Phytochemistry, Vol. 27, No. 8, pp. 2429–2433, 1988.
Printed in Great Britain.

0031–9422/88 $3.00 + 0.00

CUCUMOPINE—A NEW T-DNA-ENCODED OPINE IN HAIRY ROOT AND CROWN GALL

ELISABETH DAVIOUD,†* ANNIK PETIT,* MAX E. TATE,‡ MAARTEN H. RYDER‡ and JACQUES TEMPÉ*

*INRA, Département de Génétique et d'Amélioration des Plantes and CNRS, Unité Associée 136. Institut de Microbiologie, Bât 409, Universite de Paris-sud, 91405-Orsay, France; †Institut de Chimie des Substances Naturelles, CNRS, 91190—Gif sur Yvette, France; ‡Agricultural Biochemistry. Waite Agricultural Research Institute, 5064, Glen Osmond, South Australia

(*Received* 22 *February* 1988)

Key Word Index—*Agrobacterium tumefaciens*;*Agrobacterium rhizogenes*; Ti plasmid; Ri plasmid; crown gall; hairy root; opine.

Abstract—A new opine, cucumopine, has been identified by high voltage paper electrophoresis in hairy roots induced by *Agrobacterium rhizogenes*. Cucumopine gives a positive reaction with the Pauly imidazole reagent, the phenanthrene quinone reagent and alkaline silver nitrate. This substance was also found in spontaneous grapevine crown gall tumors. It is degraded by *A. rhizogenes* and by grapevine isolates of *A. tumefaciens*. Cucumopine was purified from carrot hairy root culture supernates and grapevine crown gall tumours.

INTRODUCTION

Crown gall and hairy root are two bacterial plant diseases, elicited respectively by *Agrobacterium tumefaciens* and *A. rhizogenes*, which have recently received much attention since they result from a unique phenomenon, the transfer of genetic information from a bacterium to plant cells. [1, 2] (for review see [3]). In plant cells, expression of the transferred DNA (T-DNA) results in formation of a tumour (crown gall, *A. tumefaciens*) or of an abundant mass of highly branched roots (hairy root, *A. rhizogenes*) and in synthesis of new metabolites which have been given the generic name opines [4].

Pathogenic determinants in *A. tumefaciens* and *A. rhizogenes* are located on large plasmids called respectively Ti and Ri plasmids [3]. These encode functions involved in plant bacteria interactions, including those borne by T-DNA regions which are involved in tumour or hairy root proliferation and opine synthesis in plant cells.

Very early on, investigations on secondary metabolite composition showed the presence in crown gall tumours of these new metabolites [5, 6]. Interest in opines developed after the discovery that their synthesis in crown gall tumours elicited by *A. tumefaciens* is strain-specific [7, 8] and that they can be degraded with the same specificity by the pathogen [8]. This suggested a nutritional relationship between the pathogen and the diseased tissues of its host and a theory, known as the opine concept, describing the role of opines as nutritional mediators of parasitism was proposed [9–12].

The opine concept states that (i) opines are trophic mediators of parasitism and (ii) this function is essential for survival and propagation of Ti and Ri plasmids. Thus, according to this theory, every one of these plasmids should carry genes involved in opine synthesis in transformed plant cells and opine degradation by bacterial cells which harbour this plasmid. This theory has received substantial experimental support from the discovery of new opines in crown gall tumours [13, 14].

More recently, the opine concept was extended to hairy root [15]. Three types of Ri plasmids have been identified [16]. Two of these have been characterized by their opine type but no known opine was associated with the third type of plasmid. We report here the identification of a new compound that possess the attributes of an opine and is characteristic for the third type of Ri plasmids. This compound has also been found in grapevine crown gall tumours incited by *A. tumefaciens* [17]. The trivial name cucumopine which recalls the origin of the corresponding rhizogenic strains originally isolated from diseased cucumber plants (D. Stead, personal communication) is proposed for this compound.

