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Regeneration of Transformed Forage Legume Plants by Agrobacterium rhizogenes R1601

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إنتاج نباتات بقولية علفية محولة وراثياً بواسطة بكتريا R1601

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تم انتاج نباتات بقولية من النوع العلفي (الجت والبرسيم) محولة وراثيا بوساطة بكتريا Agrobacteium rhizogenes R1601 المهندسة وراثيا ، مباشرة من بادرات النباتات البذرية بعد قطع جزئها الخضري عند نقطـــة ارتـــباط الأوراق الفلقية بالساق وتلقيح موقع القطع بالبكتريا ، ومن الجذور الشعرية المتكونة على السيقان تحت الفلقية لبادرات النباتات العلفية ، امتازت النباتات الناتجة بزيادة عدد افرعها الخضرية وتجعد بعضا من أوراقها قياسا بعينة المقارنة (النباتات البذرية). فضلا عن امتلاكها للحامض الاميني agropine الذي تم الكشف عنه بتقنية الرحلان الكهربائي عالي الفولتية.

Keywords: *Alfalfa, Clover, Agrobacterium , Transformation, Regeneration , Electrophoresis.*

ABSTRACT

Transformed forage legume plants (alfalfa & clover) by genetic engineering bacteria Agrobacterium rhizogenes R1601,were regenerated directly from seedling plants after inoculating the wounded site formed by cutting the plantlet stem at the cotyledon attachment site , and from hairy roots which formed on the hypocotyledon stem of these seedlings . Results show transgenic plants have large number of transformed shoots , with curled leaves compared to the control samples (seedlings plants),which were assayed by investigating the presence of the agropine using high voltage paper electrophoresis.

Introduction

Legumes are protein-rich plants widely cultivated for food or forage. Growth of legumes is independent of external addition of reduced nitrogen due to the fixation of dinitrogen in symbiosis with rhizobia [1]. Moreover, legumes used for the study of plant-pathogen interaction like Agrobacterium rhizogenes, a soil pathogen which generates adventitious, genetically (Ri T-DNA) transformed hairy-roots at the site of inoculation in many dicots [2].This mediates transformation in legume plants such as Lotus corniculatus [3 , 4] and Medicago truncatula [5], by integrating the T-DNA in plant chromosomes [6] and carry different sets of eukaryotic genes.

Our aim was to regenerate the transformed forage legumes (Alfalfa and Clover) by Agrobacterium rhizogenes R1601, and assess the expression of foreign genes (Agropine) using high voltage paper electrophoresis.

Materials and Methods

Bacterial Strain

Agrobacterium rhizogenes R1601 was provided by E.W. Nester, University of Washington, USA, which is contained pRiA4b, with a chimaeric NPTII gene from Tn5 inserted into HindIII fragment 21 of the TL – DNA, together with pTK291 in trans conferring a super virulent phenotype [7], and was maintained on APM agar medium [8] containing 100 (g/ml kanamycin and 100 (g/ml carbenecillin. Bacterial suspensions were prepared by inoculating 20 ml of liquid APM medium with the appropriate single bacteria colony from an agar plate, incubated for 3 days, at 27°C, 150rpm, which are harvested by centrifugation (1600 rpm) and resuspended in appropriate liquid medium at a density of (3 (108 bacteria/ml).

Plant material and Plant growth conditions

Seeds of alfalfa (Medicago sativa) and Clover (Trifolium repens) were surface sterilized for 10 min in a mixture of 2% sodium hypochlorite (NaOCl) and 0.01% mercuric chloride HgCl2 [9] and finally washed 6 times with sterile water, and germinated on the surface of agar-solidified nitrogen-free (NF) medium [10] at 27°C in the dark.

Plant Transformation

Sterile seedlings, 5-days old,of plantlet stem were cut at the cotyledon attachment site and inoculated by a loopful of Agrobacterium rhizogenes suspension on the wounded site, then placed on MSM/2 medium (half-concentration MS medium), [11], within a few days (7 days), shoots appeared at the

inoculated site. They were carefully excised placed on to MSM/2 medium supplemented with 500 (g/ml cefotaxime (claforan) for 4 weeks [12].

In a second experiment, the other method for transformation, was curried out by infecting the hypocotyleden stem with many sites (4-6 sites) by Agrobacterium rhizogenes, using sterilized needle (Co. Ltd Korea, 28 G x1/2n) .

The control seedlings were injected with sterile distilled water. All seedlings were placed on NF medium in petri-dish (9 cm in diameter) with average of (4 seedlings / petri-dish). These petri-dishs were partially sealed with parafilm seal allowing gas exchange, and placed vertically in a growth chamber under the following conditions : at 25°C
for 24h in the dark .Then transferred to 16 photoperiod, light intensity 1500-2000 lux.

Hairy roots were first observed approximately one week after A. rhizogenes inoculation. After two weeks these hairy roots were excised and subcultured every 3-4 weeks in petri-dish on MS medium supplemented with 100 (g/ml Carbenecillin and the antibiotic cefotaxime at successive concentrations of (500, 400, 300, 200 (g/ml), for 2 weeks in each concentration at 25°C in the dark, then transferred to MS medium suplemented with 100 ug/ml of Carbenecillin only and subcultured every one month.

Agropine Analysis

The extracts of normal roots, transformed hairy roots, transformed and non – transformed plants were prepared and analyzed by high voltage paper electrophoresis as described [13].

