Int. J. Dev. Biol. 35: 265-268 (1991)

The influence of plant growth regulators on callus induction in pumpkin (*Cucurbita pepo* L.) hairy roots

VESNA KATAVIĆ* and SIBILA JELASKA

Department of Molecular Biology, Faculty of Science, University of Zagreb, Republic of Croatia, Yugoslavia

ABSTRACT Following *in vitro* infection with *Agrobacterium rhizogenes* wild strain (mannopine, 8196) and two *A. tumefaciens* transconjugant strains (C58C1 pArA4abc and C58C1 pArA4b) transformed (hairy) roots were induced in pumpkin (*C. pepo* L.) cotyledons. The presence of pRi T-DNA in pumpkin long-term hairy root cultures was determined by Southern hybridization. The influence of plant growth regulators on callus induction in root explants from hairy root lines, which differed mutually in morphology and growth rate, was tested by the addition of growth regulators to basal nutrient medium; while 2.4-D inhibited root proliferation in all hairy root lines tested, callus induction depended both on plant growth regulators and the root line.

KEY WORDS: *Cucurbita pepo L., pRi, genetic transformation, hairy roots, transgenic callus*

Introduction

The unique trait of different bacterial strains in the genus *Agrobacterium* to genetically transform plant cells causing tumorous diseases is due to pathogenic plasmids: tumor-inducing (Ti) plasmid of *Agrobacterium tumefaciens* and root-inducing (Ri) plasmid of *Agrobacterium rhizogenes* (White and Nester, 1980; Gheysen *et al.*, 1985). In the case of *A. rhizogenes* the expression in plant cells of DNA (T-DNA) sequences originally located on the Ri plasmid results in organized cell proliferation leading to abundant root production and the so-called «hairy root» disease (Riker and Banfield, 1932). The relative ease of establishing axenic culture lines from hairy roots and their specific physiological and phenotypic characteristics make them an interesting object for fundamental research (Hanisch ten Cate *et al.*, 1987; De Vries-Uijtewaal *et al.*, 1988; Ottaviani *et al.*, 1990), as well as plant biotechnology (Rhodes *et al.*, 1987). The hairy root system and Ri-plasmid-derived gene vectors are nowadays widely used in genetic manipulations with many dicot species and especially for the improvement of agricultural plants (Trulson *et al.*, 1986; David and Tempe, 1988; Visser *et al.*, 1989). In our research we have followed the transformation of pumpkin cells with two *A. rhizogenes* wild strains and two *A. tumefaciens* transconjugant strains bearing Ri plasmids. We report here our data on the virulence of these bacterial strains with regard to their ability to infect pumpkin cells and induce hairy root proliferation, physiological and phenotypic characteristics of long-term hairy root lines cultivated on hormone-free nutrient medium, and the influence of different plant growth regulators on callus induction in pumpkin hairy root cultures.

Results

In the axenic culture of pumpkin (*Cucurbita pepo* L.) cotyledons hairy roots were induced with *A. rhizogenes* strain 8196 and both *A. tumefaciens* transconjugant strains, while strain 15834 did not promote root development (Table 1).

The long-term (more than two years in culture) pumpkin hairy root lines differed mutually in morphology and growth rate but compared to normal pumpkin roots (control) they all grew vigorously on nutrient medium without hormones, with a high accumulation of rootmass in a short time (2-3 weeks). The analyses of opine content were positive for all strains tested (Katavic *et al.*, 1990), while Southern hybridization analysis proved the presence of pRi T-DNA in pumpkin hairy-root genomic DNA (Fig. 1).

Fig. 2 (A,B) shows the influence of different plant growth regulators on root growth and on callus induction in three different long-term pumpkin hairy root lines. It is obvious that 2.4-D alone in different concentrations or in combination with cytokinins entirely inhibited elongation and lateral branching of root explants from all lines tested, while the intensity of callus induction differed depending on the root line. In explants cultured on nutrient media supplemented with 2.4-D callus formation spread over the entire explant; calli proliferated but in a short time turned necrotic.

Abbreviations used in this paper: AS, adenine sulfate; BA, benzyladenine; 2.4-D, 2.4-dichlorophenoxyacetic acid; IBA, indole-3-butyric acid; KN, kinetin; NAA, 1-naphthaleneacetic acid; TOH, indole-3-ethanol (tryptophol); Z, zeatin.

