

Ti PLASMID TRANSFER IN AGROBACTERIUM TUMEFACIENS AND ITS DETECTION SEROLOGICALLY

EL – KADY,S EMERAN , A.A ; GABR .M . AND ALA* EL –DEIN , O

Agric . Bot . Dept ., Fac . of Agric ., Kafr El – Sheikh University.

* PL . Disease Inst . Agric . Res . Stat ., Sakha . Egypt

ABSTRACT

Two pathogenic isolates (nos . 7 and 10) of *Agrobacterium tumefaciens* were isolated from tumoured apple seedlings in Egypt . The two isolates were subjected to the thermotherapy treatment at 37^oc for three days for losing their pathogenicity. Two non – pathogenic mutants (M7 and M 10) were obtained. The conjugation between the mother pathogenic isolates and their non – pathogenic resistant mutants were carried out on the tumoured apple seedlings inoculated before with the mother pathogenic isolates . The conjugative pathogenic derivatives were selected and compared serologically with their parants .

Crossed immuno electrophoresis (CIE) showed that three antigens were lost in the non – pathogenic mutants and were found in both the pathogenic mother isolates and their pathogenic derivatives (conjugatives)

INTRODUCTION

The bacterium *Agrobacterium radiobacter* sub sp. *tumefaciens* (Smith and Townsend) keane *et al* . 1970 , causes crown gall disease in a wide range of plants (Cubelo *et al* .1999 , Parrott *et al* . 2002 and Penyalver *et al* . 2004) .

A tumefaciens are almost indistinguishable from strains of the non – pathogenic species *A . radiobacter* , except that the former contains a large tumour – including Ti plasmid (Van Larebeke *et al* . 1974 and Zaenen *et al* . 1974) and are capable of induceng crown gall tumours on a variety of hosts . The pathogenic may transfer its Ti

plasmid to the non – pathogenic which becomes tumorigenic (Lopez – Lopez , 1999 , Juta Bohne *et al* . 1998 , Judd *et al* . 2005 and Minhang *et al* .2007) . This conjugative transfer takes place in tumours or *in vitro* in presence of opines (Kerr *et al* . 1977 and Ellis *et al* . 1982) .

The serological difference between pathogenic and their non-pathogenic mutants in some genera of bacteria were demonstrated in *A . tumefaciens* (EL . Kady and Sule , 1982 and Brown and New , 1986) , *Agrobacterium vitis* (Mahmoodzadah *et al* , 2003 and Kawagachi *et al* . 2008) , *Pseudomonas syringae pv . phaseolicola* (EL – Kady *et al* . 1986) . The aim of the present study is to apply quantitative immunoelectrophoretical techniques to differentiate between the pathogenic isolates of *A . tumefaciens* and their pathogenic and non – pathogenic derivatives .

MATERIALS AND METHODS

A . Isolation of A . tumefaciens .

Sterile samples of tumours formed on apple seedlings collected from Alexandria . and El – Gharbia governorates were used for *A . tumefaciens* isolation.Nutrient broth medium were used . Pathogenicity tests were carried out according to Kerr and Panagopoulos , 1977 by inoculating young tomato and sunflower seedlings using a thick suspension containing $10^7 - 10^8$ cells / ml . Galls formation were assessed after three weeks from the inoculation .

B . Loosing and acquisition of the pathogenicity in *A . tumefaciens*

loosing the pathogincity of the pathoginc isolates of *A . tumefaciens* was carried out using the thermotherapy treatments at 37 °C for three continuous days. The incubated liquid cultures, were shaken in the incubator and renewed daily, the treated isolates were tested for pathogenicity on tomato and sunflowers seedlings . The non – pathogenic mutants were isolated and used for the conjugation and serological studies .

Acquisition of the pathogenicity (Ti plasmid transfer) of *A . tumefaciens* was carried out on the tumoured tomato seedling , inoculated before with the isolated pathogenic isolates (no 7 or 10) of *A . tumefaciens*. The non – pathogenic mutants showing resistance to

500 $\mu\text{g m}^{-1}$ streptomycin and 25 $\mu\text{g m}^{-1}$ rifampicin(Brown and New 1986) were selected for the conjugation process . Acquisition of the pathogenicity (Ti plasmid transfer) were carried out on the tumoured tomato seedlings inoculated before with one of the pathogenic isolates . The resistant non-pathogenic mutants were inoculated on the tumoured seedlings . The transconjugants were selected by plating on the mating antibiotic selective medium and tested for pathogenicity on tomato and sunflower seedlings.

