Purification of a protein from *Agrobacterium tumefaciens* strain A348 that binds phenolic compounds

Florence DYÉ and Francis M. DELMOTTE*

Glycobiologie, Centre de Biophysique Moléculaire, C.N.R.S. et Université d'Orléans, rue Charles Sadron, 45071 Orléans cedex 02, France

In order to induce tumours on dicotyledonous plants, the bacterium *Agrobacterium tumefaciens* needs to be able to sense signal molecules, i.e. phenolic compounds. In order to identify putative chemoreceptors or environmental sensors involved in *vir* gene induction, we undertook the purification of a phenol-binding protein by affinity chromatography on a syringamide–Ultrogel A4 column equilibrated at pH 5.6. A mild extraction of bacterial proteins with a Tris/HCl buffer at pH 9.0 led to the

purification of a 39 kDa protein (Pbp39) with a pI of 4.3 after specific elution of the affinity matrix with sodium syringate. When the affinity chromatography was performed at neutral pH, barely any protein was isolated, indicating the importance of an acidic pH for optimal affinity. A microplate binding experiment revealed that both syringyl–biotinylated-BSA and sinapyl–biotinylated-BSA bound at pH 5.6 to the plate coated with Pbp39.

INTRODUCTION

The soil bacterium *Agrobacterium tumefaciens* causes formation of the neoplastic disease crown gall on many dicotyledonous plants and on some monocots. The early step of infection is the attachment of the bacteria to wounded plant cells [1,2]; the possible involvement of bacterial lectins in this interaction has been considered [3]. The infection results from transfer and integration of a piece of DNA (the T-DNA) from the bacterial Ti plasmid into the nucleus of plant cells [4]. Expression of T-DNA, which encodes enzymes involved in cytokinin and auxin synthesis, leads to uncontrolled growth of plant cells [5,6].

A key event for tumorigenesis is the induction of virulence (*vir*) genes of the Ti plasmid. *vir* gene products mediate excision and transfer of T-DNA into the nucleus of the plant cell ([7]; for review see [8]). Virulence genes are specifically activated by phenolic compounds [9–11] in synergy with sugars [12–14], or by phenyl glycosides [15–17]. An acidic extracellular pH is also important for efficient induction [9]. Three bacterial proteins play crucial roles in *vir* gene induction. The Ti-plasmid-encoded VirA and VirG proteins are essential for a response to any of the three stimuli [18]. A chromosomally encoded periplasmic sugar-binding protein called ChvE is required for the potentiation of induction by monosaccharides [13], but is not required to sense phenolic compounds or acidic pH. VirA and VirG belong to a gene family of two-component regulatory systems. The VirA histidine protein kinase is located in the inner membrane of bacteria and is designated as the environmental sensor [19,20]. Periplasmic and transmembrane regions of VirA are dispensable for induction by phenolic compounds [21,22]. However, the periplasmic domain is essential for the enhancement of *vir* gene induction by monosaccharides [13,21]. The potentiation induced by sugars requires the interaction of ChvE and VirA [23,24].

The binding site for phenolic inducers has been an area of active investigation, but direct interaction between VirA and phenolic compounds has never been demonstrated. Bromoacetosyringone, used as an inhibitor of virulence genes, can

irreversibly block the induction of these genes [25]. A model of phenol–receptor interaction has been proposed which takes into account all structural features of inducers (i.e. phenol ring, *o*-methoxy group, *p*-carbonyl-containing functional group) [25,26]. Two small soluble phenol-binding proteins (p10 and p21) were identified by using a radiolabelled analogue of the bromoacetovanillone, but this technique did not label VirA [27]. These results suggest that intermediate phenol-binding proteins could be involved in phenol detection, which in turn activate VirA.

In the present paper, we report the purification and characterization of a phenol-binding protein isolated from *A. tumefaciens* strain A348, on the basis of its binding to an Ultrogel A4 column derivatized with the phenolic inducer syringic acid and its specific elution from this affinity matrix. The phenol-binding properties of the purified protein were assessed by a microplate assay using biotinylated BSA bearing syringyl or sinapyl residues.

