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The *Rhodococcus fascians*-plant interaction: morphological traits and biotechnological applications

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Abstract. *Rhodococcus fascians* is a Gram-positive bacterium that infects dicotyledonous and monocotyledonous plants, leading to an alteration in the normal growth process of the host. The disease results from the modulation of the plant hormone balances, and cytokinins are thought to play an important role in the induction of symptoms. Generally, on the aerial parts of the plants, existing meristems were found to be most sensitive to the action of *R. fascians*, but, depending on the infection procedure, differentiated tissues as well gave rise to shoots. Similarly, in roots not only actively dividing cells, but also cells with a high competence to divide were strongly affected by *R. fascians*. The observed symptoms, together with the determined hormone levels in infected plant tissue, suggest that auxins and molecules of bacterial origin are also involved in leafy gall formation. The complexity of symptom development is furthermore illustrated by the necessary and continuous presence of the bacteria for symptom persistence. Indeed, elimination of the bacteria from a leafy gall results in the further development of the multiple embryonic buds of which it consists. This interesting characteristic offers novel biotechnological applications: a leafy gall can be used for germplasm storage and for plant propagation. The presented procedure proves to be routinely applicable to a very wide range of plants, encompassing several recalcitrant species.

Key words: Cell cycle – *Digitalis* – Leafy gall – *Nicotiana* – Micropropagation – Regeneration

Introduction

The Gram-positive phytopathogenic Actinomycete *Rhodococcus fascians* interacts with a vast array of plants resulting in the induction of malformations. Extensive studies on the molecular aspects underlying the *Rhodococcus fascians*-plant interaction revealed that essential virulence determinants are located on a linear plasmid pFi (Crespi et al. 1992, 1994; Stange et al. 1996; Vereecke 1997). One of the genes within the *fas* locus encodes an isopentenyl transferase (IPT) that is typically involved in cytokinin biosynthesis. The IPT is homologous to isopentenyl transferases from *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *savastanoi*, and the enzymatic activity of the IPT protein has been demonstrated (Crespi et al. 1992). The previously accepted model was that production of cytokinins was the main cause of symptom development. Indeed, at least 11 different cytokinins have been found in the supernatant of *R. fascians* cultures. However, these cytokinins are formed constitutively by the bacteria irrespective of their pathogenicity (Eason et al. 1996). Our current working hypothesis is that novel compounds produced by *R. fascians* are involved in disrupting plant hormone balances, ultimately leading to disease. The malformations produced vary from deformed leaves, to malformed multiple shoots or witches' brooms, to the most typical symptom known as leafy galls. Leafy galls were first described by Lacey (1936) as the horizontal spreading of very short hypertrophied shoots that appear at the crown of infected plants, until a large gall-like mass is produced. The continuous presence of the bacteria is important for symptom persistence (Lacey 1936).

We describe the morphological effects induced by *R. fascians* on plants and physiological traits of a leafy gall. To further comprehend these data, cell-cycle gene expression was followed using *Arabidopsis thaliana* (L.) Heynh. as a model, because much cell-cycle research has been focused on this plant and several cell-cycle regulatory genes are available as molecular markers (for a review, see Burssens et al. 1998). More specifically, we

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Abbreviations: GUS = β-glucuronidase; ELISA = enzyme-linked immunosorbent assay

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have monitored changes in cell-cycle gene expression upon *R. fascians* infection by using the promoters of *CDC2aAt*, Arath;*CycA2;1*, and Arath;*CycB1;1* fused to *β*-glucuronidase (*gus*) in both roots and shoots of infected transgenic *Arabidopsis thaliana* plants. By taking advantage of the interaction between *R. fascians* and plants and of the morphological analysis of leafy galls, we have established a novel approach for in-vitro regeneration of plants using leafy galls as a source of multiple shoots suitable for plant propagation. We finally compare the new procedure with the conventional micropropagation technique and discuss the possibility of using a leafy gall as an experimental model for studies related to the identification of molecular markers associated with growth and development of plants and, more specifically, with the imposition and release of apical dominance.

Materials and methods

Plant material and culture method. Several plant species from different botanical families were investigated for this study, including *Nicotiana plumbaginifolia* L., *Nicotiana tabacum* L. W38, and *Atropa belladonna* L. (Solanaceae); *Helianthus annuus* cv. California, *Artemisia annua* L., and *Anthemis nobilis* L. (Compositae); *Digitalis lanata* L. (Scrophulariaceae); *Catharantus roseus* (L.) G. Don f (Apocynaceae); *Arabidopsis thaliana* (L.) Heynh. C24 (Cruciferae); *Sesbania rostrata* Brem (Fabaceae); *Papaver somniferum* L. (Papaveraceae); *Populus tremula* × *P. alba* (INRA, No. 717 1-B4; Salicaceae); and *Zea mays* cv. Helix (Graminae). All seeds were obtained from personal stocks (Jardin Expérimental Jean Massart at the Université Libre de Bruxelles, Belgium), except for the sunflower seeds which were purchased in a local garden center.

Axenic seedlings were obtained after seed sterilization with calcium hypochlorite (5%, w/v), followed by washing of the seeds three times with sterile distilled water. The seedlings were cultured on solid Murashige and Skoog basal mineral medium (MS; Murashige and Skoog 1962) and incubated at 22 °C in a 16-h light per day photoperiod (70 μmol m^{-2} s^{-1}, cool-white fluorescent lamp, Osram). Dissected leafy galls that were used to regenerate shoots were grown on solid MS medium supplemented with 15% sucrose and 500 mg l^{-1} cefotaxime (Duchefa, Haarlem, The Netherlands).

Bacterial strains. The *R. fascians* strains used in this study were grown in solid or liquid YEB medium (Miller 1972) for 2 d at 28 °C. The *R. fascians* strains were D188 (pathogenic) and D188-5 (plasmid-free non-pathogenic strain Desomer et al. 1988).