RESULTS AND DISCUSSION

Identification of the new opine. Aqueous extracts from *in vitro* cultured hairy root lines from various plant species inoculated with *A. rhizogenes* strains NCPPB2655, 2657 or 2659 were analysed by high voltage paper electrophoresis (HVPE). None of the previously described opines was seen in these extracts but an unidentified spot staining with either the Pauly imidazole reagent [18], alkaline silver nitrate [19], or the phenanthrenequinone reagent [20] was detected (Fig. 1). At pH 1.9, the mobility relative to histidine of this spot was $M_{his} = +0.20$. The new spot was neither seen on electrophoregrams of root extracts incited by previously characterized *A. rhizogenes* strains, nor of extracts prepared from normal roots of any plant species. Its presence in extracts was often correlated with a conspicuous decrease of the histidine spot (Fig. 1). In earlier experiments, acid extracts from the same material showed two Pauly-positive spots, $M_{his} = 0.20$ and $M_{his} = 0.26$ [17], of which the faster moving was later shown to correspond to an acid

* In memory of Tony Swain, 1922–1987.

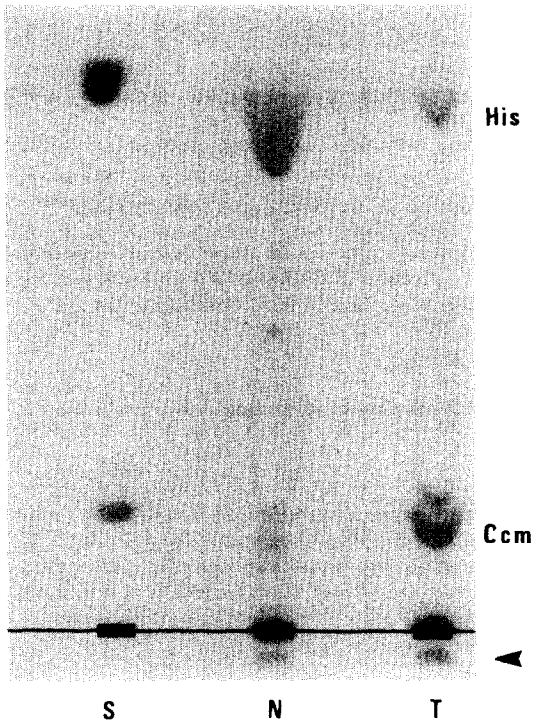


Fig. 1. Electrophoregram of extracts from *L. corniculatus* roots. Each extract spotted corresponded to 5 mg of roots (fr. wt), electrophoresis was for 10 min at 4000 V (100 V/cm). Staining was with Pauly imidazole reagent. S: standard with histidine and synthetic cucumopine, N: extract from normal roots, T: extract from transformed roots. His: histidine, Ccm: cucumopine, arrow shows an unknown Pauly-positive compound. Note the small amount of endogenous histidine in the extract containing cucumopine.

degradation product of the slow moving compound. In order to test the possibility that the new compound present in aqueous extracts from hairy roots was an opine, partially purified carrot hairy root extracts were used as a bacterial culture medium which was inoculated with *A. rhizogenes* NCPPB2659 and several other *Agrobacterium* strains. These extracts contained both the new compound and its degradation product. Visual inspection of the cultures after three days indicated good growth of some of the bacterial strains and HVPE showed that these had utilized the Pauly-positive products present in the extract (Fig. 2). Among *A. rhizogenes* strains only NCPPB 2659 and two other isolates, NCPPB2657 (not shown) and 2655 degraded the products. These were also utilized by two *A. tumefaciens* strains, originally isolated from grapevine tumours. These results confirmed the new compound of $M_{his} = 0.20$ as an opine which was called cucumopine.

Spontaneous grapevine crown gall tumours contain octopine and cucumopine

Extracts prepared from spontaneous grapevine tumours (supplied by M. Ridé, INRA, Angiers, France) were analysed by HVPE, and shown to contain octopine and cucumopine in large amounts.

