Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Giovanna Vitali

Weakly virulent mutants of Agrobacterium rhizogenes induced by ethidium bromide

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **74** (1983), n.3, p. 182–187.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1983_8_74_3_182_0>

Biologia molecolare. — *Weakly virulent mutants of* Agrobacterium rhizogenes *induced by ethidium bromide.* Nota di GIOVANNA VITALI (*), presentata (**) dal Socio G. MONTALENTI.

RIASSUNTO. — L'*A. rhizogenes* 1855 biotipo 2 e l'*A. rhizogenes* 2659 biotipo 1 sono stati trattati con il bromuro di etidio.

È stato possibile isolare mutanti debolmente virulenti da tutte e due i ceppi batterici.

Nel caso dell'*A. rhizogenes* 1855, l'87% delle colonie trattate induceva la rigenerazione di piccoli, indifferenziati calli al posto di una proliferazione di radici, nelle piante che erano state infettate. Dall'analisi elettoforetica su gel del DNA di uno di questi mutanti è stato osservato che il suo plasmide è più piccolo di quello che è presente nel ceppo selvaggio.

Agrobacterium rhizogeens induces tumours known as hairy root disease on dicotyledons plants. These tumours are characterized by roots emerging from the infected area [1].

A. rhizogenes belongs to the family of *A. tumefaciens* which induces crown gall tumours.

As for *A. tumefaciens* the virulence of *A. rhizogenes* is carried by a large plasmid [2, 3, 4].

When the plasmid is cured with ethidium bromide, the bacterium loses its infectivity [2].

We report here that after prolonged treatment with ethidium bromide of *A. rhizogenes* NCPPB mutants that show significant differences in tumour induction have been isolated.

Most of them were unable to induce root proliferation and caused little growth of unorganized tissue. All mutants analyzed harboured a plasmid smaller than that of the wild type.

MATERIALS AND METHODS

Bacterial strains.

Strain 1855 biotype 2 and 2659 biotype 1 were from the National Collection of Plant Pathogenic Bacteria. Plasmid DNA weights were 157×10^6 D and 193×10^6 D respectively [4]. All Agrobacterium cultures were grown on YMB

(*) Dipartimento di Genetica e Biologia Molecolare della Facoltà di Scienze, University of Rome, Piazzale Aldo Moro 5, Rome, Italy.

(**) Nella seduta del 12 marzo 1983.

medium described by Hooykaas *et al.* [5]. For the agar plates the medium was solidified with 1,8% (w/v) Difco Bacto-Agar. The temperature was 29 °C. Exponential cultures of *A. rhizogenes* 1855 diluted to 10^3 cell/ml were treated with 25, 50, 75, 100, 250, 500, μM ethidium bromide for 48 h using the method of Lin and Kado [6].

The bacteria were inoculated in a shaking water bath in the darkness.

A. rhizogenes 2659 has been treated with 50 μM ethidium bromide only.

Virulence assays.

Peas used for the virulence assays were cultivar Rondo.

They were sterilized by immersing in H_2SO_4 for 10', washed 10 times in distilled water, placed in Petri dishes containing 1,7% w/v agar for 5 days at 28 °C in darkness, during which time the seeds were allowed to germinate. After this period 0.1 ml of a bacterial culture of 10^9 cell/ml of *A. rhizogenes* 1855 was inoculated on the decapitated epicotyl. The plantlet was allowed to grow in a large test tube containing agar at 28 °C. The same test was carried out in quadruplicate when the results were not clear.

The virulence of the *A. rhizogenes* 2659 Biotype 1, was tested on *Daucus carota* because the above mentioned was not positive.

Carrots from the local market were cut eliminating 1 cm from the end, peeled, washed in 5% NaClO and rinsed with five changes of sterile water. The carrot root was sectioned in 10 discs 0.5 cm thick which were inoculated by pouring 0.1 ml of a culture of 10^9 cell/ml.

A. rhizogenes 2659 and one of the mutants 2659–14 were tested on 20 discs of carrot. Discs were scored for root formation four weeks after infection.

Plasmid extraction.

The plasmid DNA was extracted from 50 ml of bacterial culture according to the procedure of Casse *et al.* [7]. The DNA was loaded (30 μl per well) on 0.7% agarose gel and electrophoresed at 3,0 V/cm for 20 h. Electrophoresis buffer was 36 mM Tris HCL, 30 mM EDTA pH 7.7. Gels were stained with 2 μg/ml ethidium bromide and were photographed under U.V. light.

RESULTS

Fig. 1 shows the growth curves of *A. rhizogenes* NCPPB 1855 in the presence of various concentrations of ethidium bromide.

As can be seen the dye inhibits progressively the growth of the bacteria. After 48 hrs treatment forty colonies were cultivated on 2 ml of YMB and tested for virulence on peas.