C. Preparation of antigens and immunization:

Bacterial cells were harvested from 3-days -old shaken broth cultures containing peptone and glucose by centrifugation at 8000 r.p.m for 20 mins. The precipitate was washed three times with 0.85 % NaCl then the bacterial cells were sonicated by MSE ultrasonic disintegrator for 10 min , and the protein contents were determined according to Lowry *et al.* (1951) and adjusted to 20 mg / ml .

Antigens of *A.tumefaciens* isolates 7 and 10 were injected in the rabbit after mixing with the complete adjuvant (1:1 ratio).

Each rabbit received 8 injections (subcutaneously followed by intramuscularly) with one ml and increasing by 0.5 ml for the next injection . The rabbit was bled one week after the last injection

Antiserum was prepared and immunoglobulins were isolated according to Axelsen *et al.* (1973) .

The antibodies absorption was carried out according to Kiraly *et al.* (1970) .

D. Crossed immunoelectrophoresis (CIE) technique :

CIE technique was used according to Axelsen *et al.*, (1973) using 1.5 mm layer of 1% agarose and barbital buffer, pH 8.6 , ionic strength 0.02 . Application wells had a diameter of 4.0 mm. Ten μl antigen samples were applied in the wells . First dimension electrophoresis was carried out at 10 V/cm for 1 h . , second - dimension run was made applying 1 V/cm for 16 h at 15°C Second - dimensional gels contained 15 μl concentrated purified antibody per 1 cm . Immunoprecipitates were stained with Coomassie brilliant blue R - 250.

RESULTS

A. The pathogenic isolates of *A. tumefaciens* and their non-pathogenic derivatives :

Two non-pathogenic mutants of *A. tumefaciens* (nos. 7 and 10) were obtained by subjecting their mother pathogenic isolates (nos 7 and 10) to the thermotherapy treatment . The mutants failed to give any tumour symptoms on tomato and sunflower seedlings . On the other hand , two pathogenic conjugate derivatives of

A. tumefaciens were obtained by the conjugation between the mother pathogenic isolates (nos. 7 and 10) and their non – pathogenic antibiotic resistant mutants . The conjugatives showed tumour symptoms on the tomato and sunflower seedlings (Table 1)

B. Serological comparisons between the pathogenic mother isolates of *A. tumefaciens* and their non- pathogenic (conjugatives) derivatives:

Crossed immunoelectrophoresis (CIE) revealed that the antigenic structures of the pathogenic mother isolates (nos. 7 and 10) of *A. tumefaciens* were 17 and 20 precipitation peaks (antigens) , respectively , while the non-pathogenic mutants (M7 and M 10) showed 14 and 17 antigens , respectively (Figs 1 and 2) .

On the other hand , the new pathogenic conjugate derivatives (X7 and X10) gave 17 and 20 antigens , respectively as in the pathogenic mother isolates (the same number of antigens) . Results showed clearly that the non-pathogenic mutants of *A. tumefaciens* lost three antigens while their new pathogenic conjugate derivatives acquired again these antigens from the mother pathogenic isolates of *A. tumefaciens* (Table 2 and Figs . 1 and 2). Also , the results indicated that five and two antigens were found specific to *A. tumefaciens* isolates nos.7 and 10 , respectively while 15 antigens were found common (Table2).