MATERIALS AND METHODS

Reagents, bacterial strains and culture conditions

Reagents were of analytical grade, and were purchased from Merck (Nogent-sur-Marne, France) unless otherwise indicated. Benzamidinium chloride, (1-ethyl-3,3-dimethylaminopropyl)-carbodi-imide (EDAC), hexamethylene diamine, *N*-hydroxy-succinimide, 2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulphonic acid, BSA, Extravidin/peroxidase and chicken egg albumin (grade V) were purchased from Sigma (L'Isle d'Abeau Chesnes, France). Acrylamide, bisacrylamide, ampholytes (Bio-Lyte 3/10), SDS and proteins used as molecular mass standards were purchased from Bio-Rad (Ivry-sur-Seine, France). Sinapic acid and syringic acid were purchased from Aldrich Chimie (L'Isle d'Abeau Chesnes, France). Trisacryl GF05 (synthetic gel from *N*-acryloyl-2-amino-2-hydroxy-1,3-propanediol) and Ultrogel A4 (agarose) were purchased from BioSeptra (Villeneuve La Garenne, France). CNBr and sulphosuccinimidyl-6-(biotin-

Abbreviation used: EDAC, (1-ethyl-3,3-dimethylaminopropyl)carbodi-imide.

* To whom correspondence should be addressed.

Figure 1 Schematic protocol for synthesis of the affinity chromatography matrix

See the Materials and methods section for details.

amido)hexanoate (NHS-LC-biotin) were from Pierce (Rockford, IL, U.S.A.). Tween 20 was purchased from Prolabo (Fontenay-sous-Bois, France).

A. tumefaciens strain A348 (C58 chromosomal background) harbouring the octopine Ti plasmid pTiA6 was used throughout this study [28]. The strain was maintained on MG/L medium at 25 °C [29]. *A. tumefaciens* strain C58C1 (C58 lacking the Ti plasmid) was cultured on the same medium.

Extraction of bacterial proteins

In order to promote cell growth and protein synthesis, bacteria were grown overnight at 25 °C in 5 × 1.5 litres of MG/L liquid medium at pH 7.0 in five 5 litre Erlenmeyer flasks with gentle stirring. Cells in the exponential growth phase were harvested by centrifugation at 4000 *g* for 30 min.

In order to release proteins from the bacterial envelope, a mild extraction was performed using a procedure previously described for the extraction of bacterial lectins [3]. Briefly, a crude protein extract was obtained by stirring the bacterial pellet (10 g) with 50 ml of 50 mM Tris/HCl buffer, pH 9.0, supplemented with 20 mM benzamidinium chloride and 0.2 M MgCl₂. After stirring for 90 min at 25 °C, the bacterial suspension was centrifuged at 7700 *g* for 40 min at 4 °C. The supernatant was defined as the crude protein extract.

A second type of extraction procedure was performed to selectively obtain periplasmic proteins. The protocol was adapted from that of Manoil and Beckwith [30]. A bacterial pellet (5 g) was resuspended in 50 ml of cold buffer (100 mM Tris/HCl, pH 8.0, supplemented with 0.5 M sucrose, 0.5 mM EDTA and 20 mM benzamidinium chloride), kept on ice for 5 min and centrifuged for 5 min at 12000 *g* at 4 °C. The pellet was warmed

at room temperature and resuspended in 50 ml of ice-cold water by vigorous shaking. After 1 min, MgCl₂ was added to a final concentration of 1 mM and the suspension was kept for 5 min on ice. The bacteria were then centrifuged for 5 min at 12000 *g*, and the supernatant was defined as the periplasmic protein fraction.

