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Proximity of *Agrobacterium* to living plant tissues induces conversion to a filamentous bacterial form

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Abstract Changes in *Agrobacterium* colony and cell morphology were observed following co-culture of the bacterium with a variety of different plant tissues. Bacterial colonies grown in the presence of plant tissue became opaque and appeared to grow as a thick mat of cells. A single bacterial colony would often grow to fill an entire 100-mm-diameter petri dish. Ultrastructural observations of the bacteria in these colonies revealed the formation of a predominantly filamentous form of the bacterium. The bacteria ranged from 5 to 100 μm in length as compared to 2 μm in the non-filamentous form. The filamentous form was observed 2–3 days after co-culture and only if the bacteria were either in direct contact with or in close proximity (<5 mm) to living plant tissues. The filamentous form was observed with both wild-type and engineered *Agrobacterium* strains. As proximity of the bacteria to living tissue was necessary for induction, plant tissues apparently produce inductive compounds that are either very labile or have their effect only at high concentrations.

Keywords *Agrobacterium* · Filamentous form · Swarming · Transformation · Bacterial morphology

Introduction

Agrobacterium tumefaciens is a gram-negative, soil-borne plant pathogen that has been used extensively over the past 15 years as a vector for the transformation of plant cells. Since the first report of transformation of tobacco, tomato and petunia plants using *Agrobacterium* (Horsch et al. 1985), there have been numerous reports of *Agrobacterium*-mediated transformation of many other plants (Hinchee et al. 1988; McGranahan et al. 1988; Bechtold et al. 1993), including rice (Hiei et al. 1994), wheat (Cheng et al. 1997) and maize (Ishida et al. 1996), which are not considered to be natural hosts for this bacterium. The molecular aspects of the T-DNA transfer process were studied and reviewed by Zambryski (1988), and enhancements in transformation rates were subsequently found to result from using bacteria modified to either contain additional copies of *vir* genes (Hiei et al. 1994) or express the *vir* genes constitutively (Hansen et al. 1994). In addition, acetosyringone (Stachel et al. 1985), which is a powerful inducer of some of the *vir* genes, is often used to increase transformation efficiency.

Although much is known about the molecular biology of T-DNA processing and transfer, very little is known about the physical relationship between the bacterium and the target host cell. Early studies on *Agrobacterium* binding indicated that the bacterium primarily infects wounded plant tissue (Lippincott and Lippincott 1969) and apparently binds to a component of the middle lamella (Rao et al. 1982). Bacterial attachment may be aided by the production of cellulose fibrils (Matthysse 1983), which appear to anchor the bacteria to the host cell. In a more recent study, *Agrobacterium* cells were shown to rapidly colonize infected, partially wounded plant cells and possibly transform the adjacent intact cells (Trick and Finer 1998). 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 *Agrobacterium* colonization and infection, the initial events upon which gene transfer between plant and host are ultimately dependent.

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In this study, a filamentous form of *Agrobacterium* was observed following inoculation of a variety of plant tissues with both wild-type and engineered bacterial strains. The filamentous form was initially observed 2–3 days following co-culture of the bacterium with the plant tissue, only when cultured on a plant tissue culture medium. Changes in colony morphology were also associated with conversion to the filamentous form.

Materials and methods

Agrobacterium preparation

Agrobacterium tumefaciens EHA105, kindly provided by Elizabeth Hood (Hood et al. 1993) was used for the majority of experiments in this work. Bacteria were grown in Luria-Bertani (LB) liquid medium with no antibiotics for 18–24 h at 27°C until an OD_{600nm} of approximately 0.8 was reached. Bacterial cultures were centrifuged at 4,300 *g* for 10 min, the supernatant was discarded and the bacteria were resuspended in liquid medium (OMS-liquid) containing MS salts (Murashige and Skoog 1962), B5 vitamins (Gamborg et al. 1968) and 3% sucrose (pH 5.7). Other *Agrobacterium* strains that were evaluated for induction of the filamentous form included A281, C58, B6-806, LBA4404, 15955 (all *A. tumefaciens*) and R1000 (*A. rhizogenes*).