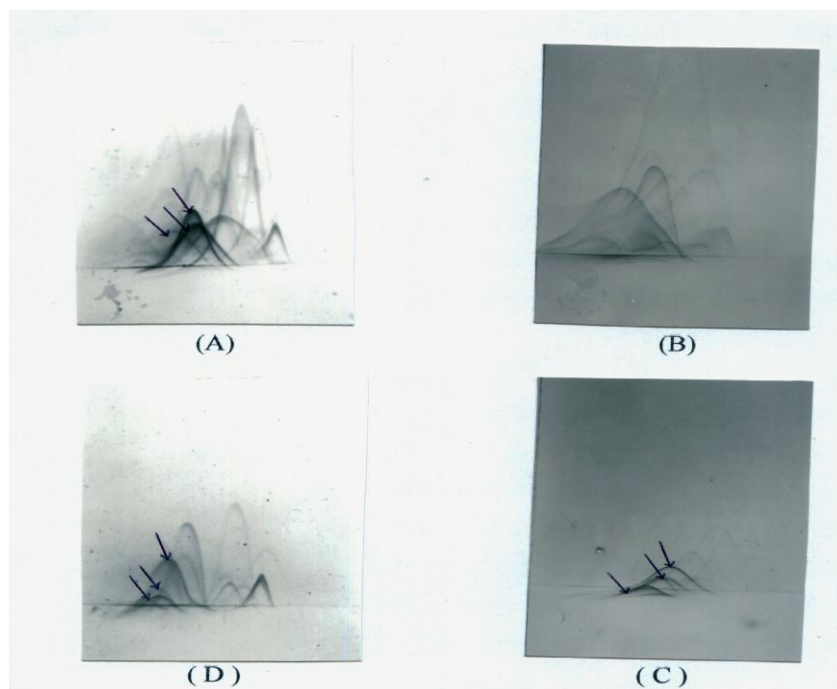
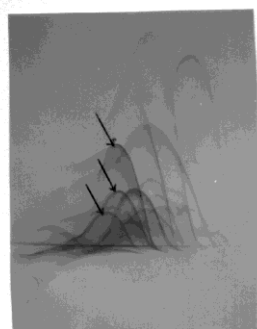
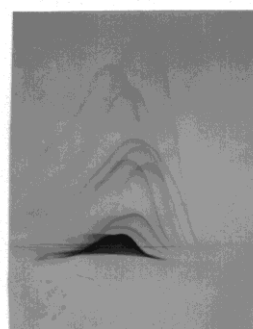


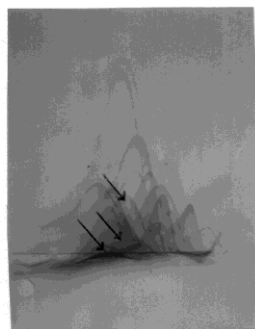
Fig .1. CIE of *A . tumefaciens* isolates no.7 and its non-pathogenic and pathogenic derivatives. Antibodies of isolate no.7 were electrophorized with antigens of . (A) isolate no.7 (B) mutant M7 and (C) conjugative X7 . Absorbed antibodies of isolate no.7 electrophorized with antigens of isolate no.7 or its pathogenic conjugative X7. Arrows indicate Ti – Plasmid (pathogenic) associated antigens of *A . tumefaciens* .



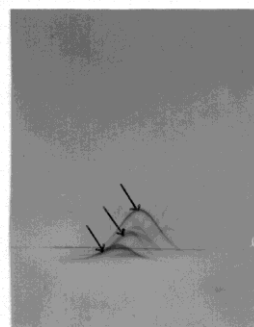
(A)



(B)



(C)



(D)

s of

Isolate no.10 were electrophorized with antigens of : (A) isolate no.10 (B) mutant M10 (C) conjugative X10 Absorbed antibodies of isolate no.10 electrophorized with antigens of isolate no.10 or conjugative X10 . Arrows indicate Ti – plasmid (pathogenic) associated antigens of *A . tumefaciens*.

Table 1 . *Agrobacterium tumefaciens* isolates and their non- pathogenic and pathogenic derivatives

<i>A . tumefaciens</i> derivatives <i>A . tumefaciens</i> Isolates	Non – pathogenic <i>A . tumefaciens</i> mutants	Pathogenic <i>A . tumefaciens</i> conjugatives
Isolate no.7 Isolate no.10	M7 M10	X7 X10

Table 2. *Serological comparison between the pathogenic isolates of A . tumefaciens and their non-pathogenic and pathogenic derivatives using CIE*

Antigens	Nos. of precipitin peaks (antigens) detected					
	Path. Isol. (7)	Non patho- genic mutant (M 7)	Path. Conj. (X7)	Path. Isol. (10)	Non patho- genic mutant (M 10)	Path. Conju. (X 10)
<i>A . tumefaciens</i> pathogenic isolates						
(7)	20(17+3*)	17	20 (17+3*)	15(12+3*)	12	15(12+3*)
(10)	15(12+3*)	12	15(12+3*)	17(12+3*)	14	17(14+3*)

* : Specific antigens for pathogenicity (Ti plasmid) in *A . tumefaciens*.