Results and Discussion

Pathogenesity of Agrobacterium rhizogenes

Infection of wounded dicotyledonous legume plants (alfalfa and clover), with A. rhizogenes, cause prolific root formation (hairy roots disease) at the site of hypocotyleden stem infection. The type of newly developing tissue are characterized by the acquistion of bacterial DNA fragment from a large root inducing (or Ri-) plasmid of A. rhizogenes [14]. The T-DNA gene expression products are responsible for hairy roots development by altering plant hormone related processes [15]. The formation of hairy roots is due to the mediation this bacteria to transfer oncogenicity (onc) genes which are responsible for abnormal division of infected cells [16]. Root loci (rol) genes which are responsible for genetic transformation of normal cell to hairy roots [17, 18] and virulence (vir) genes, which controlled transferring the above genes to plant genome [19].

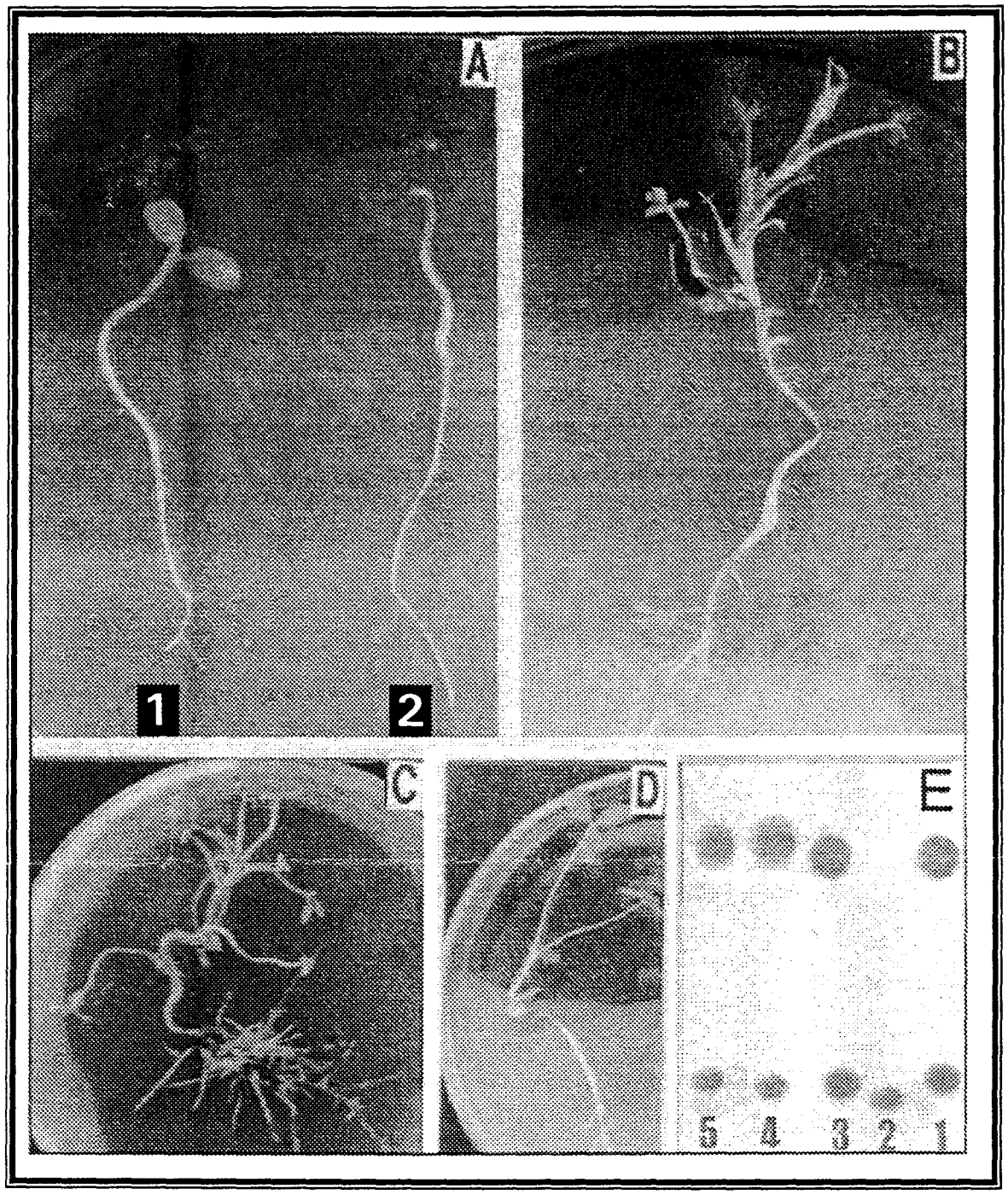

Fig. 1: Genetic transformation of clover plants

A- 1- clover seedlings, 5-days old.
2- seedling of 1, excised the main stem at the cotyleden attachment site and inoculated by A. rhizogenes.
B- Shoots generation from A.
C- Shoots spontaneously generated on hairy roots.
D- Clover seedling.
E- Detection of genetic transformation by standard agropine preperated from:
1-Standard agropine
2-Normal roots
3-Hairy roots
4-Shoots generation from hairy roots
5-Shoot regenaretion from direct transformation technique.

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