Plant tissue preparation

Cotyledons from immature embryos of soybean (*Glycine max* cvs. Jack and Defiance) were used as the co-culture tissue for most of this work. Immature pods were collected from greenhouse-grown soybean plants at about 14–21 days post-pollination. Pods were surface-sterilized in a 20% commercial bleach solution containing 0.5% Tween-20 for 20 min and washed four to five times with sterile, distilled water. Seeds measuring 3–5 mm were aseptically excised, and the cotyledons were removed for culture as described previously (Santarém et al. 1997). Soybean cotyledons were co-cultured with the *Agrobacterium* on OMS medium solidified with 0.2% Gelrite (Schweizerhall, South Plainfield, N.J.).

To evaluate the effects of tissue viability on the induction of the filamentous form of *Agrobacterium*, excised immature cotyledons were pretreated with low and high temperatures. Prior to co-culture, four excised cotyledons were placed in a 1.5-ml microfuge tube and placed at either –80°C, –20°C, 4°C or 65°C for 1 h.

In a series of experiments designed to evaluate the effects of different soybean tissues on the induction of the filamentous form of *Agrobacterium*, immature zygotic embryos were either left intact or the cotyledons were removed and used for co-culture. In addition, leaves, cotyledonary tissue and stem tissues were excised from 12-day-old aseptically germinated seedlings (seeds sterilized as described above and germinated on OMS medium solidified using 0.2% Gelrite). Proliferative embryogenic D20 soybean tissue, initiated and maintained as described earlier (Santarém et al. 1997), was also evaluated.

Arabidopsis leaves and roots were obtained from 2-week-old seedlings. Seeds were sterilized as described above and germinated on OMS medium solidified with 0.2% Gelrite.

Embryogenic callus of wheat (*Triticum aestivum* L. cv. Bob White) was initiated and maintained following the culture of immature embryos on a medium containing MS salts, B5 vitamins, 3% sucrose and 1 mg/l 2,4-D (2,4-dichlorophenoxyacetic acid) (Weeks et al. 1993).

Embryogenic callus of Ohio buckeye (*Aesculus glabra* Wendl.) was initiated and maintained following the culture of seedling hypocotyl tissue on a medium containing MS salts, B5 vitamins, 3% sucrose, 2 mg/l 2,4-D, 2 mg/l kinetin and 0.2% Gelrite (pH 5.7) (Trick and Finer 1999).

Although wheat cultures were maintained at 27°C in the dark, the remaining cultures were maintained at 27°C under a 23-h (day) photoperiod at a light intensity of 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Inoculation and co-culture

Plant tissues (described above) were inoculated with a 2- μl suspension of *Agrobacterium* resuspended in OMS-liquid medium. For most experiments, the *Agrobacterium* suspension was placed directly on top of the plant tissue. In some cases, the tissues were dipped into the *Agrobacterium* suspension for 1 min and then blotted on dry filter paper before co-culture. To evaluate proximity effects, we placed a droplet of *Agrobacterium* suspension on the medium at 3, 8, 15 and 24 mm from the excised cotyledonary tissue.

To evaluate co-culture medium effects, immature cotyledons of soybean were inoculated with *Agrobacterium* and placed on OMS or LB medium, with and without 100 μM acetosyringone, at either pH 5.7 or pH 7.

Microscopy

For the majority of observations on the filamentous form, samples of bacteria were placed on a glass microscope slide, covered with a cover slip and viewed on an Olympus IMT-10 inverted microscope using the 40 \times objective. For collection of light microscopic images, slides of bacteria were observed and photographed using an Olympus BX 60 microscope equipped with Nomarski DIC optics.

For scanning electron microscopy, bacteria were blotted onto 13-mm polycarbonate track-etch screen membrane filters (Osmonics, Livermore, Calif.), fixed in 0.1 *M* potassium phosphate buffer (pH 7.4) containing 3% glutaraldehyde, 2% paraformaldehyde and 1.5% acrolein for 0.5 h at room temperature and then washed three times with 0.1 *M* potassium phosphate buffer (pH 7.4). Samples were then dehydrated in an ethanol series (12.5–100% ethanol, 10 min each step), critical point-dried, sputter-coated with platinum and viewed on an ISI40 scanning electron microscope as described earlier (Trick and Finer 1997).