***Address for reprints**: Department of Molecular Biology, Rooseveltov trg 6, POB 933, YU 41001 Zagreb, Yugoslavia. FAX: 41.432-526

0214-6282/91/$03.00

Printed in Spain

TABLE 1

VIRULENCE OF *AGROBACTERIUM* STRAINS USED FOR INOCULATION IN PUMPKIN *(C. PEPO* L.) COTYLEDON CULTURE

Strain	Plasmid content	Opine-type	Virulence on *C.pepo* cotyledons
Agrobacterium rhizogenes			
8196	pAr8196a pAr8196b=pRi8196 pAr8196c	mannopine	+++
15834	pAr15834a pAr15834b=pRi15834 pAr15834c	agropine	–
Agrobacterium tumefaciens			
C58C1 (pArA4abc)	pArA4a,b,c	transconjugant strain (agropine)	++++
C58C1 (pArA4b)	pArA4b=pRiA4	transconjugant strain (agropine)	++++

++++, very strong; +++, strong; –, no response

On media supplemented with other auxins (IBA, NAA, TOH), alone or in combination with cytokinins, the growth of root explants depended on the root line. In the case where lateral branching was not suppressed (*e.g.* line 4 on IBA; NAA + AS; TOH) calli developed only at the cut (end) sides of explants and they proliferated poorly.

Discussion

Our results show that induction of hairy roots on excised pumpkin cotyledons depended on the bacterial strain used. Both *A. tumefaciens* transconjugant strains caused more abundant hairy root induction and proliferation than the *A. rhizogenes* strain 8196. This could be explained by differences in chromosomal virulence regions (chv genes), as well as specific organization of pRi T-DNA regions (agropine-type pRi T-DNA versus mannopine-type pRi T-DNA). In contrast to normal pumpkin roots, the transformed root cultures were easily established and maintained on MS hormone-free medium. Because of the relative ease of maintenance in simple nutrient medium without hormones, enhanced biomass production and genetic stability, hairy root cultures have recently been intensively investigated as a suitable system for the production of secondary metabolites (Constabel *et al.*, 1988; Davioud *et al.*, 1989). Pumpkin cells synthesize a tetracylic terpenoid – *cucurbitacin* (Lavie and Glotter, 1971) that could be of interest to the pharmaceutical industry. Hairy root cultures are equally interesting for fundamental research of transformation events, as well as the role and metabolic pathways of plant hormones (auxin) in root tissue differentiation, growth and development (Shen *et al.*, 1988; Croes *et al.*, 1989). The appearance of each transformed root has been attributed to a unique transformation event (Tepfer, 1984). Perceived differences in growth rate and pattern among pumpkin long-term hairy root lines might be caused by the variation in length, copy number and site of insertion of pRi T-DNA. This could cause different endogenous hormonal levels in each transformed root line, which might influence the sensitivity to exogenous hormones leading to variations in the response of each root line to plant growth regulators and their ability to produce callus.

The regeneration in the pumpkin juvenile (seedling) tissues through somatic embryogenesis (SE) has already been reported (Jelaska, 1972, 1974, 1986). In pumpkin, hairy root regeneration of SE or shoots was achieved neither spontaneously nor via callus formation. Further investigations will be necessary before it becomes possible to state the conditions for SE or caulogenesis in pumpkin hairy root tissue.

Materials and Methods

Plant material

Excised cotyledons of pumpkin (*Cucurbita pepo* L.) from 6-8-day-old seedlings germinated *in vitro* were used in this study.

Bacterial strains

Agrobacterium rhizogenes wild strains: 8196 (mannopine-type) and 15834 (agropine-type) as well as two *A. tumefaciens* transconjugant strains bearing Ri plasmids: C58C1 (pArA4abc) and C58C1 (pArA4b) were used for plant inoculation.

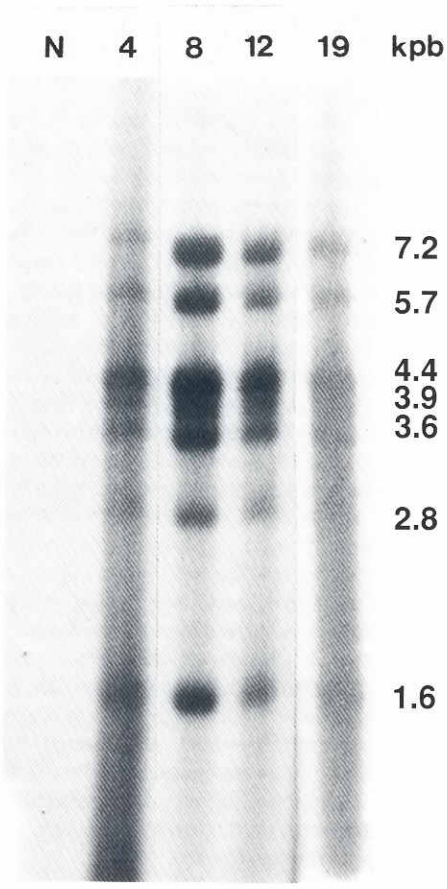

Fig. 1. Southern blot analysis of DNA prepared from hairy root (pRi8196) cultures. *BamHI digests from lines 4, 8, 12, 19 and from normal pumpkin roots (N) were hybridized with a clone 118. The size (kpb) of fragments is indicated.*