Infections. For all infections, late-exponential 2-d-old bacterial cultures were used. Most of the infections were achieved either by decapitation or by infiltration. In the first approach, the apical and the first axillary meristems of 4-week-old plants were removed and a drop of bacterial suspension was applied at the cutting site; in the second method, 3-week-old plants were submerged in a bacterial culture that had been washed and resuspended in water, submitted to vacuum (water pump) for 2 min, and finally replanted in MS medium. Other infection methods were (i) seedling infection in which seeds were germinated on MS medium and a drop of bacterial suspension was applied to the seeds as soon as the radicle emerged; (ii) infection at the apical site without wounding, which consisted of applying a drop of bacterial suspension to the apical meristem of 2- to 3-week-old plants; and (iii) infection via watering, in which the plants were grown in soil or vermiculite and watered with a bacterial culture. The plants were photographed with a Leica M2 camera with a Fotocar 50 mm lens and a Leica Reprovit IIa reprocolumn. The films used were Kodak Ektachrome 64T. Sufficient light was provided by four Photolita-S lamps (Philips) under an angle of 45°.

For the scanning-electron-microscopic analysis, samples were fixed overnight at 4 °C with 4% formaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After two washes with the cacodylate buffer, a post-fixation was performed overnight at 4 °C with 2% osmium tetroxide in water. Subsequently, the samples were washed twice with the cacodylate buffer and dehydrated in an ethanol series at 4 °C (30%, 50%, 70%, 85%, 95% ethanol in water, mininum of 3 h per step). For the microscopic analysis, the samples were submitted to critical-point drying with CO_2, coated with gold, and examined with a JSM-840 scanning electron microscope (JEOL) at 15 kV. Photographs were taken with Mamiya optics.

Histochemical GUS assays on A. thaliana plants. Transgenic *Arabidopsis thaliana* (L.) Heynh. C24 plants containing the Arath;*CycB1;1-gus* (Ferreira et al. 1994), Arath;*CycA2;1-gus* (Shaul et al. 1996; data not shown), or *CDC2aAt-gus* (Hemerly et al. 1992) cassette were grown in sterile conditions on K1 germination medium (Valvekens et al. 1988). After 1 or 2 weeks, the plants were infected with *R. fascians* strain D188-5 or D188. The bacteria were washed and resuspended in a 0.2-M sodium phosphate buffer (0.05% Na-citrate, 0.025% $MgSO_4$, 0.001% thiamine, pH 5.7). The plants were submerged in the bacterial suspension, submitted to vacuum (water pump) for 2 min, and washed twice with the same buffer. As controls, plants vacuum-infiltrated with the sodium phosphate buffer without bacteria and non-treated plants were included. After infiltration, the plants were transferred to K1 medium containing half the percentage of sucrose. Subsequently, the GUS assays were performed as described by Beeckman and Engler (1994) 2, 5, 10, 28, and 42 d after infection. For visualization, the tissues were cleared with chlorallactophenol (Beeckman and Engler 1994) and photographed with Normaski optics.

For the root analysis, 2-week-old plants were infected as described above and planted on vertical plates containing K1 medium with 0.5% sucrose. For each condition (control, D188, and D188-5), 20 plants were analyzed 10 and 18 d after infection. The lengths of the roots were measured and the total number of roots per plant was counted as viewed under binoculars Sterni SV11 (Zeiss). The data were statistically analyzed by one-way analysis of variance (ANOVA); probability (*P*) values for the differences between the three conditions are given in Table 2.

Hormone analysis. Indole-3-acetic acid (IAA) was determined by using enzyme immunoassay kits purchased from Mayoly Spindler. (Phytosciences Department, Chatou, France). The enzyme-linked immunosorbent assay (ELISA) procedure was performed according to the manufacturer's instructions. A standard curve was included on each microtiter plate and methylated IAA was used as standard. The monoclonal antibody that was used, anti-IAA-Met AC312 Ephyscience, had 100% cross-reactivity with methylated IAA.

The plant material was extracted and pre-purified as follows. Typically, 20 mg powder of lyophilized plant material was extracted by maceration overnight at 4 °C in 5 ml of methanol:H_2O (80:20) containing 40 mg l^{-1} of butylhydroxytoluene (extraction solvent). After centrifugation (10 000 *g* at 4 °C), the pellet was resuspended in 2 ml of the extraction solvent, and centrifuged again. The combined supernatant was filtered through a Mediakap filter (pore size 0.2 μm) and then applied to a Sep-pak C18 column that was pre-equilibrated with 5 ml of methanol:H_2O (80:20). The column was eluted with 5 ml of methanol:H_2O (80:20). The eluate was evaporated at room temperature under vacuum to remove the organic solvent and then lyophilized.

For IAA analysis by ELISA a methylation step is required. Therefore, the lyophilized residue was dissolved in 2 ml of dry acetone, treated with diazomethane for 2 h, and evaporated under reduced pressure. The residue was dissolved in 0.5 ml of H_2O, diluted with an appropriate volume of phosphate-buffered saline (0.01 M phosphate, 0.15 M NaCl, pH 7.2–7.4), and subjected to ELISA analysis.

Transformation of A. belladonna. The transformation procedure used was that previously described by Jaziri et al. (1994) with the *Agrobacterium rhizogenes* strain NCIB 8196.

Results

Host range of R. fascians and infection methods. *Rhodococcus fascians* has a very broad host range encompassing 28 families and 59 genera (Bradbury 1986). During the course of this study, however, even more plants species were found to be sensitive to *R. fascians* infection (Fig. 1): *Helianthus annuus* (Fig. 1A), *Anthemis nobilis* (data not shown), *Artemisia annua* (data not shown), *Arabidopsis thaliana* (Fig. 1B), *Atropa belladonna* (Fig. 1C), *Nicotiana plumbaginifolia* (data not shown), *Digitalis lanata* (data not shown), *Sesbania rostrata* (Fig. 1D), *Papaver somniferum* (Fig. 1E), *Populus tremula* × *P. alba* (Fig. 1F), *Catharantus roseus* (data not shown), and *Zea mays* (Fig. 1G), bringing the current host range to 32 families and 70 genera. Therefore, the option was taken to test different hosts to evaluate several aspects of the interaction.

The degree of symptom development on plants is a consequence of multiple factors: the host plant, including variety and/or cultivar (Lacey 1939; Eason et al. 1995), the age of the plant (Roussaux 1965; Faivre-Amiot 1967), the bacterial strain (Eason et al. 1995), and the growth conditions used (Faivre-Amiot 1967). Evaluation of symptom development on plants inoculated by different infection methods showed that the inoculation procedure was also of great importance. For this analysis, *Nicotiana tabacum* W38 was used because this plant has been one of the model hosts for studying the *R. fascians*-plant interaction (Crespi et al. 1992; Vereecke 1997).