Affinity chromatography columns

Ultrogel A4 matrix was activated with CNBr according to March et al. [31]. Hexamethylene diamine was coupled to this activated support to generate a linker arm with a free amino group [32] (Figure 1). Syringic acid (0.2 mmol; 40 mg in 1 ml of *N,N*-dimethylformamide) and 40 mg of EDAC were added to 54 ml of decanted [(6-aminohexyl)iminomethylene]-Ultrogel A4. This slurry was adjusted to pH 5.0 and shaken for 24 h at 4 °C. A second cycle of activation was performed by adding 40 mg of EDAC, adjusting to pH 5.0 and shaking for a further 24 h at 4 °C. The slurry was then filtered on a scintered glass filter and washed with distilled water. The gel was resuspended in an equivalent volume of 0.2 M NaOH and the remaining free amino groups were blocked by the dropwise addition of 600 μ l of acetic anhydride at 0 °C. The slurry was then stirred for 24 h at 4 °C. Another cycle was performed by adding a further 600 μ l of acetic anhydride and stirring for 24 h at 4 °C. The gel was then extensively washed with distilled water and stabilized in 0.1 M sodium acetate buffer, pH 5.6. The proportion of syringic acid fixed on the gel was determined approximately by titration of the unbound syringic acid recovered in the filtrate. It was found that 2 μ mol of syringic acid was coupled to 1 ml of decanted [(6-aminohexyl)iminomethylene]-Ultrogel A4 under such conditions.

Protein purification

The entire purification procedure was performed at 4 °C in a cold room. Bacterial proteins (crude protein extract or periplasmic protein fraction) were passed over a Trisacryl GF05 gel-filtration column (20 cm × 5 cm) equilibrated in 0.1 M sodium acetate buffer, pH 5.6. The protein fraction eluted at the column void volume was submitted to affinity chromatography. The material was first loaded on to a precolumn of free Ultrogel A4 (10 cm × 1.1 cm) to minimize non-specific binding, before being injected on to a column of [syringic (6-aminoethyl)imino-methylene]-Ultrogel A4 (hereafter called syringamide-Ultrogel A4) (10 cm × 1.1 cm).

The affinity column was specifically eluted with a gradient of sodium syringate, and then with solutions of increasing ionic strength (0.15 and 0.30 M NaCl in 0.1 M sodium acetate buffered to pH 5.6). Sodium syringate was prepared by adding syringic acid to NaOH. After freeze-drying, a stock solution of 0.1 M sodium syringate was prepared and adjusted at pH 5.6 with diluted acetic acid. This stock solution was then diluted in 0.1 M sodium acetate buffered to pH 5.6 in order to obtain the desired concentration. Each eluted fraction was dialysed against distilled water, freeze-dried and analysed by SDS/PAGE. In order to determine the importance of pH for the interaction of the putative phenol-binding proteins with the affinity matrix, gel filtration and affinity chromatography were also performed in 50 mM sodium phosphate buffer, pH 7.0.

SDS/PAGE and isoelectrofocusing

SDS/PAGE was performed according to Laemmli [33] using a 12.5 % acrylamide slab gel (16 cm × 18 cm). The marker proteins used were: phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa) and egg white lysozyme (14.4 kDa). The gels were stained with Coomassie Blue G250.

Isoelectrofocusing PAGE was carried out in glass tubes (0.5 cm × 10 cm) packed with 8 % polyacrylamide gel (see Wrigley [34]) containing 2.4 % (v/v) carrier ampholytes in the pH range 3.0–10.0. Upon focusing, the gels were immediately fixed with 2.5 % trichloroacetic acid for 48 h to eliminate the ampholytes, and then stained with Coomassie Blue G250.

Synthesis of macromolecular ligands

N-Hydroxysuccinimide (150 mg, 1.3 mmol) and *N,N'*-dicyclohexylcarbodi-imide (300 mg, 1.45 mmol) were added slowly to a solution of syringic acid (218 mg, 1.1 mmol in 10 ml of *N,N*-dimethylformamide) at 0 °C. This mixture was then stirred at room temperature. After 4 h, the solution was cooled down at 0 °C and a further 300 mg (1.45 mmol) of *N,N'*-dicyclohexylcarbodi-imide was added. After 8 h of reaction at room temperature, syringic acid (R_f 0.21) had been completely consumed, as shown by TLC on silica gel (Merck 60F₂₅₄) in chloroform/ethyl acetate (1:1, v/v). The solution was kept at –20 °C overnight to allow precipitation of the *N,N'*-dicyclohexylurea produced during the reaction. The precipitate was removed by filtration, and the filtrate containing the *N*-hydroxysuccinimide ester of syringic acid (R_f 0.48) was stored at –20 °C.