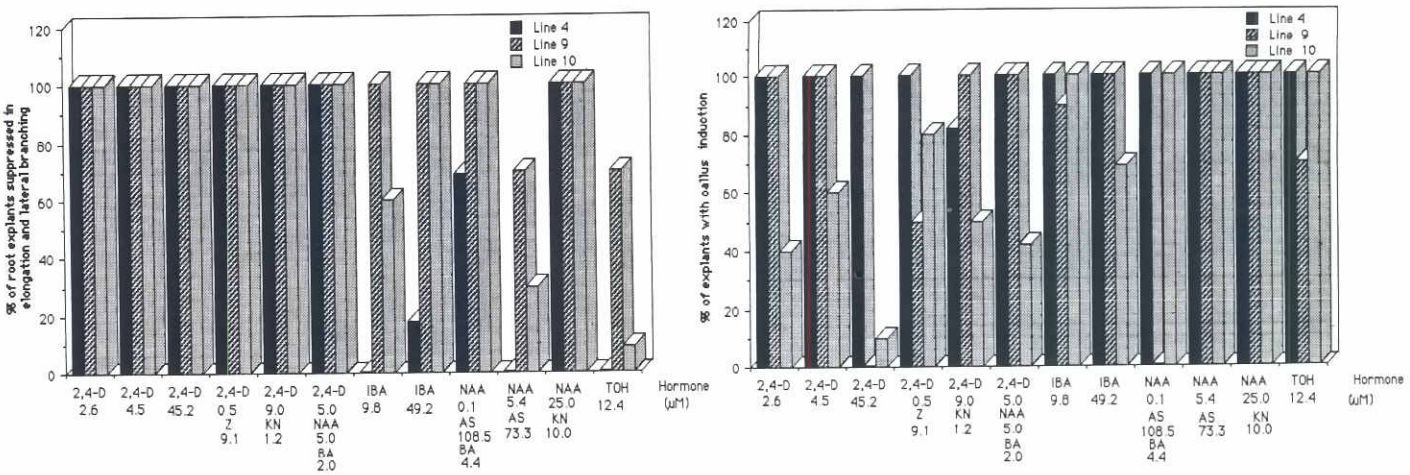

Fig. 2. (A) *The influence of growth regulators on elongation and lateral branching of root explants from pumpkin hairy root (pRi8196) lines.* **(B)** *The influence of growth regulators on callus induction in root explants from pumpkin hairy root (pRi8196) lines.*

Inoculation with bacteria

Pumpkin cotyledons were detached and injured by diagonal incisions to the left and the right of the midrib and the wounds were inoculated with bacteria.

Establishment of hairy root cultures

Primary hairy roots which emerged in the inoculated wound area were explanted and cultured on an agarified MS (Murashige and Skoog, 1962) medium (0.9% agar, 3% glucose) without hormones, but with carbenicillin (500 mg/l). Established axenic hairy root cultures were further subcultured monthly on MS medium without hormones and carbenicillin.

DNA isolation

Genomic DNA was isolated from hairy root cultures according to the procedure of Dellaporta *et al.* (1983).

Blotting

Gels were prepared for blotting as described by Southern (1975).

Probe labeling.

The probe was a clone 118 (from a library constructed with partial BamHI fragments from pRi 8196 cloned into pBR328) labeled with ^{32}P by oligolabeling (Pharmacia kit).

Callus induction

Explants (1cm) from three different pumpkin hairy root lines were cultured on a solid MS medium supplemented with plant growth regulators in twelve different combinations and/or concentrations – twenty explants were tested on each combination. Cultures were incubated in a growth-room at 25°C under artificial light (1600 lux) and a 16/8h light-dark cycle.

Acknowledgments

DNA isolation and Southern blotting were carried out at the Institute for Molecular Genetics and Genetic Engineering, Belgrade, Yugoslavia. We are indebted to Prof. Dr. Ana Savic and colleagues from the Institute for their help and advice. We thank Dr. David Chantal, Institut Jacques Monod, Paris, for providing A. tumefaciens *transconjugant strains, Dr. Marijana Krsnik-Rasol and Dr. Branka Pevalek-Kozlina (from our Dept.) for critical reading of the manuscript. This research was supported by the Scientific Research Council of Croatia, Grant No. 2.04.01.01.09. and Federal GIBIT Grant No. 1.6.1.2.*

References

CONSTABEL, C.P. and TOWERS, G.H.N (1988). Thiarubrine accumulation in hairy root cultures of *Catharanthus douglasii. J. Plant Physiol. 133:* 67-72.