Infection of germinating seedlings led to a complete growth inhibition of both the roots and the aerial plant

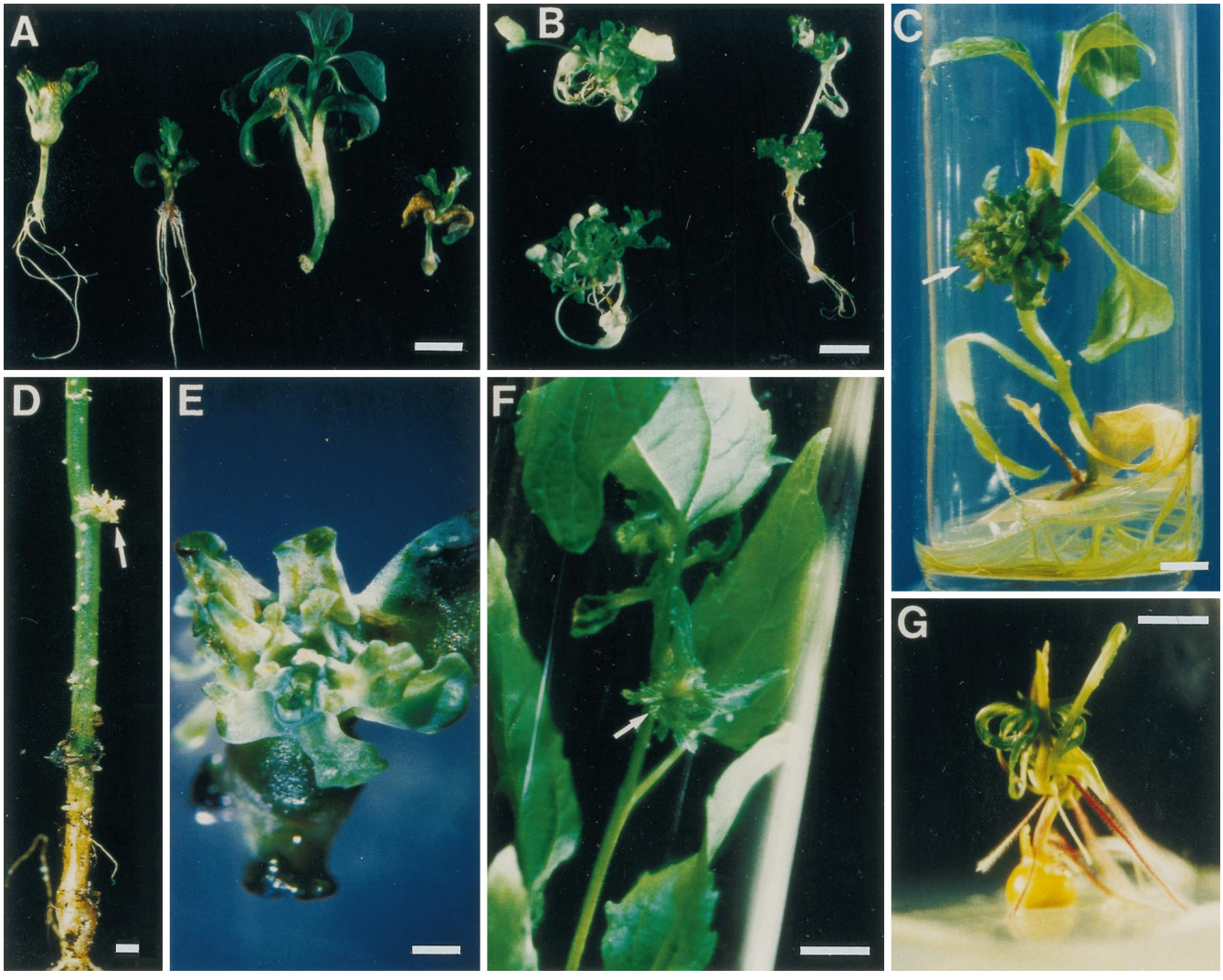


Fig. 1A–G. Extension of the host range of *R. fascians*. **A** *Helianthus annuus*; note thickened hypocotyl and cotyledonary node from where multiple deformed shoots arise. Bar = 12.5 mm. **B** *Arabidopsis thaliana*; the normal rosette structure of the plant is completely lost. Bar = 10 mm. **C** *Atropa belladonna*; leafy gall is formed from an axillary meristem. Bar = 10 mm. **D** *Sesbania rostrata*. Bar = 2.5 mm. **E** *Papaver somniferum*; close-up of a shoot on a leafy gall. Bar = 1 mm. **F** *Populus tremula* × *P. alba*. Bar = 5 mm. **G** *Zea mays*. Bar = 5 mm. **A,D,G** Symptoms after seedling infection; **B,C,E,F** Symptoms upon infection of decapitated plants; leafy galls are indicated by an *arrow*

parts, with the occasional development of a leafy gall at the apical meristem (Fig. 2A). The addition of a bacterial culture to vermiculite in which 4-week-old plants were grown did not affect the general development of the plant. Nevertheless, malformed shoots or witches' brooms developed at the crown (Fig. 2B).