BSA (204 mg, 3 µmol) was dissolved in 0.1 M NaHCO₃ (15 ml) at 4 °C. The *N*-hydroxysuccinimide ester of syringic acid (120 µmol) was added dropwise. The reaction was performed at 4 °C for 18 h. This mixture was then filtered in order to eliminate the insoluble residue produced. The filtrate was then dialysed against distilled water and freeze-dried.

The average number of syringyl residues bound to the BSA

was calculated from measurement of the absorbance at 268 and 280 nm. About five syringyl residues were bound per molecule of BSA (syringyl-BSA).

The preparation of synapyl-BSA was performed using the same conditions as described above.

Biotinylation of ligands

The macromolecular ligands were biotinylated using sulpho-succinimidyl-6-(biotinamido)hexanoate. BSA, syringyl-BSA or synapyl-BSA (10 mg, 150 nmol) was dissolved in 50 mM Na₂CO₃/NaHCO₃ buffered to pH 9.2 (1 ml) at 4 °C. Biotin ester (3.3 mg, 6 µmol) was added and the reaction was performed at 4 °C, with stirring, for 150 min. The solution was then submitted to gel filtration over a Trisacryl GF05 column (15 cm × 1.5 cm) equilibrated in 0.1 M sodium acetate buffer, pH 5.6. The excluded fraction was divided into aliquots and stored at –20 °C.

Microplate binding assay

After mild extraction in 50 mM Tris/HCl buffer, pH 9.0, supplemented with 20 mM benzamidinium chloride and 0.2 M MgCl₂ and affinity chromatography on syringamide-Ultrogel A4, the fraction eluted by 5 mM sodium syringate was submitted to ultrafiltration in order to concentrate the sample, to eliminate the sodium syringate for further interaction and to stabilize this protein sample in a neutral buffer, which favours microplate coating.

The 96-well microtitre plate (Maxisorp; Nunc, Vienna, Austria) was coated with protein samples serially diluted in PBS (100 µl final volume) by incubation overnight at 4 °C. After washing the plate with 200 µl of PBS supplemented with 0.05 % Tween 20, non-specific sites were blocked with 100 µl of 1 % ovalbumin in PBS/Tween 20 for 1 h at 37 °C. The plate was then washed as described above and 100 µl of biotinylated ligand (BSA, syringyl-BSA or synapyl-BSA) at 50 µg/ml in 0.1 M sodium acetate buffer, pH 5.6, containing 0.05 % Tween 20 (acetate-T) was added prior to a 1 h incubation at 37 °C. After washing the wells with 200 µl of acetate-T, 100 µl of an Extravidin/peroxidase solution, prepared by 1:1000 dilution in acetate-T from a 2 mg/ml stock solution, was added to each well and incubated for 30 min at 37 °C. The plate was then washed with acetate-T and incubated with substrate [2,2'-azinobis-(3-ethylbenzthiazoline)-6-sulphonic acid; 10 mg in 50 mM citrate/phosphate buffer, pH 5.6, containing 20 µl of hydrogen peroxide]. After 20 min, the reaction was stopped by adding 2 % oxalic acid. The peroxidase activity bound to the plate was measured spectrophotometrically at 405 nm on a microtitre plate reader (Multiskan, Labsystems).

All experiments were performed in duplicate, and the results were expressed as mean values. The phenol-binding protein was omitted from the control well for use as a blank.