Isolation of cucumopine from spent growth medium of carrot hairy root cultures

Spent culture media on which carrot hairy root cultures had been grown were concentrated (× 10) and analysed by HVPE. The use of various stains showed cucumopine as the most abundant organic cation in these. Therefore culture media were used as a source of cucumopine. Organic cations were recovered from spent medium by ion exchange. At this stage HVPE showed that an important fraction of cucumopine had been degraded to the faster moving compound during the ion exchange process. The cationic extract was cleaned up by biological purification [13]. This involves selective bacterial degradation of contaminants present in the extract. Following this step, organic cations were recovered as before. At this stage, electrophoresis indicated only three major cationic components: cucumopine, its acid degradation product and a fast moving ninhydrin-positive contaminant. This material was further purified by anion exchange chromatography on DEAE Sephadex. Electrophoretically homogeneous cucumopine and its acid rearrangement product were obtained in low yield.

Purification of cucumopine from grapevine tumours

Dry, lignified grapevine stems covered with tumours were ground and the resulting powder treated with water. The suspension was filtered and organic cations recovered by ion exchange. The mixture, which contained octopine, cucumopine (its acid degradation product) and a number of ninhydrin-positive amino acids, was biologically purified. Most of the contaminants were used during bacterial growth resulting in efficient purification of cucumopine and its degradation product. The cations were recovered from the culture supernate by ion exchange. Cucumopine and its degradation product were further purified by anion exchange chromatography.

Analytical properties of cucumopine

Cucumopine was completely converted to the faster moving compound by treatment with 1 M acetic acid (110°, 30 min). On electrophoregrams, cucumopine reacts with alkaline phenanthrenequinone to give an orange fluorescent spot (clearly distinguishable from arginine or octopine) under short wavelength (254 nm) UV light, the spot corresponding to the acid degradation product giving a bluish-violet spot. Cucumopine gives a pink to orange colour with the Pauly imidazole reagent. The second product gives a greenish-yellow reaction with the same reagent. Both substances react slowly with the silver nitrate reagent to give a grey colour which can be enhanced by steaming. However these spots fade out upon fixation.

Cucumopine is a condensation product of L-*histidine and* α-*ketoglutaric acid*

The structure of cucumopine and its acid degradation product were shown by mass and NMR spectrometry (Davioud *et al.* in preparation) to be respectively 4-carboxy, 4-(2-carboxyethyl)-spinacine or 4,5,6,7-tetrahydroimidazo(4,5-c) pyridine 4-propanoic, 4,6-dicarboxylic acid (cucumopine, **1**) and the lactam (**2**) formed between the 4-carboxyethyl substituent and the α-imino-nitrogen.