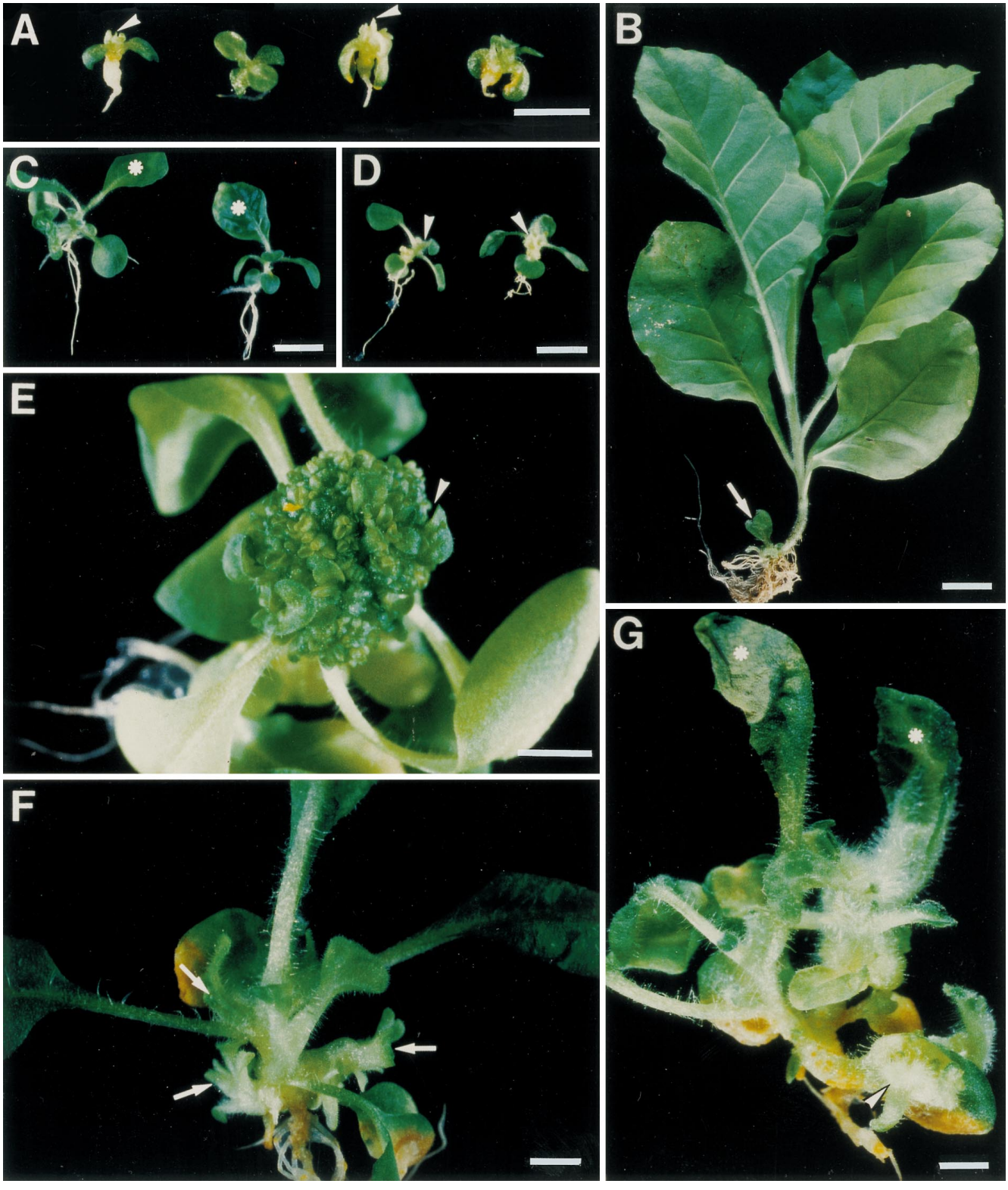


Fig. 2A–G. Overview of the symptoms that are induced by *R. fascians* on tobacco after different infection procedures. **A** Infection of seedlings. Bar = 5 mm. **B** Infection by watering. Bar = 30 mm. **C,D** Infection at the apical site without wounding. Bar = 10 mm. **E** Infection after decapitation. Bar = 5 mm. **F,G** Infection via vacuum infiltration. Bar = 5 mm. For infection procedures, see *Materials and methods*. *Asterisk*, deformed leaf; *arrowhead*, leafy gall; *arrow*, deformed shoot

Table 1. Effect of *R. fascians* and cytokinins on the three major meristematic areas present in a shoot

Function of the meristematic area	Normal plant	*R. fascians*	Exogenous cytokinin
Leaf formation	+	+ + +	+ +
Lateral expansion	+	+ +	+
Elongation of intercallary meristem	+	− − −	±

The intensity of the effects is marked by + or − and is relative for the three compared systems

Whereas infection of sterile 3-week-old plants at the apical bud without wounding led to an array of phenotypes ranging from malformed leaves (Fig. 2C) to small leafy galls (Fig. 2D), infection of the apical site of similar plants after decapitation of the apical meristem consistently led to the formation of a leafy gall (Fig. 2E). Finally, infection of plants of the same age by vacuum-infiltrating a bacterial culture into the plant led to a strong growth inhibition of the aerial parts and root system, to the activation of all axillary meristems (Fig. 2F), and to severe malformations and swelling of the leaves (Fig. 2G).