RESULTS

Protein extraction and purification

Proteins of the bacterial envelope were released from a bacterial pellet with 50 mM Tris/HCl buffer, pH 9.0, supplemented with 20 mM benzamidinium chloride and 0.2 M MgCl₂ used as protease inhibitor and chaotropic reagent respectively. This crude protein extract was first passed over a Trisacryl GF05 gel-filtration column equilibrated in 0.1 M sodium acetate, pH 5.6, and was then loaded on to an Ultrogel A4 precolumn coupled to the syringamide-Ultrogel A4 affinity column also equilibrated in 0.1 M sodium acetate, pH 5.6. Specific stepwise elution of the

Figure 2 Affinity chromatography on syringamide–Ultrogel A4 at pH 5.6 after extraction by the Tris/HCl buffer method: stepwise elution with sodium syringate

SDS/PAGE analysis (12.5% acrylamide) is shown of protein fractions from *A. tumefaciens* strain A348 stained with Coomassie Blue G250. Crude protein extract was obtained using a Tris/HCl buffer extraction method. Affinity chromatography on syringamide–Ultrogel A4 was performed in 0.1 M sodium syringate, pH 5.6. Lanes: 1, sodium syringate solution as a control; 2, unbound material (material that passed through both columns); 3–6, material specifically eluted from the syringamide–Ultrogel A4 column at pH 5.6 with 5, 10, 25 and 50 mM sodium syringate respectively; 7, molecular mass markers (indicated on the right in Da); 8 and 9, material eluted from the syringamide–Ultrogel A4 column at pH 5.6 with 0.15 and 0.30 M NaCl respectively.

syringamide affinity matrix with 5 and 10 mM sodium syringate led to the isolation of a major protein of molecular mass 39 kDa (Figure 2, lanes 3 and 4). At higher concentrations of sodium

Protein binding in 0.1 M sodium acetate buffer at pH 5.6
Specific elution of the Pbp39 with 5 mM sodium syringate

Figure 3 Purification of Pbp39

Figure 4 Affinity chromatography on syringamide–Ultrogel A4 at pH 5.6 after extraction by osmotic shock: stepwise elution with sodium syringate

SDS/PAGE analysis (12.5% acrylamide) is shown of protein fractions from *A. tumefaciens* A348 stained with Coomassie Blue G250. The periplasmic protein fraction was obtained after osmotic shock and was submitted to affinity chromatography on syringamide–Ultrogel A4 in 0.1 M sodium acetate buffer, pH 5.6. Lanes: 1, unbound material; 2–4, material specifically eluted from the syringamide–Ultrogel A4 column at pH 5.6 with 5, 10 and 25 mM sodium syringate respectively; 5, molecular mass markers (indicated on the right in Da).

syringate, and on increasing the ionic strength to 0.15 and 0.30 M NaCl, two other proteins of molecular mass 33 and 32 kDa were eluted (Figure 2, lanes 5, 6, 8 and 9). Increasing the ionic strength also allowed elution of a smaller protein of 17 kDa (Figure 2, lanes 8 and 9).

Isolation of the 39 kDa protein (named Pbp39, for phenol-binding protein of 39 kDa) was extremely reproducible when affinity chromatography was performed at pH 5.6 with elution with 5 mM sodium syringate, as summarized in Figure 3. Approx. 10 µg of purified Pbp39 was isolated from a 10 g bacterial pellet. We observed very poor binding of protein when the affinity column was equilibrated in 50 mM sodium phosphate, pH 7.0. Isoelectrofocusing PAGE of Pbp39 showed a single band at a pI of 4.3 (results not shown).

Purification by affinity chromatography at pH 5.6 was also performed using a periplasmic protein fraction obtained by osmotic shock. Specific elution of the syringamide–Ultrogel A4 column by 5 and 10 mM sodium syringate also revealed the 39 kDa protein (Figure 4, lanes 2 and 3). It appeared that both extraction procedures yielded several common proteins, as shown by electrophoresis analysis (Figure 2, lane 2, and Figure 4, lane 1).

An alternative specific elution of the syringamide–Ultrogel A4 column was assessed using 10 mM sodium sinapate, another inducing phenol. Under such conditions, sodium sinapate appeared to be less effective than sodium syringate for selective and specific elution of Pbp39 (results not shown).