DISCUSSION

Ti plasmid is a large plasmid in *A. tumefaciens* contains many genes, some of them are associated to the pathogenicity (Judd *et al.* 2005 and Minhang *et al.* 2007). The losing of pathogenicity of *A. tumefaciens* is associated with the losing Ti plasmid or at least the large part of it that contains the pathogenic genes. The present results clearly showed that the two non-pathogenic mutants (M7 and M10) of *A. tumefaciens* lost both the pathogenicity and three antigens by comparison with the mother pathogenic isolates (nos.7 and 10). Similar results were obtained in *A. Vitis* as reported by Mahmoodzadah *et al.*2003 and Kawaguchi *et al.* 2008. On the other hand, the conjugate derivatives (X7 and X10) of *A. tumefaciens* resulting from the conjugation between the pathogenic isolates (nos.7 and 10) and their non-pathogenic antibiotic resistance mutants (M7 and M10) acquired both the pathogenicity and the three antigens (that lost in M7 and M10) by comparison with the mother pathogenic isolates. This finding means that, there is a strong association between the pathogenicity and these acquired (or lost) antigens that located on the Ti plasmid of *A. tumefaciens*. So, the Ti plasmid could be transferred from a pathogenic donor to a non pathogenic recipient by conjugation in tumoured plant seedlings and CIE was able to detect the losing or acquisition the plasmid.

REFERENCES

- Axelsen N. H; Kroll, J. and Weeke , B .1973.** Quantitative Immuno-electrophoresis. Universities, Forlaget Oslo.
- Brown,A. C. and New, B.P.1986 .** Immuno-electrophoretic comparison of cell envelope antigens in pathogenic and non-pathogenic *Agrobacterium tumefaciens*. *Physiol Mole. Pl. Pathol.* 28 , 149-159.
- Cubelo , H; Mortinez , C.M.; Liopand , P.and Lopez , M.M. 1999** A simple and efficient PCR method for detection of *Agrobacterium tumefaciens* in plant tumour . *J . Appl Microbiol* 86 , 591-602 .
- El kady , S. and Sule , S. 1982.** Serological comparison between strains of *Agrobacterium tumefaciens* *Acta Phytopath. Acad. Sci. Hung .* 17,1-6.
- El Kady , S. ; Somlyai , G; Hevesi , M. and klement, Z.1986.** Differences in the antigenic structure between the wild type and non-pathogenic mutants of *Pseudomonas syringae* pv. *phaseolicola* induced by Tn5 transposon insertion . *Physiol . Mol. Plant Pathol.* 29,381-392.
- Ellis , J.G;kerr A.; Petit, A . and Tempe ,J. 1982 .** Conjugal transfer of nopaline and agropine Ti-plasmids- the role of agrocinopines . *Molecular and General Genetics* 186,269-274.
- Judd, P.K.; Mahli , D. and Das , A. 2005 .** Molecular characterization of the *Agrobacterium tumefaciens* DNA transfer protein vir B6 . *Microbiology.* 151, 3483-3892.
- Jutta Bohne, Andy Yim and Andrew N. Binns . 1998 .** The Ti plasmid increases the efficiency of *Agrobacterium* as a recipient in vir B mediated conjugal transfer of an Inc Q plasmid *Microbiology.* 95,7057-7062.
- Kawaguchi , A; Sawada, H . and Ichinose , y . 2008.** Polygenetic and serological analysis reveal genetic diversity of *Agrobacterium vitis* strains in Japan . *Plant Pathology*, published on line 12 , Marc . 2008.
- Keane , P. J. ; Kerr , A . and new B.P. 1970** Crown gall of stone fruit II. Identification and nomenclature of *Agro- bacterium* isolates . *Austral . J boil Sci* 23 , 569 – 572 .