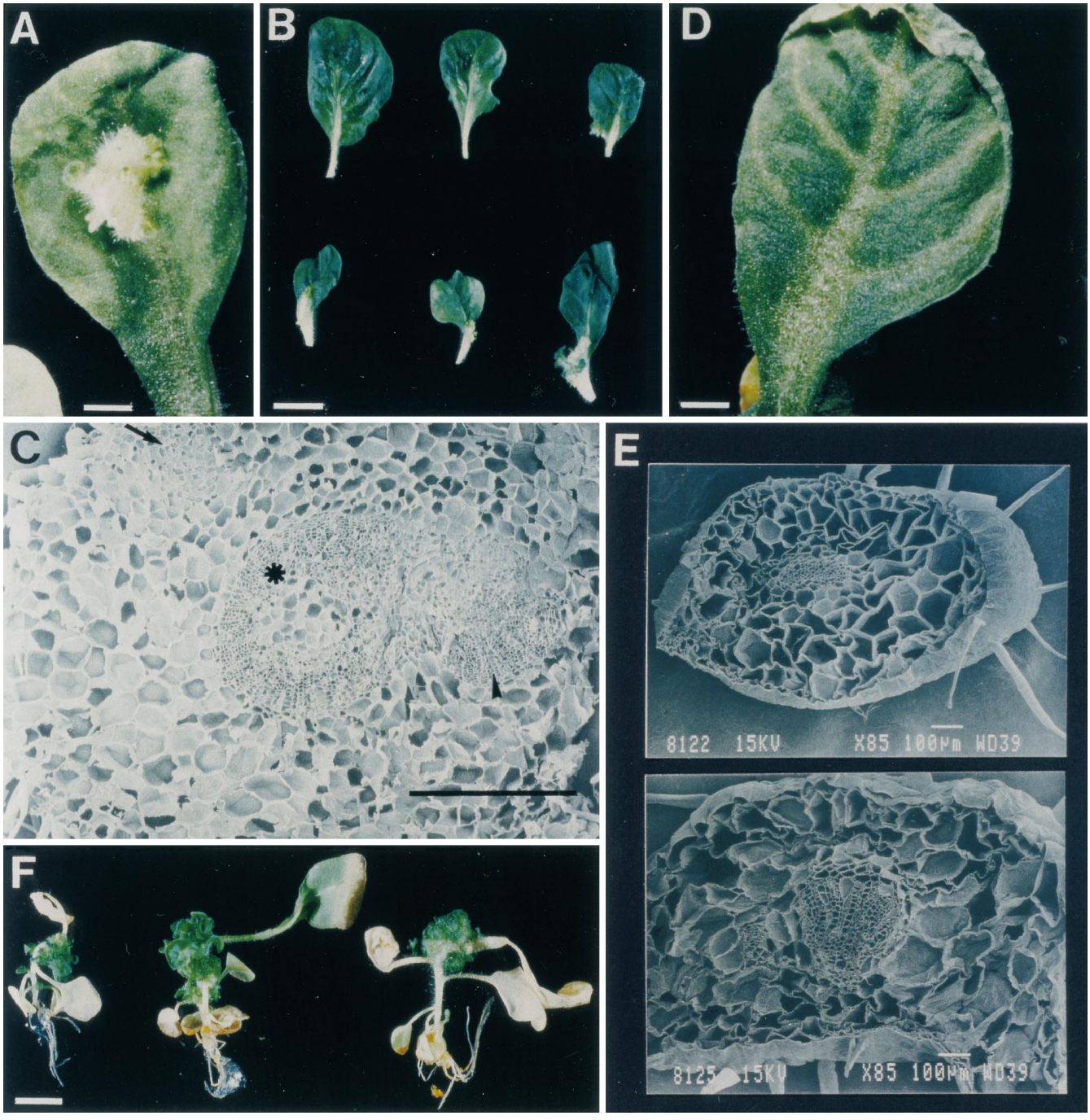
Neoformation of shoot meristems. It was initially observed that somatic tissues consisting of differentiated cells with arrested mitotic activity were less sensitive to *R. fascians* when compared to actively dividing cells present in meristematic tissues. Moreover, there was no evidence for neoformation of meristems (Lacey 1939; Baker 1950; Roussaux 1965, 1975). When the three major meristematic locations in a shoot apex were compared in *Atropa belladona* control plants, leafy galls, and excised shoot apices cultured on cytokinin-containing medium, the interaction with *R. fascians* and the addition of exogenous cytokinins had similar effects (Table 1). Although both *R. fascians* and cytokinins affected the leaf primordia and the lateral expansion areas positively and the elongation areas negatively, the effect of *R. fascians* was much more pronounced. However, upon infection via infiltration of tobacco plants, leafy galls formed on leaf margins and veins (Figs. 2G and 3A,B). These data suggest that under certain conditions shoots can form from tissues that retain the potential to divide. Moreover, in the cortex of the crown of naturally infected tobacco plants, regions with very small cells could occasionally be observed. These regions are most probably at the origin of the multiple adventitious shoots that are formed upon infection with *R. fascians* (Fig. 3C).

Alterations in cell-cycle gene expression upon R. fascians infection of Arabidopsis thaliana. From the above mentioned, it is clear that molecular markers associated with cell division must be activated during the interaction between *R. fascians* and the host plant. The effects of *R. fascians* on plant growth and development were monitored by following the changes in expression of two cyclins (Arath;*CycB1;1* and Arath;*CycA2;1*) and one cyclin-dependent kinase (*CDC2aAt*) in transgenic *Arabidopsis* plants that carry the respective promoter-*gus* fusions. The promoter activity of these three cell-cycle genes analyzed by *gus* expression was shown to be strongly correlated with the expression of the genes (Hemerly et al. 1993; Ferreira et al. 1994). Expression of Arath;*CycB1;1* is restricted to G2 and M phases and to actively dividing cells (Ferreira et al. 1994; Shaul et al. 1996); Arath;*CycA2;1* expression is a marker for S, G2, and M phases (Shaul et al. 1996); and *CDC2aAt* is constitutively transcribed during the cell cycle (Hemerly et al. 1993). The expression of Arath;*Cyc2A;1* and *CDC2aAt* is not only correlated with active cell division, but also with competence to divide (Hemerly et al. 1993; data not shown).

Upon vacuum-infiltration of 10-d-old *A. thaliana* plants with *R. fascians*, the pathogenic strain D188 induced abundant shoot formation with loss of apical dominance and fasciation of the flower stem with the formation of multiple flowers (Fig. 4A,B). The non-pathogenic strain D188-5 had no effect on the aerial plant part. During the time-course experiments, both in roots and in shoots, a differential expression was observed for the three cell-cycle genes upon *R. fascians* infection compared to that of control plants. However, an important difference was that the effects on the shoots could only be imposed by the pathogenic strain, whereas the effects on the roots were induced by both strains.

Expression of cell-cycle genes in the shoots was comparable for uninfected control and D188-5-infected plants. However, strong induction of Arath;*CycA2;1* expression was detected in the shoot apex, 5 d after infection with D188 (Fig. 4C,D). After 10 d, expression of Arath;*CycB1;1* and *CDC2aAt* (data not shown) was also strongly induced, which correlated with abundant shoot formation. When plants were infected at a younger stage (7 d after germination), the symptoms were basically similar to the changes induced on 10-d-old plants, but the alterations were faster and more severe. This observation confirms the direct correlation between the age of the plant at the time of infection and the rate and severity of the subsequent morphological and developmental changes.

In the roots, from 2 d after infection with the strains D188 and D188-5, an increasing decline in promoter activities of *CDC2aAt*, Arath;*CycB1;1*, and Arath;*CycA2;1* was visible. Moreover, *gus* expression for all three cell-cycle markers decreased appreciably in the root meristems, illustrating a rapid decrease in mitotic activity, which is in agreement with the observed retardation of the main root growth and the inhibition of lateral root growth (Fig. 4E–J). Nevertheless, new lateral roots could be initiated at four different locations, although lateral root outgrowth was often arrested upon infection by *R. fascians*. Preferentially, roots were formed on sites near an inhibited lateral root (Fig. 4H,J) that had lost meristematic activity as visualized by the loss of Arath;*CycB1;1* expression (Fig. 4J). Secondly, root primordia were generated from the vascular stele of young inhibited lateral roots that still showed Arath;*CycB1;1* promoter activity in the vascular cylinder but not in the apical meristem (Fig. 4J). Sometimes,



lateral roots were formed that were not in the direct neighborhood of existing degenerating lateral roots, but in sectors of the root that retained a high competence for lateral root formation, as indicated by the presence of *CDC2aAt* and Arath;*CycA2;1* expression (data not shown). Finally, adventitious roots were formed from the vascular stele of the hypocotyl (Fig. 4K,L), which was in accordance with de-novo expression of Arath;*CycA2;1* (Fig. 4L).