Binding experiments

To study phenol–receptor interactions, a microplate assay was used in order to preserve the structure of the putative receptor and to favour interactions. In this experiment, the eluted Pbp39 (extracted by the Tris/HCl buffer method and specific elution on affinity chromatography by 5 mM sodium syringate) was coated on to a microplate. After blocking unbound solid-phase sites with 1% ovalbumin, ligands such as syringyl- and sinapyl-

Figure 5 Binding of biotinylated ligands on Pbp39

Pbp39 (extraction by the Tris/HCl buffer method and specific elution from affinity chromatography by 5 mM sodium syringate) was coated on to the plate. Biotinylated ligands (syringyl-biotinylated-BSA, synapyl-biotinylated-BSA and biotinylated-BSA alone) were incubated at 50 µg/ml at pH 5.6 for 1 h at 37 °C. Binding was revealed at pH 5.6 by peroxidase coupled to avidin.

biotinylated-BSA were added to the microplate. After washing, binding was revealed using avidin coupled to peroxidase. Binding was clearly demonstrated with both biotinylated-BSA ligands when the incubation was performed at pH 5.6 (Figure 5). No binding was observed with biotinylated-BSA alone used as a control. The same experiment performed at neutral pH showed no binding of either biotinylated ligand. As expected, the binding assay results were affected by the protein concentration used for coating the microplate. This kind of sensitive microplate assay was previously used to reveal interactions between glycoproteins and biotinylated lectins [35].

DISCUSSION

In addition to the role of phenolic wound exudates as *vir* gene inducers in agrobacteria, monocyclic phenolic compounds such as acetosyringone can serve as chemoattractants or nutrients for *A. tumefaciens* (for review, see [36]). The mechanisms of detection of phenolic compounds by *A. tumefaciens* remain unclear. Chemoreceptors involved in chemotaxis towards phenolic compounds have not yet been identified in *A. tumefaciens*. Whether VirA protein directly senses phenols or whether intermediate protein(s) are required is still unknown. The essential role of VirA in signal transduction has been shown by numerous studies based on molecular genetic approaches [20,21,37–39]; however, a direct interaction between VirA and phenols has yet to be demonstrated by specific labelling or isolation of an inducer–VirA complex. The search for a phenol-binding protein associated with the bacterial envelope of *A. tumefaciens* strain A348 yielded a major protein of 39 kDa, Pbp39, that was purified by affinity chromatography on syringamide–Ultrogel A4 at pH 5.6. The choice of such an affinity matrix was made in view of the high induction levels of the virulence genes by ethylsyringamide due to its amide function [39a]. Pbp39 appeared as a monomer, since electrophoresis under both reducing and non-reducing

conditions revealed a unique band. The choice of the pH used for purification was guided by the fact that optimal induction of virulence gene products occurs at pH 5.5–5.6. When purification was performed at neutral pH, little protein bound to the affinity matrix.

Other minor proteins of molecular mass 33 and 32 kDa could be specifically eluted from the syringamide–Ultrogel A4 column by sodium syringate. A 17 kDa protein was also eluted by increasing the ionic strength. The possibility cannot be excluded that several proteins with different specificities, based on phenol structure, could exist within bacteria. In *Rhizobium*–legume interactions, differing responses to inducers have consistently been linked to the existence of different forms of the NodD protein, the putative receptor of the flavonoid signal molecules [40].

Pbp39 is clearly different from the p10 and p21 soluble proteins previously revealed by labelling with α -bromo-¹²⁵I-iodoacetovanillone [27]. Such a difference in the proteins isolated could be due to the structures of the phenolic derivatives used. Pbp39 was also present in an extract prepared from strain C58C1, which is a *A. tumefaciens* strain lacking the Ti plasmid (results not shown). This result confirmed that Pbp39 is chromosomally encoded, in contrast with the VirA protein which is encoded by a Ti-plasmid gene.