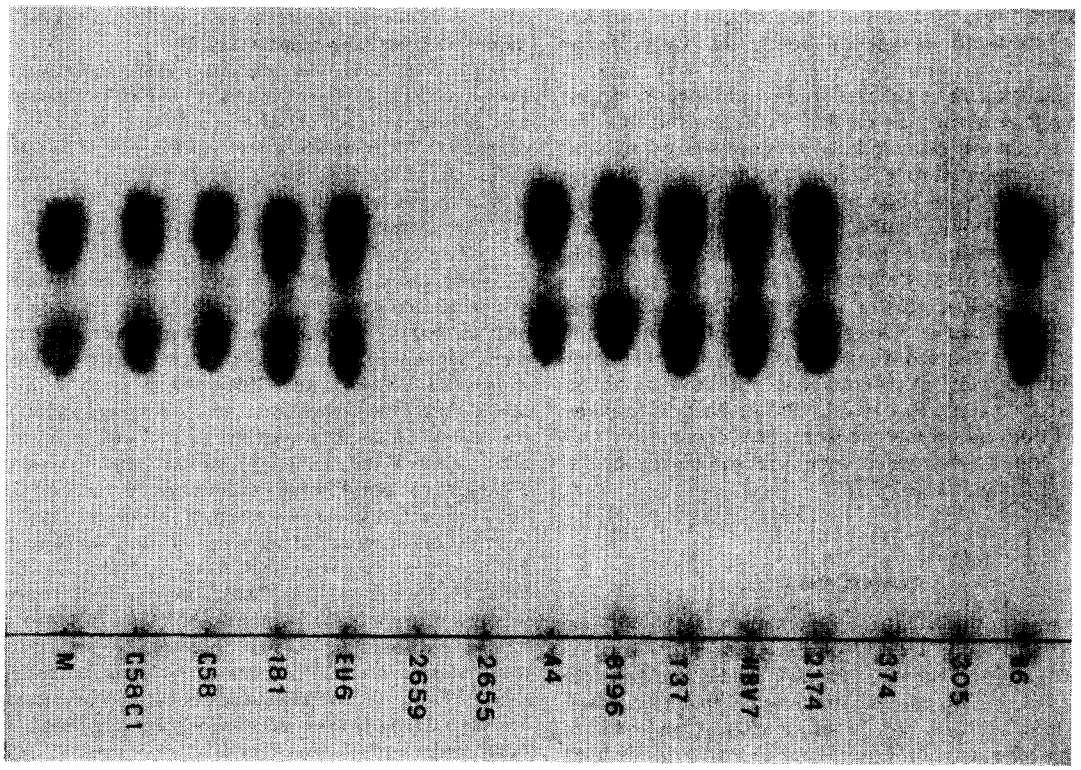
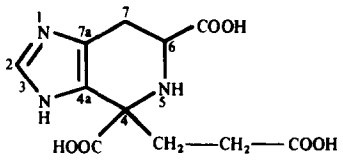


Fig. 2. Utilization of cucumopine and its acid degradation product by *Agrobacterium* strains. A partially purified extract was used for preparing a bacterial culture medium which was inoculated with several strains. After three days, the cultures were centrifuged and the supernates analysed by HVPE. Strain names are indicated, M: uninoculated culture medium.

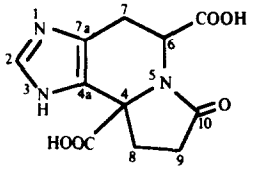
Cucumopine was synthesized by base-catalysed condensation of α-ketoglutaric acid and L-histidine (Pictet–Spengler reaction [21]). Synthetic cucumopine was converted to the lactam (**2**) by hot acid treatment. Both compounds were used as sole carbon source by *Agrobacterium* strains which incited cucumopine containing tumours or hairy roots.

The work presented here demonstrates that the Pauly positive compound named cucumopine detected in hairy roots incited by *A. rhizogenes* strains isolated from cucumber is an opine in the sense commonly accepted for this word. That is a selective growth substrate whose production is a consequence of the interaction between the plant and the microorganism. In the case of *Agrobacterium*, opine synthesis is the result of the activity of T-DNA-encoded enzymes (opine synthases) [3]. Genetic studies have shown that this is also the case for cucumopine in hairy roots [22]. Thus this work constitutes a new extension of the concept according to which opine-related functions are essential features of the pathogenic plasmids of *Agrobacterium*. Because cucumopine readily forms from histidine and α-ketoglutaric acid in aqueous solution, it is reasonable to assume that it is formed *in vivo* by an enzymatic reaction mimicking the chemical reaction. In connection with this, it is interesting to remark that except for agrocinopines [23] all the presently known opines are iminoacids obtained by chemical or enzymatic reactions performed on Schiff bases formed between aminoacids and sugar or ketoacids [12].

Opine-related properties constitute the basis for the current classification of *Agrobacterium* strains and their Ti or Ri plasmids. So far five classes of *Agrobacterium*/Ti plasmids and three classes of *A rhizogenes*/Ri plasmids have been identified [17]. These accommodate the majority of pathogenic *Agrobacterium* strains. However, a few strains still escape this classification (A. Petit,



1



2

unpublished results) and this raises the question whether further investigations will lead to the discovery of new opines.