Although the induction of lateral root formation and the inhibition of cell-cycle gene expression appeared similar for both strains, the inhibition of root development was much stronger with D188 than with D188-5. Indeed, when the length and number of roots were determined, it became obvious that D188-5-infected plants could form more developed roots, whereas root development in D188-infected plants was completely blocked (Table 2).

Auxin effects during symptom development. Evidently, gall formation results from the modulation of the regular hormone balances in the infected plant tissues. To evaluate relative changes in the IAA equilibrium, a semi-quantitative phytohormone analysis, as assayed by ELISA, was performed on tissue from *Atropa belladonna* control plants and from plants carrying a leafy gall. Because of the link between *R. fascians*-induced symptoms and true fasciations, similar analyses

Fig. 3A–F. Effect of *R. fascians* on tobacco. **A–C** Leafy gall formation from non-meristematic tissues in tobacco. **A** Leafy gall on the vein of a leaf. Bar = 2.5 mm. **B** Deformation of leaves and formation of leafy galls on leaf margins and veins. Bar = 10 mm. **C** Section through the crown of an infected plant showing the stem vascular bundle (*asterisk*), the differentiated vascular bundle of a deformed shoot (*arrowhead*), and a region in the cortex from which adventitious shoots can be formed (*arrow*). Bar = 1 mm. **D,E** Thickening of petioles and veins is the result of swelling of the cells and secondary differentiation of the vascular bundle. **E** D188-5-infected control (*upper panel*); D188-infected plant (*lower panel*). Bar (**D**) = 2 mm; bar (**E**) = 100 μm. **F** Leafy gall as an isolated physiological unit on a decaying plant. When infected plants are allowed to grow for a very long time under sterile conditions, the leafy gall tissue remains dark green and shows no symptoms of decay, whereas the normal plant tissue appears dead. We regard this phenomenon as delayed senescence. Bar = 10 mm

were performed on transgenic *A. belladonna* plants that were obtained via transformation with a wild-type *Agrobacterium rhizogenes* strain, which showed spontaneous fasciation, and on transgenic plants that appeared normal (data not shown). Interestingly, the level of IAA in the plants carrying a leafy gall was 40-fold higher than that of normal shoots cultured in hormone-free medium (Table 3). This result is in complete agreement with the morphological analysis of the leafy gall structure: it is the most extreme form of apical dominance with all the embryonic buds in the dormant state. Moreover, the thickening of the veins and the petioles observed in plants upon infiltration with *R. fascians*, proved to be the combined result of swelling of the cells and secondary differentiation of the vascular tissue (Fig. 3D,E), which are also typical auxin effects (Aloni 1987). Finally, compared to the normal shoot culture and to the non-fasciated transgenic plant, the spontaneously fasciated transgenic *A. belladonna* plant showed 12-fold higher IAA levels (Table 3).

Leafy gall structure as a source of multiple shoots. The unique property of *R. fascians* to amplify shoots through the modulation of specific hormone balances is useful for the establishment of a routine regeneration program for a wide range of host plants including ornamental, medicinal, crop, and woody plants. Plantlets from several plant species propagated in vitro were infected after decapitation and were subcultured on hormone-free, mineral, plant tissue culture medium. The phenotypic characteristics associated with leafy galls induced by *R. fascians* were observed after a few weeks, depending on the plant species. For example, in the case of *Atropa belladonna* (Fig. 1C), *Digitalis lanata*, or *Artemisia annua*, a well developed leafy gall was observed 4 weeks after the infection.

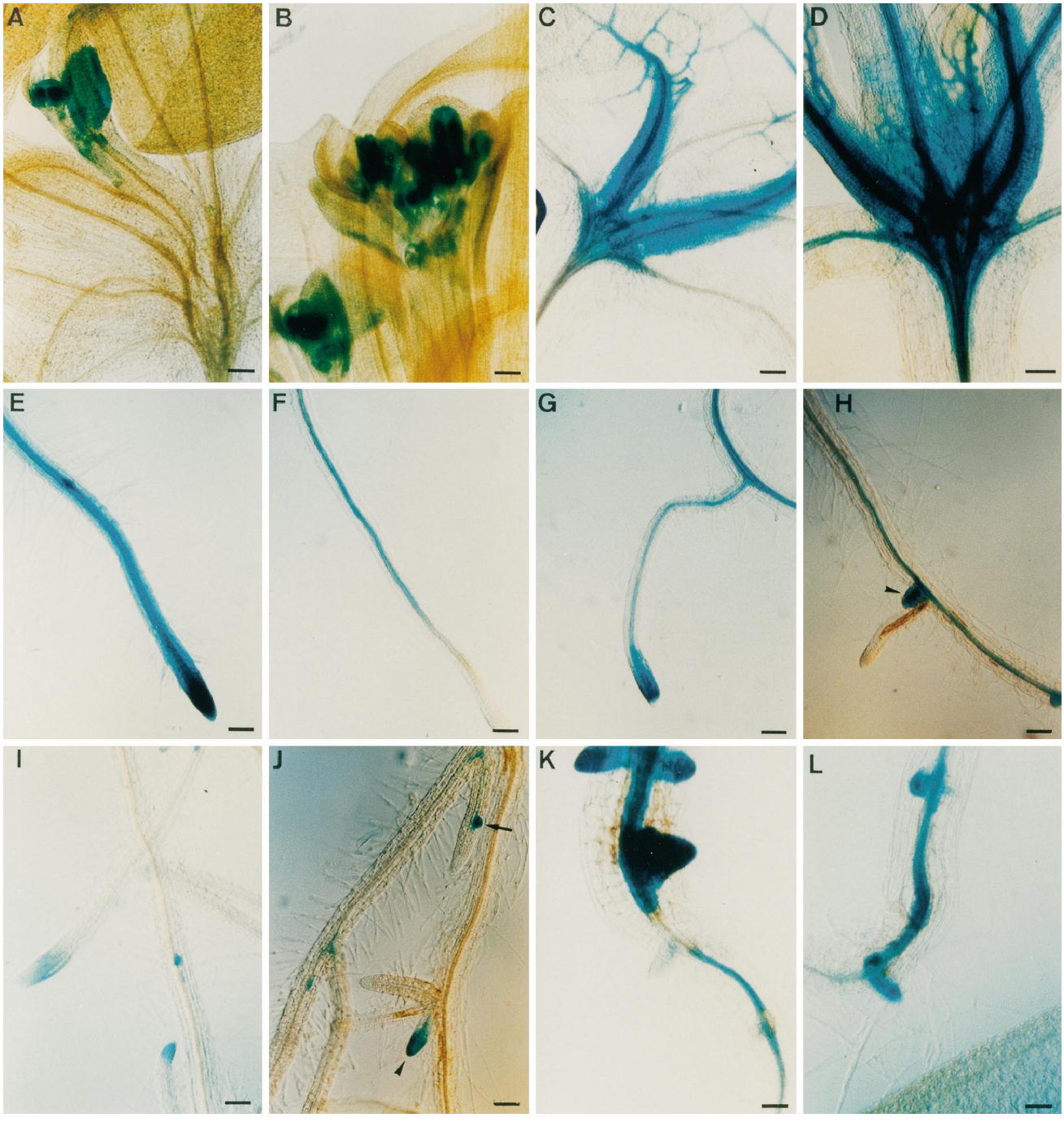
When the leafy galls were dissected from the initial plants and transferred to fresh culture medium, different observations were made depending on the presence or absence of antibiotics in the culture medium. When the leafy galls were regularly subcultured on fresh medium without antibiotics, the bacterial cells as well as the plant cells still grew but at an extremely reduced rate. This observation is relevant with regard to plant conservation because compact small structures with multiple dormant shoots, such as a leafy gall, are suitable for germplasm storage. When the cultures were stored at low temperatures (4 °C), the capability of the shoots to develop was maintained for more than two years.