Results and discussion

Agrobacterium, co-cultured in the presence of soybean cotyledons, showed marked differences in colonial morphology and structure compared with bacteria cultured on media without plant tissue (Fig. 1). These differences were first apparent after 2–3 days of co-culture. Bacterial colonies grown on bacterial culture medium in the absence of plant tissue grew to 1–2 mm in diameter, with a uniform surface density (Fig. 1A). Bacterial colonies grown in the presence of plant tissue on a plant tissue culture medium grew much larger, were darker in color and displayed a large variation in surface opacity (Fig. 1B; note difference in scales). In the presence of a single cotyledon, the colony often grew to fill a 100-mm-diameter Petri dish after 1–2 weeks (Fig. 1C, left), whereas bacterial colonies which were not cultured in the presence of plant tissue showed no or minimal growth and were translucent (Fig. 1C, right). The rapidly proliferating, co-cultured bacterial colonies grew 1.5–6 mm outwardly per day. Although the edge of the growing colony was smooth, the colony surface was convoluted, at approximately 1 mm behind the growth front (Fig. 1D).

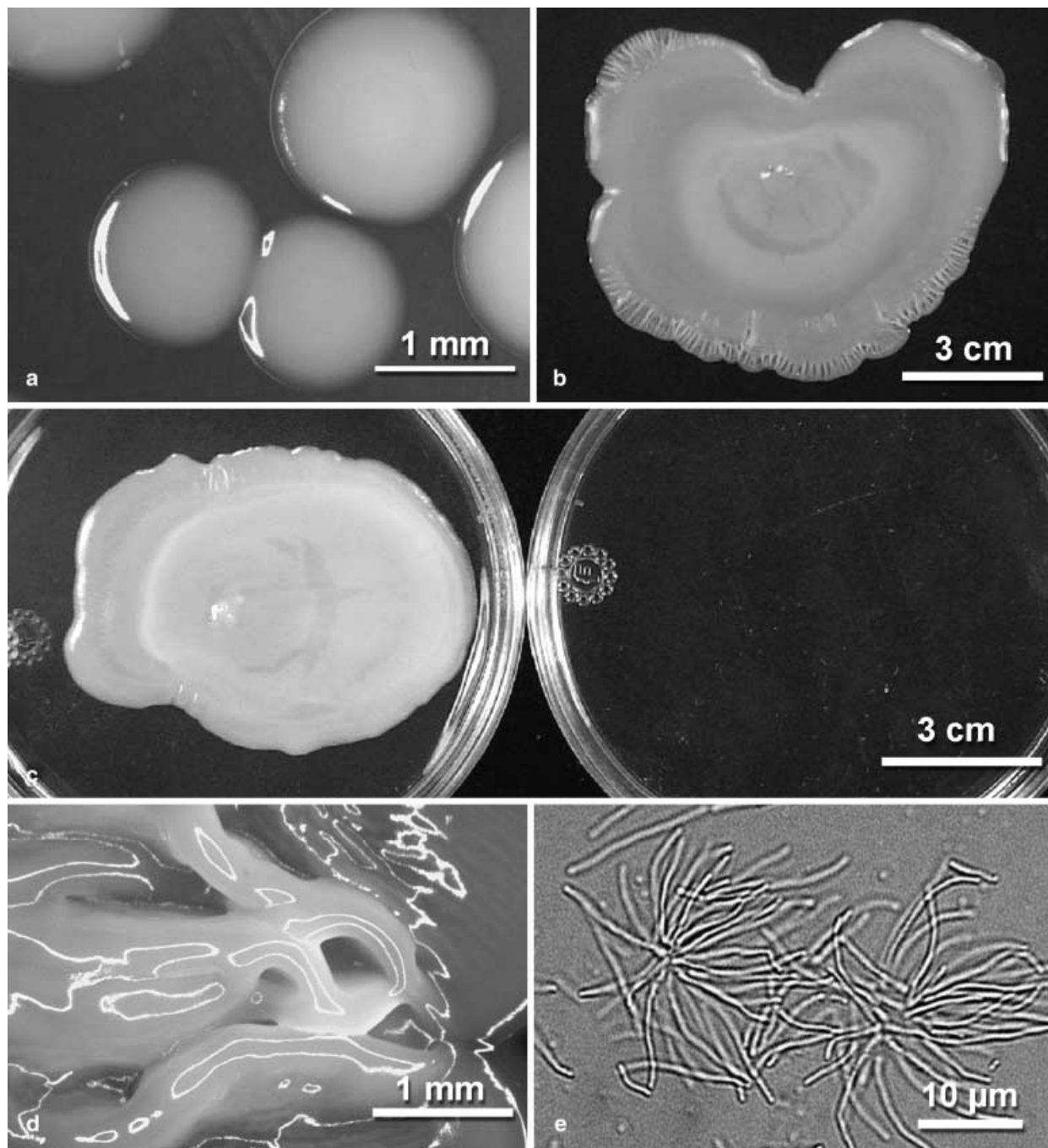


Fig. 1A–E Bacterial morphology. **A** Colony of *Agrobacterium* cultured on LB medium. **B** Formation of swarming bacterial colony after co-culture with soybean immature cotyledon. **C** Bacterial growth following inoculation of bacteria into 100-mm petri dishes containing a single immature soybean cotyledon (*left*) and no plant tissue (*right*). **D** Higher magnification of **B** showing convolution at the periphery of the colony. **E** Light micrograph of filamentous bacteria

Observations of the bacteria in these rapidly growing colonies using the light microscope revealed an unusual bacterial morphology. The bacteria were quite elongated and appeared filamentous (Fig. 1E). Often the bacteria appeared to be centrally connected and radiated out-

wards in a starburst pattern (Fig. 1E). Although the elongated form of the bacteria was predominant, smaller single bacteria were also observed under the light microscope.

Electron microscopy revealed the filamentous form of *Agrobacterium* in more detail (Fig. 2). Bacteria that were not cultured with plant tissue showed a typical rod-shaped structure (Fig. 2a), while those resulting from inoculation on the soybean immature cotyledons on OMS medium were very elongated (Fig. 2b, c). In some instances, cells became excessively elongated and tangled (Fig. 2d).

Although this type of colony formation and growth has been reported previously with *Agrobacterium* (Shaw