The fact that in every case studied pathogenic strains of *Agrobacterium* or their resident Ti/Ri plasmid could be shown to possess opine-related functions is strong support for an essential role of these functions in the survival and propagation of Ti/Ri plasmids. The mechanism by which these opine-related functions operate is very simple. The presence of plants susceptible to crown gall or hairy root results by plant transformation in creating a source of selective growth substrates for the bacterial host of the plasmid. The question whether other soil bacteria have evolved similar systems to benefit from their interactions with plants has not been much investigated. Nevertheless, in one case at least, *Rhizobium*-induced nodules on lucerne were seen to contain strain specific compounds which were also specifically degraded by the bacteria [24, 25].

EXPERIMENTAL

Biological material. Hairy root cultures were established from plant material inoculated with *A. rhizogenes* NCPPB2659 as described [15, 26]. They were cultured in solid or liquid medium as described [15, 26]. Bacterial strains were kept on agar slants of LB medium [27]. Freshly grown cultures on the same medium were used for the experiments reported here. The following strains were used: *A. rhizogenes* NCPPB2655, 2657, 2659 (obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts., U.K.) *A. tumefaciens* C58 and C58C1, its Ti plasmid-less derivative [28] (obtained from J. Schell, State University of Ghent, Belgium), grapevine isolates *A. tumefaciens* 305 and 374 (obtained from A. Kerr, Waite Agricultural Institute, Adelaide, South Australia), *A. tumefaciens* T37α, a constitutive mutant for nopaline/octopine degradation [29] and all other strains were from our collection (*A. tumefaciens*: 181, EU6, T37, 2174, IIBV7, B6; *A. rhizogenes*: A4, 8196). Liquid cultures in 5 ml tubes or in glass bottles were incubated at 28° on an orbital shaker (190 rpm).

Preparation of extracts and HVPE. Extracts from roots were prepared by hot acid treatment (0.12 HCl, 100°, 10 min) of samples, or more recently by H_2O treatment (100°, 10 min) when it was realized that acid treatment resulted in degradation of cucumopine. HVPE and staining of electrophoregrams were performed as described in ref. [15]. Electrophoretic mobilities (M_{his}) were computed with respect to mobilities of, respectively, histidine taken as 1, and mannitol, taken as 0.

Partial purification of cucumopine from carrot hairy root cultures. Roots (10 g fr. wt) were treated with 40 ml of 0.1 M HCl at 100° for 10 min. The resulting soln was evapd to dryness *in vacuo* at 37°. The residue was dissolved in 40 ml H_2O, adjusted to pH 7.5 with NaOH, and filter-sterilized (Millipore, porosity 0.45 μ). This soln was diluted with one vol. of sterile H_2O and inoculated with *A. tumefaciens* C58C1. The culture was incubated on an orbital shaker at 28° for 3 days. This step (biological purification) resulted in drastic reduction of organic components in the culture medium, with the exception of cucumopine and its degradation product which were not affected. The spent medium was freed from bacteria by centrifugation and concd *in vacuo* to *ca* 10 ml, the resulting soln was adjusted to pH 7.5 and brought to 15 ml with H_2O, filter-sterilized and dispensed in culture tubes. These were inoculated with various *A. tumefaciens* and *A. rhizogenes* strains except for one that was kept uninoculated as a control. After 3 days, aliquots of the cultures were centrifuged and 2 μl from each supernate were analysed by HVPE and stained with the Pauly reagent (Fig. 2).

Isolation of cucumopine from hairy root culture supernates. Pooled media (1.4 l) from cultures of NCPPB2659 carrot hairy root cultures were percolated over a Dowex 50–X8 (H^+ form) column (15 × 4.0 cm) which was washed with H_2O (1660 ml). Cations were then desorbed with 1.5 M NH_4OH (2 l) and concd at 37° *in vacuo* to 50 ml.