On the other hand, when the bacteria were killed in the gall tissues through treatment with bactericidal agents, the role of *R. fascians* in the phenomenon of suppression of elongation and alteration of apical dominance was exemplified. Growth of detached galls on hormone-free medium with the addition of antibiotics to kill the bacteria resulted in the release of the inhibition of the amplified primordia and the ensuing outgrowth of numerous independent shoots from the gall tissue. The elongation could be facilitated by treating the galls in the dark where the release of the shoots was accompanied by etiolation, leading to masses of elongated sprouts. Upon transfer of the newly regenerated plantlets to hormone-free medium, rooted plants were obtained.

By using the method described for *Digitalis lanata*, an average of 60 regenerated shoots per explant was obtained, compared to 12 regenerated shoots using a conventional micropropagation method in which exogenous cytokinins and auxins are added to the culture medium (Schöner and Reinhard 1982). The plantlets thus obtained were acclimatized and then individually transferred to greenhouse conditions, which resulted in the production of fertile plant clones. Quantitative and qualitative analysis of secondary metabolites (cardenolides) in the regenerated *D. lanata* plants showed a distribution similar to that of the mother plant (data not shown). In addition, the morphological traits, such as leaf distribution and shape, and the branching of the regenerated plants were also similar to those of the mother plant, indicating a reduced variability as compared to the classical micropropagation technique.

Discussion

We were able to provoke symptom development on 12 plant species that did not belong to the known host range of *R. fascians*, indicating that the capacity of *R. fascians* to interact with different plant genera and families is much broader than recognized to date. Further, we have shown that the infection procedure determines the outcome of the interaction. Symptoms will range from deformation of leaves, to witches' brooms, activation of all meristems, and finally leafy galls. Leafy galls are non-autonomous structures and, until now, no evidence has been found for the occurrence of stable transfer of bacterial DNA to the plant (data not shown). The formation of leafy galls is characterized by an alteration of the apical dominance, resulting in the development of multiple lateral embryonic buds situated in the axils of leaf primordia. The outgrowth of the buds is inhibited shortly after their formation. In a normal plant, the shoot apex controls lateral bud outgrowth and, upon removal of the apical meristem, an axillary shoot will take over the control.



This correlative inhibition is known as apical dominance (for a review, see Cline 1997). However, when decapitation is followed by infection with *R. fascians*, numerous lateral shoots are formed, each of which inhibits the outgrowth of the other shoots. As a consequence, a leafy gall that represents a center of elongation inhibition can also be defined as the most extreme form of apical dominance.

Meristematic tissue and buds are most sensitive to the action of *R. fascians*. However, microscopical analysis showed that *R. fascians* could also induce shoot formation from other tissues. The formation of adventitious buds on leaf surfaces and edges is also observed in viviparous leaves obtained upon genetic transformation with agrobacteria. Bud formation in such leaves is the result of cytokinin overproduction that induces cell division in vascular parenchyma, thus overriding the developmental plan of these differentiated cells (Estruch et al. 1991; Jaziri et al. 1994). Furthermore, by using transgenic *A. thaliana* plants that carried a *gus* fusion to one of three cell-cycle gene markers, data were obtained suggesting that *R. fascians* acts on pericycle cells, which are mitotically active (Arath;*CycB1;1*) or in which the machinery for rapid activation of the cell cycle is present (*CDC2aAt* and Arath;*CycA2;1*). This observation broadens the action range of *R. fascians* beyond meristems to all cell types that are competent to divide.