- Kerr , A; Manigault , P . and Tempe , J . 1977.** Transfer of virulence *in vivo* and *in vitro* in *Agrobacterium*. Nature , London 265 , 560-561.
- Kerr , A . and Panagopoulos, G.C. 1977** Biotypes of *Agrobacterium radiobacter* var *tumefaciens* and their biological control. Phytopath.Z. 90 , 172-179 .
- Kiraly , Z . , Klement , Z. , Solymosi , F . and Voros , J . 1970.** Methods in Plant Pathology. Akademiai kiado , Budapest.
- Lopez – Lopez, M.J. ; Vicedo , B. ; Orenllana , N.; Piquer J. and Lopez , M.M.1999.** Behavior of a virulent strain dlerived from *Agrobacterium radiobacter* strain K84 after spontaneous Ti plasmid acquisition. Phytopathology 89 , 286 -292 .
- Lowry O.H.; Rosebrough Nira , J . , A.L. and Randall Rose , J . 1951** Protein measurement with the folin phenol reagent . J. Biol . chem.. 193, 265-275.
- Mahmood Zadah , H., . Nazemieh , A., Magidid, T., Paggami, I. and Khalighi , A.2003.** Effects of thermotherapy treatments systemic *Agrobacterium* visit in dormant Grape cutting .j. phytopathology 151,481-484 .
- Minhang Guo, Shouguaug Jin, Deqing Sun, Choyl.Hew and Shen Q.Pan . 2007 .** Recruitment of conjugative DNA transfer substrate to *Agrobacterium* type. IV secretion apparatus . PNAS, 104 (50) 20019 – 20024 .
- Parrott, D.L., Anderson , A.J.and Carman , G.J. 2002 .** *Agrobacterium* induces plant cell death in wheat (*Triticum aestivum* L.) Physiol . Molecul . Plant Pathol . 60 – 59 – 69 .
- Penyalver , R., sonchez, J.J. ; Petit, A; Salcedo, I.C. and Lopez, M.M.2004 .** Tumorigenic *Agrobacterium* sp. Isolated from weeping fig in spain . plant Disease, 88 P.428 .
- Van Larebeke, N., Engler, G., Holsters, M., Van den Elsacker, S., Zaenen, I. Sehilperoort, R.A and Schell, J. 1974** Large plasmid in *Agrobacterium tumefaciens* essential for crown gall inducing ability . Nature , London 252, 169- 170 .
- Zaenen, I.; Van Larebeke, N.;Teuchy, H.;Van Montagu, M. and Schell, J. 1974 .** supercoiled circular DNA in crown – gall inducing *Agrobacterium* strains . J. Molecul. Biol. 86, 109 – 127 .

الملخص العربي

نقل بلازميد T في بكتريا أجروباكتيريوم تيوميباشنس والكشف عنها سيرولوجيا

السيد القاضي ، عمر عمران ، محمد جبر ، أميمة علاء الدين*
قسم النبات الزراعي - كلية الزراعة - جامعة كفر الشيخ
* معهد أمراض النباتات - محطة البحوث الزراعية - سخا- مصر

تم عزل عزلتين ممرضتين (رقم 7 و 10) من البكتريا أجروباكتيريوم تيوميباشنس المسببه لمرض التدرن التاجي من شتلات تفاح بمحافظتى الإسكندرية والغربية بمصر . أمكن الحصول على طفرتين غير ممرضتين من تلك العزلات هما M10 – M7 وذلك بالمعاملة على درجة حرارة 37م لمدة 3 أيام متوالية مع التجديد اليومي للمزرعة والهز بالهزاز داخل الحضان . كذلك أمكن الحصول على أفراد ممرضة ناتجة من التزاوج بين العزلات الممرضة الأم وطفراتها الغير ممرضة (المقاومة لمضادات حيوية) وذلك على تورمات درنية على شتلات التفاح ناتجة من العدوى الصناعي بالعزلات الام (M10–M7) وبالمقاومة السيرولوجية باستخدام تقنية الإمينواليكتروفوريسيس بين تلك العزلات الممرضة (الام) ومشتقاتها الممرضة والغير ممرضة وجد غياب ثلاثة أنتيجنيات فى الطفرات الغير ممرضة بينما كانت موجودة فى كل من العزلات الممرضة (الام) ومشتقاتها الممرضة الناتجة من التزاوج (X10, X7) .