Electrophoresis at pH 1.9 indicated acid catalysed degradation of cucumopine. For biological purification, the concentrated eluate was neutralized, filter sterilized and incorporated into a mineral salt soln as described [24]. The resulting culture medium (100 ml) which contained no carbon or nitrogen source other than those present in the extract, was then inoculated with *A. tumefaciens* C58C1. After 3 days of incubation at 28° on an orbital shaker the bacteria were removed by centrifugation and the organic cations recovered by ion exchange as above. The total weight of the biologically purified cationic residue was 275 mg. It contained, besides cucumopine and its acid degradation product, one major fast-moving unidentifed ninhydrin-positive contaminant. 240 mg of this mixture was further purified by anion exchange using DEAE Sephadex A25 (acetate form), 40–120 micron (10 g, 35 meq), bed vol. 75 ml. The column was eluted with 0.5 M pyridine–1 M HOAc (pH 4.5) at 1.4 ml/min, and 5 ml fractions were collected. The ninhydrin positive contaminant eluted in fractions 7–12 was concentrated *in vacuo* (190 mg). Electrophoretically homogeneous cucumopine acid degradation product was obtained as a glassy solid (54 mg) from fractions 15–18. Fractions 19–21 contained a mixture of cucumopine and its degradation product, and fractions 22, 23 were homogeneous with respect to cucumopine. After combining homogeneous cucumopine fractions from two more DEAE anion exchange separations performed on the unresolved material, cucumopine was isolated as a fawn microcrystalline solid (8.3 mg).

Isolation of cucumopine from grapevine crown gall tumours. Dry grapevine tumours (3.3 kg) were powdered in a mechanical grinder and extracted 3 times with H_2O (70°, 24 l, 30 l, 30 l). The filtered extracts were combined and evapd *in vacuo* to 4 l. This soln was batch treated with Dowex 50-X8 (H^+, 1 l) resin with moderate stirring for 12 hr. The resin was recovered on a sintered glass filter and transferred to a column. After washing with H_2O (3.8 l) the organic cations were eluted with 1 M NH_4OH (5 l), the eluate was concd *in vacuo* to 200 ml. Electrophoretic analysis of this fraction showed the presence of cucumopine and its degradation product besides octopine and several ninhydrin positive contaminants. The extract was incorporated into a mineral salt soln (10 l) [24] and biologically purified with *A. tumefaciens* T37α. Organic cations in the culture supernate were recovered by ion exchange. Vacuum concn of the eluate yielded a brown soln which was decolorized by treatment with charcoal before evapn to dryness. A total amount of 1.57 g of a white powder was obtained, which consisted mostly of the acid degradation product of cucumopine. This mixture was purified by anion exchange as described above. The pooled products of 12 separations amounted to 119 mg of cucumopine and 963 mg of its degradation product. These samples were used for structural studies without further purification (Davioud *et al.* in preparation).

Acknowledgements—We thank M. Fétizon and J. C. Tabet (Ecole Polytechnique, Palaiseau, France) for providing mass spectrometry data. This work was supported by the Ministère de l'Industrie et de la Recherche and the Biotechnology Action Programme of the Commission of the European Communities (contract BAP 0015–F). ED and MET were recipients of fellow-

ships from respectively Ministère de la Recherche et de la Technologie and Centre National de la Recherche Scientifique.