Fig. 4A–L. Effect of *R. fascians* infection on cell-cycle gene expression in transgenic *Arabidopsis thaliana* plants. Cell-cycle gene expression in flowers (**A,B**), in shoots (**C,D**), in roots (**E–J**), and in the hypocotyl (**K,L**). The plants were infected after 2 weeks and analyzed after 6 weeks (**A,B**), after 5 d (**C–H** and **K,L**), or after 10 d (**I,J**). **A** Control shoot: Arath;*CycB1;1* expression; formation of one flower bud. **B** D188-infected shoot: Arath;*CycB1;1* expression; multiple flower formation. **C** Control/D188-5-infected shoot: Arath;*CycA2;1* expression. **D** D188-infected shoot: enhanced Arath;*CycA2;1* expression in the apex. **E** Control root: *CDC2aAt* expression. **F** Infected root: down-regulation of *CDC2aAt* expression in the root apical meristem. **G** Control root: Arath;*CycA2;1* expression. **H** Infected root: down-regulation of Arath;*CycA2;1* expression in the lateral root meristem and lateral root initiation near such roots (*arrowhead*). **I** Control root: Arath;*CycB1;1* expression. **J** Infected root: no Arath;*CycB1;1* expression in meristems; de-novo lateral root formation near a root that retained Arath;*CycB1;1* expression in the vascular tissue (*arrow*), and near a root in which the cell-cycle genes are no longer expressed in the meristem (*arrowhead*). **K** Hypocotyl of infected plant: down-regulation of *CDC2aAt* expression in the lateral roots and root primordia; de-novo lateral root formation from the vascular tissue of the hypocotyl. **L** Hypocotyl of infected plant: Arath;*CycA2;1* expression; primordium formation on hypocotyl, and de-novo expression in the vascular tissue. Bars = 100 μm

Table 2. Effect of *R. fascians* infection on root growth of *Arabidopsis thaliana* C24 plants

Time after infection	Strain	Root length (cm) ± SD[a]	Roots per plant (number) ± SD[a]
10 d	Control	3.0 ± 1.0	11 ± 7
	D188-5	2.2 ± 1.5	11 ± 5
	D188	0.9 ± 0.6	4 ± 2
18 d	Control	6.5 ± 0.7	78 ± 29
	D188-5	3.6 ± 1.8	34 ± 9
	D188	0.9 ± 0.6	6 ± 3

[a] Statistical analysis for the difference between the control and D188-infected plants and between plants infected with D188-5 and D188 gave *P* values smaller than 0.001 for the root length and the number of roots at both time points; for the difference between control plants and D188-5-infected plants, the *P* value after 10 d was 0.039 and 0.368 for root length and root number, respectively; after 18 d the *P* value for both parameters was smaller than 0.001. $n = 20$

Table 3. Analysis of IAA in normal shoot cultures, leafy galls, and fasciated and non-fasciated transgenic *Atropa belladonna* plants. The data are the average (± SD) of two independent extractions of independent plant material

Plant material	IAA [pg (g Fw)$^{-1}$]
Shoot culture	10.70 ± 0.14
Leafy gall	393.24 ± 9.97
Non-fasciated transgenic plant	10.42 ± 0.14
Fasciated transgenic plant	125.65 ± 1.41

The experiments with the transgenic *A. thaliana* plants also showed that strain D188-5 had no effect on shoot development, whereas strain D188 induced the formation of multiple shoots, followed by the inhibition of their further development. This differential effect can be correlated with the pathogenicity genes that are located on the linear plasmid pFiD188 (Crespi et al. 1992) and that are probably responsible for the disruption of the endogenous hormonal balance of the host plants. Interestingly, D188-5 is able to partially block root development. This result is intriguing because it indicates that the non-pathogenic strain D188-5 can also interfere with normal plant development.

The symptoms provoked by *R. fascians* on plants were initially explained by the ability of the bacteria to destroy auxins (Roussaux 1965; Kemp 1978), but it was soon realized that the method of action would involve other mechanisms. Some symptoms could be counteracted by the addition of gibberellic acid (Roussaux 1975), whereas others could be mimicked by the addition of cytokinin (Thimann and Sachs 1966; Oduro and Munnecke 1975). Finally, it became generally accepted that cytokinins were the major cause of symptom development in the aerial plant parts. Nevertheless, we have shown that the auxin levels in plants carrying leafy galls are much higher than those in control plants. This result explains the observed swelling of cells, the effects of *R. fascians* on vascular tissues (Aloni 1987), and the fasciation (Gorter 1965). Moreover, the observed induction of lateral root initiation can also be obtained by auxin treatment (Celenza et al. 1995; Lakowski et al. 1995). On the other hand, the abundant shoot proliferation is a typical cytokinin effect (Davies 1995) and the induction of Arath;*CycA2;1* promoter activity in the shoot apical meristem and the surrounding vascular tissue can be mimicked by adding exogenous cytokinins to the media (data not shown). Additionally, the observed inhibition of root growth and delayed senescence (Fig. 3F) can also be explained as cytokinin-like effects. Interestingly, preliminary cytokinin analysis did not reveal any significant differences in the level of standard cytokinins between shoot cultures and leafy gall tissue (data not shown). This result is in agreement with the cytokinin analysis on pea seedlings infected with different *R. fascians* strains reported by Eason et al. (1996). These data clearly suggest that a more complex process is occurring and it is therefore postulated that *R. fascians* produces signal molecules that differ from known hormones both in structure and activity. To explore further the effect of *R. fascians* on hormone balances, a detailed quantitative hormonal analysis is currently being performed.

We have demonstrated that co-cultivation of plant tissue with *R. fascians* induces cell differentiation and organogenesis and contributes to the improvement of in-vitro plant propagation for a wide range of plant species (Goethals et al. 1998). A number of recent reports have described the beneficial effects of bacterization (co-culture of plant and bacteria), such as promotion of nodulation using *Bacillus* sp. or promotion of shoot and root biomass using *Pseudomonas* sp. (Pillay and Nowak 1997). The use of *R. fascians* for plant propagation offers several advantages compared to the classical micropropagation technique. The addition of exogenous plant growth regulators to the culture medium is not required for shoot multiplication or for rooting. Moreover, the regenerated shoots generally originate from a selected meristematic tissue avoiding

mixed cultures and therefore decreasing the risk of genetic and/or epigenetic variation. Additionally, this method does not give the vitrification problems associated with the use of exogenous synthetic cytokinin. A supplementary advantage is that fewer steps are required so that the method effectively accelerates the micropropagation process. Lastly, the method is found to be suitable for a wide spectrum of plant species including trees, crops, medicinal, and aromatic plants.

Finally, a leafy gall represents an appropriately synchronized experimental model system suitable for studies related to the identification of molecular markers associated with apical dominance because the imposition of inhibition of bud outgrowth is complete. After the elimination of the bacteria, the inhibition of bud growth is relieved for all meristems. This situation represents a second form of synchronization in which shoots start to develop. Molecular analysis on both synchronized systems will certainly contribute to the identification of markers associated with shoot initiation, inhibition of shoot growth, and shoot elongation.

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