collected by centrifugation, washed with 10 mM $MgSO_4$, and diluted to 10^9 colony-forming units per mL (OD_{600} of 1) before being sprayed onto the plants. The M9 medium used was either ad-

Table 4. *Agrobacterium tumefaciens* Strains

Bacterial Strain	Reference	Ti Plasmid[a]	Relevant Characteristics	Tumorigenicity[b]
A136	Garfinkel et al. (1981)	None	C58 chromosome, pTi cured	−
A348	Garfinkel et al. (1981)	pTiA6	A136 with wild-type octopine pTi	+
A6.1h	Thomashow et al. (1987)	pTiA6	A6 strain, chromosomal *pscA*::Tn*5* insertion, attachment deficient	−

[a] Besides the Ti plasmid, the binary plasmid pCG5 was maintained in the bacterial strains.
[b] The ability of a particular strain to produce tumors when inoculated into wounded plants is indicated by the (+). The (−) indicates no tumor formation.

justed to pH 5.5 and supplemented with 0.2 mM acetosyringone (AS) (referred to as inducing medium) or adjusted to pH 7 without AS (referred to as noninducing medium).

To test the activation of the *PIP* promoter by spraying, three kinds of aerosols were tested: (1) a 10 mM $MgSO_4$ solution; (2) M9 minimal medium, pH 5.5, and 0.2 mM AS; and (3) Agrobacterium (strain A348) suspensions, which were prepared as specified above. Approximately 200 μL of aerosol per plantlet was sprayed using sterile Erlenmeyer glassware on a sterile bench. A manual soft pump device (made from rubber like the ones used in cosmetics) was used to spray the liquid from a distance of 15 cm above the plantlets. A green safety light was used when experiments were performed in the dark. The upper epidermis from plants kept in either light or dark was painted with commercial nail polish, and the replicas were examined to determine the state of the stomata.

When required, methyl jasmonate (Sigma) was included in the MS plant culture medium at 50 to 100 μM in tight boxes to avoid interference effects. Transgenic W38 plantlets were assayed for activity of the *PIP–GUS* gene product 12 or 24 hr after treatment (e.g., with aerosol or methyl jasmonate or by wounding) in several series.

Abscisic Acid Application

Abscisic acid (ABA; 100 μM) solutions in sterile water were applied to the surface of tobacco plantlets by using the same aerial spray setup that was routinely used for bacteria. ABA was provided, under sterile conditions, 2 hr before the plantlets were sprayed with Agrobacterium suspensions, and this hormone treatment was repeated every 12 hr during the first 2 days after spraying.

Bacteria–Plant Cocultivation

Before Agrobacterium was provided, the plantlets were kept at 25°C in either continuous white light (2000 lux) or continuous darkness for 4 hr. After infection, these conditions were maintained for an additional 3 days before the determination of T-DNA transfer frequencies by histochemical GUS staining (see below). Plantlets used in the tumorigenesis assay were transferred after 3 days to the alternate 16-hr-light and 8-hr-dark regime before scoring. Plants were otherwise kept aseptically in vitro during the entire experimental period.

GUS Activity Assays

After 3 days of cocultivation with bacteria, plants were treated for histochemical analysis of GUS activity by using the X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) staining as described previously (Escudero et al., 1995). The number of GUS spots (i.e., plant cell clusters showing GUS activity) on unwounded plants was scored as individual blue spots appearing after GUS staining. The number of GUS spots on wounded plants was estimated by taking the smallest blue spot observed in that particular tissue as a reference unit. Simultaneous with the X-gluc assay, GUS enzymatic activity was more precisely quantified, at high concentration ranges, by the fluorometric 4-methylumbelliferyl β-D-glucuronide assay, as described by Rossi et al. (1993).

To minimize wound induction of the *PIP–GUS* transgene as a consequence of manipulation, transgenic PIP–GUS plantlets were fixed in 2% formaldehyde, 50 mM Na_3PO_4, pH 7, and 1 mM EDTA for 30 min at room temperature and then rinsed three times in the same buffer without formaldehyde before GUS staining.

Tumorigenesis Test

Four weeks after Agrobacterium was provided, the number of visible galls on the surface of living plantlets, grown on MS agar medium without phytohormones, was scored. The plant tissue containing tumors was then excised, washed in MS medium containing cefotaxime and vancomycin (both at 500 μg/mL), and placed on solidified (0.8% agar; Difco, Detroit, MI) MS medium with the same antibiotics to test hormone-independent growth for an additional 3-week period. Tumorigenic tissue was tested for opine production (Petit et al., 1983) and GUS activity (see above).

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