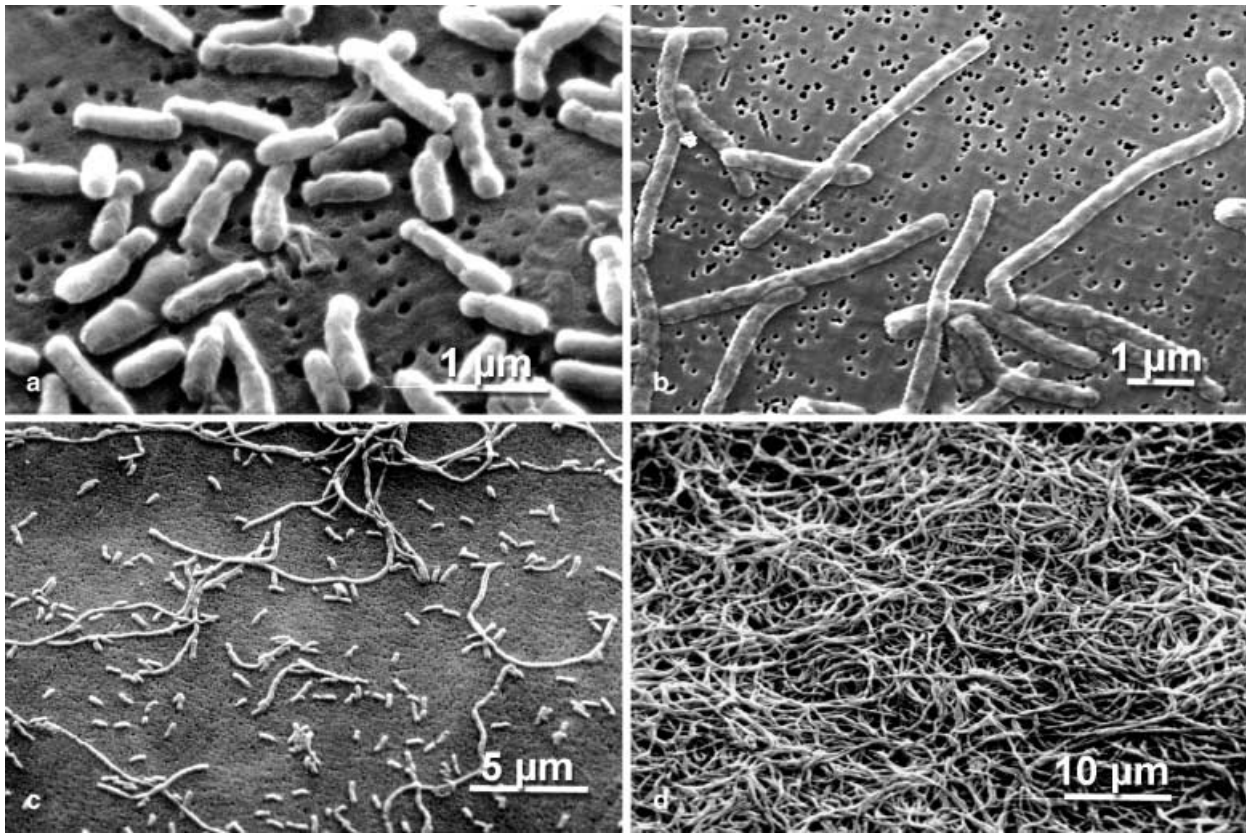


Fig. 2a–d Scanning electron micrographs. **a** Rod-shaped non-filamentous bacteria. **b** Filamentous form of bacteria. **c** Mixed rod-shaped and long filamentous form. **d** Mass of filamentous form

et al. 1991), the bacteria were cultured in the absence of plant tissue, on a bacterial culture medium that induced swarming. Bacterial swarming was first observed in 1885 in *Proteus* (Hauser 1885) and has been well studied in *Serratia liquefaciens* (Lindum et al. 1998; Eberl et al. 1999) and *Pseudomonas syringae* (Kinscherf and Willis 1999). It has often been associated with bacterial cell elongation (Eberl et al. 1999), although Shaw et al. (1991) did not observe elongation in their earlier investigation of swarming *Agrobacterium* despite extensive efforts to detect structural changes.

Filamentous forms of rod-shaped *E. coli* cells have been reported following either the treatment of cells with cell-division inhibitors or generation of division mutants (Begg and Donachie 1985). More recent findings indicate that many of these division mutants result from modifications to the *E. coli* septation initiator gene, *ftsZ* (Bi and Lutkenhaus 1991). Without formation of the septum, the bacterium cannot divide and separate, resulting in the formation of long non-septate bacterial filaments. In *Agrobacterium*, mutant screening (Fujiwara and Fukui 1974), over-expression of the *E. coli ftsZ* gene and application of cell division inhibitors resulted in the generation of branched forms (Latch and Margolin 1997), but a filamentous form of *Agrobacterium* has not previously