Binding experiments performed at pH 5.6 with syringyl- and synapyl-biotinylated-BSA confirmed the specificity of Pbp39. In this microplate assay, the affinity of Pbp39 for the putative ligand was highly preserved during both incubation steps, i.e. with the biotinylated ligand and then with avidin–peroxidase, assessed at pH 5.6. No binding of either biotinylated ligand was observed at neutral pH. This result was in agreement with the low level of Pbp39 recovered after affinity chromatography performed at pH 7.0.

Pbp39 is a new phenol-binding protein which has not been previously identified in *A. tumefaciens*. It is constitutively expressed, since bacteria used for protein extraction were grown in media without inducer (MG/L medium, pH 7.0). We do not exclude the possible involvement of Pbp39 in chemotaxis. Chemotaxis towards phenolic compounds such as acetosyringone has been reported [41,42], but nothing is known about the proteins involved in chemotaxis signal transduction pathways. The role of Pbp39 is still unknown, and may be elucidated after cloning and sequencing of its gene. Regulation of the gene encoding Pbp39 should be studied, especially in the presence of inducing compounds, since, if this protein is implicated in phenol perception or transport, its expression will be enhanced by phenols.

We give special thanks to Professor E. W. Nester (University of Washington, Seattle, WA, U.S.A.) and Dr. X. Nesme (U.R.A.-C.N.R.S. 1977, Villeurbanne, France), who provided the *A. tumefaciens* strains A348 and C58C1 (C58 plasmidless) respectively. We thank Dr. V. Piller and Dr. F. Piller for helpful discussions, Professor M. Nachtigal (University of South Carolina, Columbia, SC, U.S.A.) for his kind help in improving the English of the manuscript, and Dr. B. Guérin for her skilful contribution in bacterial cultures. This research was partly supported by grants from Arbocentre and Conseil Régional Région Centre. F.D. was the recipient of a fellowship from M.E.S.R. (Ministère de l'Enseignement Supérieur et de la Recherche).

REFERENCES

- Lippincott, B. B. and Lippincott, J. A. (1969) *J. Bacteriol.* **97**, 620–628
- Matthysse, A. G. (1994) *Protoplasma* **183**, 131–136
- Depierreux, C., Kang, H.-C., Guérin, B., Monsigny, M. and Delmotte, F. M. (1991) *Glycobiology* **1**, 643–649
- Chilton, M.-D., Saiki, R. K., Yadav, N., Gordon, M. P. and Quetier, F. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 4060–4064
- Inzé, D., Follin, A., Van Lijsebettens, M., Simoons, C., Genetello, C., Van Montagu, M. and Schell, J. (1984) *Mol. Gen. Genet.* **194**, 265–274