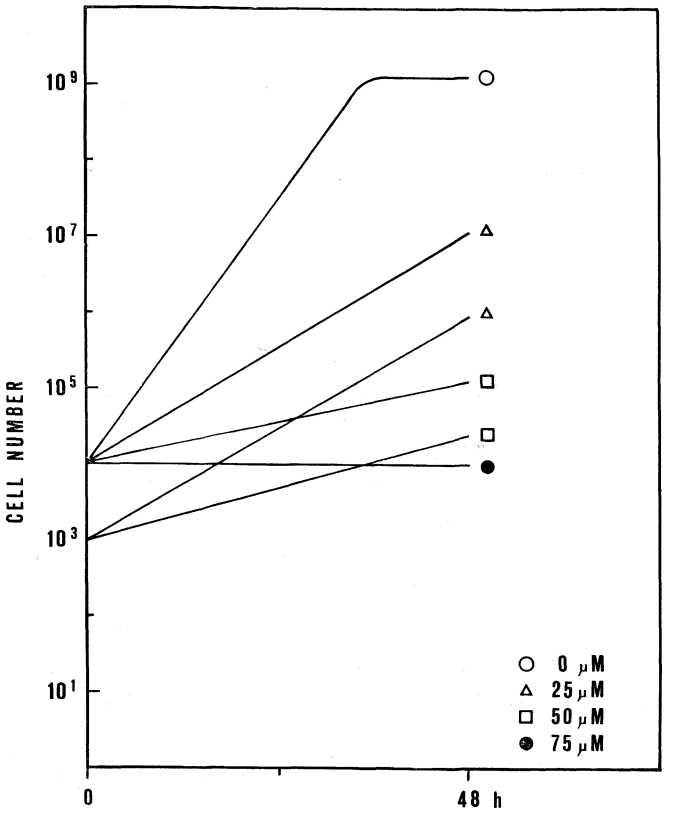

Fig. 1. - Growth curve of *Agrobacterium rhizogenes* 1855 in the presence of increasing concentrations of ethidium bromide. The culture treated with 25 μM ethidium bromide was scored from 10^3 to 2×10^6 cell per ml. The culture treated with 50 μM was scored up to 4×10^4 cell per ml. No growth was observed in the culture treated with 75 μM dye. The control culture reached stationary phase after 36 hrs. The growth inhibition was unaffected by the concentration of the inocula (10^3 or 10^4 cell per ml).

Thirty-five mutants were able to induce growth of small undifferentiated callus after 3 weeks and five were normally virulent. One hundred per cent of untreated *A. rhizogenes* 1855 induced roots on all inoculated peas about 18 days after infection (Fig. 2 A).

Uninfected controls did not show tissue overgrowth at the wound site during four weeks of observation and virulence attenuation was not observed (Fig. 2 B). One of the callus inducing mutants (1855–8) was examined in greater detail: plasmid DNA was extracted and analyzed by agarose gel electrophoresis. The plasmid of this mutant showed a faster migration than that of the wild type.

In order to verify that this mutant harbours a splasmid of smaller size than the wild type, the DNA of 5 subclones of 1855–8 was analyzed by gel electropho-

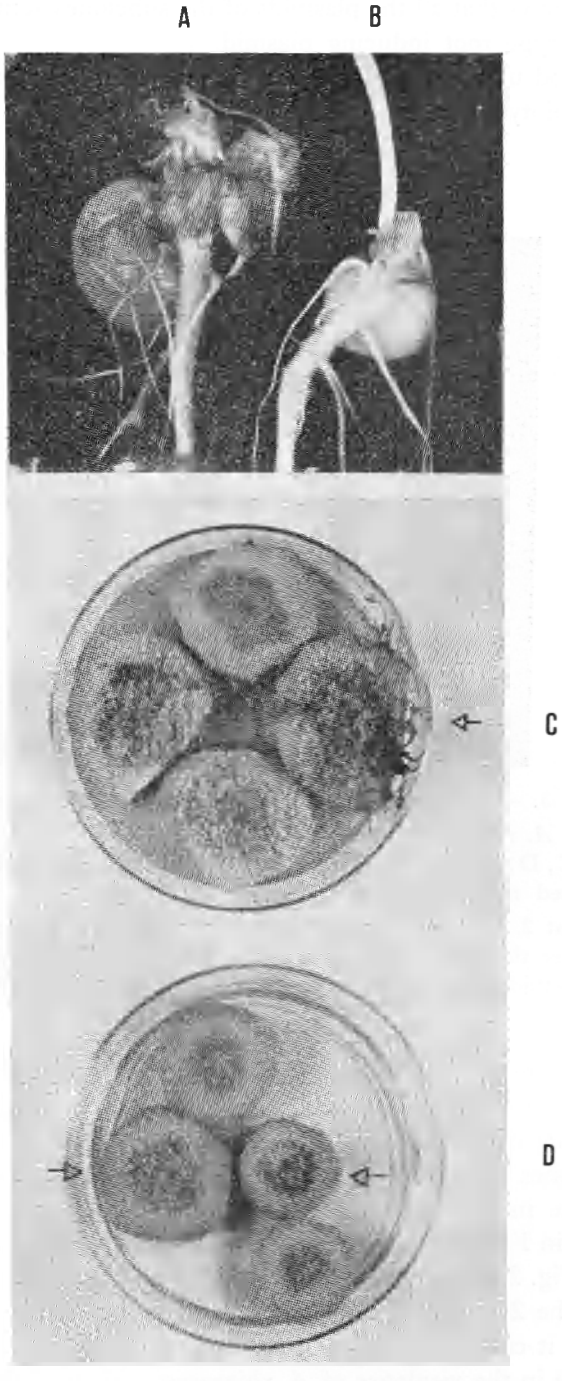

Fig. 2.