been described. Using the co-culture conditions that induced the formation of the filamentous form of *Agrobacterium*, we found that neither *E. coli* nor selected rhizobia showed swarming over the surface of the medium, although a filamentous form of *Bradyrhizobium elkanii* and an unusual branched form of *Sinorhizobium fredii* were observed (data not shown).

The conditions that were evaluated for induction of this filamentous form of *Agrobacterium* are summarized in Table 1. The formation of filamentous bacteria was absolute; if the morphology was observed, it was very clear and typically observed in the majority of cells. The filamentous form was never observed when LB medium was used for growing the bacteria (either bacteria alone or with plant tissue) or when the bacteria were cultured without plant tissue on plant tissue culture media. The addition of acetosyringone to either MS or LB medium did not lead to the formation of filamentous bacteria in the absence of plant tissue. Modifying the pH of the plant culture medium also had no effect on conversion to the filamentous form. Co-culture of *Agrobacterium* with many different plant tissues revealed that all tissues caused the formation of the filamentous form (Table 1). The types of tissues evaluated ranged from proliferative embryogenic tissue to leaf tissue, of both monocot and dicot plants. It is still unclear if the filamentous form is either positively or negatively associated with a successful transformation or compatible plant/bacterial interaction. There does appear to be a universal response of the bacterium to some component or exudate of all the plant

Table 1 The effects of various treatments on formation of filamentous *Agrobacterium*