- 6 Akiyoshi, D. E., Klee, H., Amasino, R. M., Nester, E. W. and Gordon, M. P. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 5994–5998
- 7 Stachel, S. E. and Nester, E. W. (1986) *EMBO J.* **5**, 1445–1454
- 8 Hooykaas, P. J. J. and Beijersbergen, A. G. M. (1994) *Annu. Rev. Phytopathol.* **32**, 157–179
- 9 Stachel, S. E., Nester, E. W. and Zambryski, P. C. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 379–383
- 10 Spencer, P. A. and Towers, G. H. N. (1988) *Phytochemistry* **27**, 2781–2785
- 11 Melchers, L. S., Regensburg-Tuink, A. J. G., Schilperoort, R. A. and Hooykaas, P. J. J. (1989) *Mol. Microbiol.* **3**, 969–977
- 12 Ankenbauer, R. G. and Nester, E. W. (1990) *J. Bacteriol.* **172**, 6442–6446
- 13 Cangelosi, G. A., Ankenbauer, R. G. and Nester, E. W. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 6708–6712
- 14 Song, Y.-N., Shibuya, M., Ebizuka, Y. and Sankawa, U. (1991) *Chem. Pharm. Bull.* **39**, 2613–2616
- 15 Morris, J. W. and Morris, R. O. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3614–3618
- 16 Delmotte, F. M., Delay, D., Cizeau, J., Guérin, B. and Leplé, J.-C. (1991) *Phytochemistry* **30**, 3549–3552
- 17 Delay, D., Dyé, F., Wisniewski, J.-P. and Delmotte, F. M. (1994) *Phytochemistry* **36**, 289–298
- 18 Stachel, S. E. and Zambryski, P. C. (1986) *Cell* **46**, 325–333
- 19 Leroux, B., Yanofsky, M. F., Winans, S. C., Ward, J. E., Ziegler, S. F. and Nester, E. W. (1987) *EMBO J.* **6**, 849–856
- 20 Melchers, L. S., Regensburg-Tuink, T. J. G., Bourret, R. B., Sedee, N. J. A., Schilperoort, R. A. and Hooykaas, P. J. J. (1989) *EMBO J.* **8**, 1919–1925
- 21 Chang, C.-H. and Winans, S. C. (1992) *J. Bacteriol.* **174**, 7033–7039
- 22 Turk, S. C. H. J., van Lange, R. P., Regensburg-Tuink, T. J. G. and Hooykaas, P. J. J. (1994) *Plant Mol. Biol.* **25**, 899–907
- 23 Shimoda, N., Toyoda-Yamamoto, A., Aoki, S. and Machida, Y. (1993) *J. Biol. Chem.* **268**, 26552–26558
- 24 Banta, L. M., Joerger, R. D., Howitz, V. R., Campbell, A. M. and Binns, A. N. (1994) *J. Bacteriol.* **176**, 3242–3249
- 25 Hess, K. M., Dudley, M. W., Lynn, D. G., Joerger, R. D. and Binns, A. N. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 7854–7858
- 26 Duban, M. E., Lee, K. and Lynn, D. G. (1993) *Mol. Microbiol.* **7**, 637–645
- 27 Lee, K., Dudley, M. W., Hess, K. M., Lynn, D. G., Joerger, R. D. and Binns, A. N. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 8666–8670
- 28 Garfinkel, D. J., Simpson, R. B., Ream, L. W., White, F. F., Gordon, M. P. and Nester, E. W. (1981) *Cell* **27**, 143–153
- 29 Chilton, M.-D., Currier, T. C., Farrand, S. K., Bendich, A. J., Gordon, M. P. and Nester, E. W. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3672–3676
- 30 Manoil, C. and Beckwith, J. (1986) *Science* **233**, 1403–1408
- 31 March, S. C., Parikh, I. and Cuatrecasas, P. (1974) *Anal. Biochem.* **60**, 143–152
- 32 Delmotte, F. M. and Goldstein, I. (1980) *Eur. J. Biochem.* **112**, 219–223
- 33 Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- 34 Wrigley, C. W. (1971) *Methods Enzymol.* **22**, 559–564
- 35 Duk, M., Lisowska, E., Wu, J. H. and Wu, A. M. (1994) *Anal. Biochem.* **221**, 266–272
- 36 Gelvin, S. (1992) in *Molecular signals in Plant-Microbe Communications* (Verma, D., ed.), pp. 137–167, CRC Press, London
- 37 Ankenbauer, R. G., Best, E. A., Palanca, C. A. and Nester, E. W. (1991) *Mol. Plant-Microbe Interact.* **4**, 400–406
- 38 McLean, B. G., Greene, E. A. and Zambryski, P. C. (1994) *J. Biol. Chem.* **269**, 2645–2651
- 39 Lee, Y. W., Jin, S. G., Sim, W. S. and Nester, E. W. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12245–12249
- 39a Dyé, F., Berthelot, K., Griffon, B., Delay, D. and Delmotte, F. M. (1997) *Biochimie*, in the press
- 40 Spaink, H. P., Wijffelman, C. A., Pees, E., Okker, R. J. H. and Lugtenberg, B. J. J. (1987) *Nature (London)* **328**, 337–340
- 41 Parke, D., Ornston, L. N. and Nester, E. W. (1987) *J. Bacteriol.* **169**, 5336–5338
- 42 Ashby, A. M., Watson, M. D., Loake, G. J. and Shaw, C. H. (1988) *J. Bacteriol.* **170**, 4181–4187