A – Roots induced by *A. rhizogenes* 1855 on decapitated pea epicotyls.
B – Small callus induced by mutant 1855–8.
C – Roots induced by biotype 1 *A. rhizogenes* 2659 on carrot discs.
D – Callus induced by mutant 2659–14 on carrot discs.

resis. Fig. 3 shows that all the plasmids of the subclones actually migrate faster than the wild type root inducing plasmid.

The method used to obtain weakly virulent mutants has also been successfully used on biotype 1 *A. rhizogenes* 2659 [8].

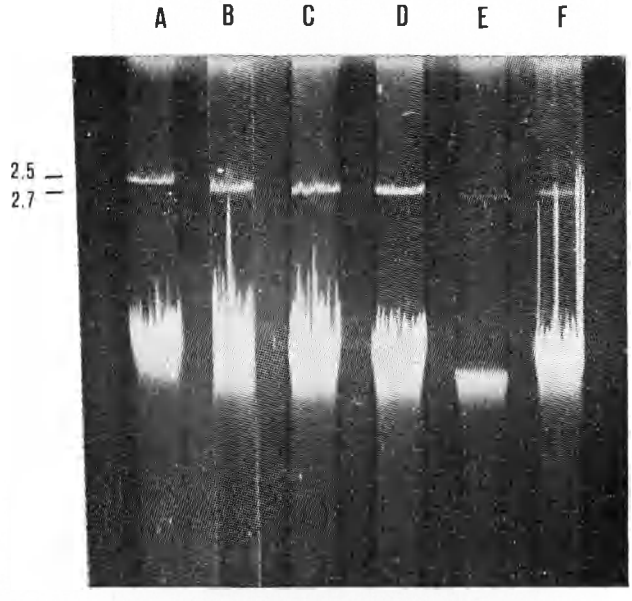

Fig. 3. - Agarose gel electrophoresis of DNA extracted from:
A - *A. rhizogenes* 1855.
B, C, D, E, F - Subclones derived from ethidium bromide treated mutant 1855-8.
Bar at 2.5 cm marks the migration of 1855 wild type plasmid. Under the same electrophoresis conditions the plasmids from the ethidium bromide treated subclones migrate at 2.7 mc.

Twenty colonies were isolated after ethidium bromide treatment and their plasmids analyzed using the rapid procedure of Birboim and Doly which does not give an exact estimate of the molecular size of the plasmid [9].

All the clones harboured a plasmid but one strain, 2659-14, showed quite a big difference in the virulence test.

As shown in Fig. 2 C and D, whereas wild strain 2659 induced abundant root proliferation (Fig. 2 C) the strain 2659-14 induced undifferentiated callus growth and roots on the 20 discs of carrots inoculated (Fig. 2 D). From the results presented here it can be concluded that ethidium bromide treatment produced deep alterations in the virulence of *A. rhizogenes*. Most of the treated colonies induce, in fact, undifferentiated outgrowth instead of root proliferation on decapitated epicotyls and carrot discs. These alterations are probably due to relatively large deletions in the regions of the root inducing plasmids that control virulence and tumor morphology.

Plasmids from various subclones of one of the mutants of *A. rhizogenes* 1855 seem in fact to be smaller than the wild type, as judged from the relative migration on agarose gel electrophoresis.

Acknowledgements. I am grateful to Dr. P. Costantino of the Assoreni, Monterotondo, Rome for helpful discussion and critical reading of the manuscript, and to R. Gargamelli for the photograpic work.

LITERATURE

[1] C. ELLIOT (1951) – « Manual of Bacterial Plant Pathogens », 2nd rev. ed. Chronica Botanica, Waltham, Mass.
[2] L. MOORE, G. WARREN and G. STOBEL (1979) – « Plasmid », *2*, 617.
[3] F. F. WHITE and E. W. NESTER (1980) – « J. Bacteriol. », *144*, 710.
[4] P. COSTANTINO, M. L. MAURO, G. RISUELO, P. J. J. HOOYKAAS and R. SCHILPEROORT (1981) – « Plasmid », *5*, 170.
[5] P. J. J. HOOYKAAS, P. M. KLAPWJIK, M. P. NUTI, R. SCHILPEROORT and A. RORSCH (1977) – « J. Gen. Microbiol. », *98*, 477.
[6] B. C. LIN and C. I. KADO (1977) – « J. Microbiol. », *23*, 1554.
[7] F. CASSE, C. BOUCHER, J. S. JULLIOT, M. MICHEL and J. DENARIÈ' (1979) – « J. Gen. Microbiol. », *133*, 229.
[8] P. J. KEANE, A. KERR and P. B. NEW (1970) – « Aust. J. Biol. Sci. », *23*, 585.
[9] H. C. BIRBOIM and J. DOLY (1979) – « N.A.R. », 7, 1513.