Treatment	Absence (-) or presence (+) of filamentous bacteria
LB	-
MS (pH 5.7)	-
MS (pH 7.0)	-
LB (100 μ M acetosyringone)	-
MS (100 μ M acetosyringone)	-
LB (+ soybean cotyledons)	-
MS (+ soybean cotyledons)	+
Soybean tissue ^a	
Immature cotyledon	+
Immature embryo	+
Leaf	+
Stem	+
Mature cotyledon	+
Proliferative "D20" embryogenic	+
Soybean immature cotyledons ^a	
-80°C (1 h)	-
-20°C (1 h)	+
4°C (1 h)	+
65°C (1 h)	-
Wheat embryogenic callus ^a	+
Buckeye embryogenic callus ^a	+
Arabidopsis leaves ^a	+
Roots ^a	+
Bacterial strain ^b	
EHA105	+
LBA4404	+
A281	+
A208	+
C58	+
B6-806	+
15955	+
R1000 (<i>A. rhizogenes</i>)	+

^a Plant tissue was cultured on MS-based medium (pH 5.7)

^b Bacteria were cultured with immature soybean cotyledons. The bacterial genus/species was *A. tumefaciens* except where noted

tissues tested, resulting in *Agrobacterium* swarming and the induction of the filamentous form.

Pretreatment studies with soybean immature cotyledons revealed that treatments resulting in tissue death did not induce the filamentous form (Table 1). Freezing the tissue at -80°C and heating to 65°C for 1 h caused rapid tissue death, as indicated by the complete loss of color from the green immature cotyledons. These treatments did not yield filamentous bacteria, while the co-culture of bacteria with cotyledons exposed to milder treatments that did not result in immediate tissue death gave rise to the filamentous form. In most cases, contact of the bacterium with viable plant tissue was necessary in order to induce elongated bacteria. However, some bacterial colonies were induced to swarm and converted to the filamentous form after growing to within 5 mm of excised immature soybean cotyledons. If the elongated form is induced by a compound produced by living plant tissue, it is either transported over only relatively short distances in the medium or it rapidly diffuses, resulting

in concentrations below a required threshold level. Crude extracts of soybean cotyledons failed to induce the filamentous form of *Agrobacterium* (data not shown), suggesting that the filamentous form is induced by the active secretion of a compound by living plant tissue. Contact of an "induced" bacterial colony with a non-induced colony also resulted in the formation of the filamentous form and the increased spreading growth (swarming) associated with this phenotype. The transfer of induced bacteria to LB medium resulted in a reversion to the rod or non-filamentous form (data not shown). The transfer of induced bacteria to OMS medium (with no co-culture plant tissue) resulted in minimal bacterial growth and, again, reversion to the non-elongated form. Although the majority of this research was performed with an engineered *Agrobacterium* (EHA105), the filamentous form was also induced with numerous wild-type strains and *A. rhizogenes* following culture with immature soybean cotyledons (Table 1).

Although *Agrobacterium* has been in use by numerous plant transformation laboratories for over 15 years, this phenomenon of *Agrobacterium* swarming and the associated filamentous form has not been previously reported. In most cases, tissues that become overgrown with *Agrobacterium* after co-culture are simply discarded. The filamentous form can also be routinely observed on the co-culture medium, a few days after the co-cultured plant tissue is normally removed and transferred to an antibiotic-containing medium (data not shown).

It is unclear what compounds induce the filamentous form, what concentrations are required, or how long these compounds remain active in the medium. Given the uniform response of different bacterial strains to various plant tissues of numerous plants, induction of the filamentous form does not appear to result from a specific bacterial/plant recognition but is a general response. The filamentous form may represent a non-infectious form of the bacterium, which is actively induced by plant tissues. Alternately, elongated rapidly growing bacteria, radiating out from a central point, are ideally suited to span long distances. The filamentous form may aid in the search for a potential entry site into the target organism. In *Serratia*, swarming cells produce an extracellular lipopeptide, which acts as a biosurfactant, reducing surface tension and allowing rapid spread of the swarming colony (Lindum et al. 1998). Surfactants have been added during *Agrobacterium*-mediated transformation of *Arabidopsis* (Clough and Bent 1998) and wheat (Cheng et al. 1997) and may allow more efficient delivery of the bacterium into dry or tight spaces in the target tissue. Although homoserine lactone derivatives can induce biosurfactant production and swarming in *Serratia*, these compounds had no effect on swarming or the induction of the filamentous form in *Agrobacterium* (data not shown). As this is the first report of *Agrobacterium* swarming and the induction of the filamentous form, much work is needed to further characterize the process and isolate the inductive compounds released by the plant tissues. In addition, the precise role of swarming

and the function of the filamentous form during *Agrobacterium*-plant interactions remain to be elucidated.

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