REFERENCES

1. Chilton, M. D., Drummond, M. H., Merlo, D. J., Sciaky D., Montoya, A. L., Gordon, M. P. and Nester, E. W. (1977) *Cell* **11**, 263.
2. Chilton, M. D., Tepfer, D. A., Petit, A., David, C., Casse-Delbart, F. and Tempé, J. (1982) *Nature* **295**, 432.
3. Gheysen G., P. Dhaese, M. Van Montagu and J. Schell (1985) in *Plant Gene Research: Genetic Flux in Plants* (Hohn, B. and Dennis, E. S., eds), p. 11. Springer, Wien.
4. Tempé, J., Petit, A., Holsters, M., Van Montagu, M. and Schell, J. (1977) *Proc. Natl Acad. Sci. U.S.A.* **74**, 2848.
5. Morel, G. (1956) *Bull. Soc. Fr. Physiol. Vég.* **2**, 76.
6. Lioret, C. (1956) *Bull. Soc. Fr. Physiol. Vég.* **2**, 76.
7. Goldmann, A., Tempé, J. and Morel, G. (1968) *C. R. Soc. Biol.* **162**, 630.
8. Petit, A., Delhaye, S., Tempé, J. and Morel, G. (1970) *Physiol. Vég.* **8**, 205.
9. Petit, A., Dessaux, Y. and Tempé, J. (1978) in *Proc. 4th Inter. Conf. Plant Pathog. Bact.* (Ridé, M, ed.) p. 143. INRA–Angers.
10. Tempé, J., Estrade, C. and Petit, A. (1978) in *Proc. 4th Inter. Conf. Plant Pathog. Bact.* (Ridé, M., ed.), p. 153. INRA–Angers.
11. Tempé, J., Guyon, P., Tepfer, D. and Petit, A. (1979) in *Plasmids of Medical, Environmental, and Commerical Importance* (Timmis, K. N. and Pühler, A., eds), p. 353. Elsevier-/North Holland Biomedical Press, Amsterdam.
12. Tempé, J. (1983) In *Chemistry and Biochemistry of Amino-Acids, Peptides and Proteins* (Weinstein, B. ed.) Vol. 7, p. 113. Marcel Dekker, New York.
13. Guyon, P., Chilton, M. D., Petit, A. and Tempé, J. (1980) *Proc. Natl Acad. Sci. U.S.A.* **77**, 2693.
14. Chilton, W. S., Tempé, J., Matzke, M. and Chilton, M. D. (1984) *J. Bacteriol.* **157**, 357.
15. Petit, A., David, C., Dahl, G. A., Ellis, J. G., Guyon, P., Casse-Delbart, F. and Tempé, J. (1983) *Mol. Gen. Genet.* **190**, 204.
16. Costantino, P., Mauro, M. L., Micheli, G., Risuelo, G., Hooykaas, P. J. J. and Schilperoort, R. A. (1981) *Plasmid* **5**, 170.
17. Petit, A. and Tempé, J. (1985) in *Molecular Form and Function of the Plant Genome* (Van Vloten-Doting, Groot and Hall, eds), p. 625. Plenum, New York.
18. Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. (1968) *Data for Biochemical Research*, 2nd Edn, p. 545. Clarendon Press, Oxford.
19. Trevelyan, W. E., Procter, D. P. and Harrison, J. P. (1950) *Nature* **166**, 444.
20. Yamada, S. and Itano, H. A. (1966) *Biochim. Biophys. Acta* **133**, 538.
21. Wellish J. (1913) *Biochem. Z.* **49**, 173.
22. Brevet J., Borowski D., and Tempé J. (1988) *Molec. Plant Microbe Interact.* (in press).
23. Ryder, M. H., Tate, M. E. and Jones, G. P. (1984) *J. Biol. Chem.* **259**, 9704.
24. Tempé, J., Petit, A., and Bannerot, H. (1982). *C. R. Acad. Sci. Paris. Série III*, **295**, 432.
25. Murphy P. J., Heycke N., Tate M. E., Banfalvi Z., Kondorosi A., Tempé J. and Schell J. (1987) *Proc. Natl Acad. Sci. U.S.A.* **84**, 493.
26. Petit, A., Berkaloff, A. and Tempé, J. (1986) *Molec. Gen. Genet.* **202**, 388.
27. Maniatis T., Frisch E. F. and Sambrook J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, New York.
28. Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I., Schilperoort, R. A. and Schell, J. (1974) *Nature* **252**, 169.
29. Petit, A. and Tempé, J. (1978) *Molec. Gen. Genet.* **167**, 147.