CROES, A.F., VAN DEN BERG, A.J.R., BOSVELD, M., BRETELER, H. and WULLEMS, G.J. (1989). Thiophene accumulation in relation to morphology in roots of *Tagetes patula.* Effects of auxins and transformation by *Agrobacterium. Planta 179:* 43-50.

DAVID, D. and TEMPE, J. (1988). Genetic transformation of cauliflower *(Brassica oleracea* L. var. *Botrytis)* by *Agrobacterium rhizogenes. Plant Cell Rep. 7:* 88-91.

DAVIOUD, E., KAN, S., HAMON, J., TEMPE, J. and HUSSON H.-P. (1989). Production of indole alkaloids by *in vitro* root cultures from *Catharanthus trichophyllus. Phytochemistry 28:* 2675-2680.

DELLAPORTA, S.L., WOOD, J. and HICKS, J.B. (1983). A plant DNA minipreparation: version II. *Plant Mol. Biol. Rep. 1:* 19-21.

DE VRIES-UIJTEWAAL, E., GILISSEN, L.J.W., FLIPSE, E., SREE RAMULU, K. and DE GROOT, B. (1988). Characterization of root clones obtained after transformation of monohaploid and diploid potato genotypes with hairy root inducing strains of *Agrobacterium. Plant Sci. 58:* 193-202.

GHEYSEN, G., DHAESE, P., VAN MONTAGU, M. and SCHELL, J. (1985). DNA flux across genetic barriers: the crown gall phenomenon. In *Genetic Flux in Plants* (Eds. B. Hohn and E.S. Dennis). Springer, Vienna, pp. 11-47.

HANISCH TEN CATE, C.H., SREE RAMULU, K., DIJKHUIS, P. and DE GROOT, B. (1987). Genetic stability of cultured hairy roots induced by *Agrobacterium rhizogenes* on tuber discs of potato cv. Bintje. *Plant Sci. 49:* 217-222.

JELASKA, S. (1972). Embryoid formation by fragments of cotyledons and hypocotyls in *Cucurbita pepo* L. *Planta 103:* 278-280.

JELASKA, S. (1974). Embryogenesis and organogenesis in pumpkin explants. *Physiol. Plant. 31:* 257-261.

JELASKA, S. (1986). Cucurbits. In *Biotechnology in Agriculture and Forestry 2. Crops I.* (Ed. Y.P.S. Bajaj). Springer-Verlag, Berlin, Heidelberg, New, Tokyo, pp. 371-386.

KATAVIC, V., JELASKA, S., PETRICIOLI-BAKRAN, T. and DAVID, C. (1990). Host-tissue differences in transformation of pumpkin *(Cucurbita pepo* L.) by *Agrobacterium rhizogenes. Plant Cell Tiss. Org.* (In press).

LAVIE, D. and GLOTTER, E. (1971). The cucurbitacins, a group of tetracyclic triterpenes. In *Progress in the Chemistry of Organic Natural Products* (Eds. W. Hertz, H. Grisebach and G.W. Kirby). Springer-Verlag, Berlin-Heidelberg, pp. 307-362.

MURASHIGE, T. and SKOOG, F. (1962). A revised medium for the rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant. 15:* 473-479.

OTTAVIANI, M.P., SCHEL, J.H.N. and HANISCH TEN CATE, C.H. (1990). Variation in structure and plant regeneration of *Agrobacterium rhizogenes* transformed and control roots of the potato cv. Bintje. *Plant Cell Tiss. Org. Cult. 20:* 25-34.

RHODES, M.J.C., ROBINS, R.J., HAMILL, J.D., PARR, A.J. and WALTON, N.J. (1987). Secondary product formation using *Agrobacterium rhizogenes* transformed «hairy root» cultures. *Newsletter IAPTC 53:* 2-15.

RIKER, A.J. and BANFIELD, W.M. (1932). Studies on the development of crown gall, hairy root and wound overgrowth in treated soil. *Phytopathology 22:* 167-177.

SHEN, W.H., PETIT, A., GUERN, J. and TEMPOE, J. (1988). Hairy roots are more sensitive to auxin than normal roots. *Proc. Natl. Acad. Sci. USA 85:* 3417-3421.

SOUTHERN, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol. 98:* 503-517.

TEPFER, D. (1984). Transformation of several species of higher plants by *Agrobacterium rhizogenes:* sexual transmission of the transformed genotype and phenotype. *Cell 37:* 959-967.

TRULSON, A.J., SIMPSON, R.B. and SHAHIN, E.A. (1986). Transformation of cucumber *(Cucumis sativus* L.) plants with *Agrobacterium rhizogenes. Theor. Appl. Genet. 73:* 11-15.

VISSER, R.G.F., JACOBSEN, E., WITHOLT, B. and FEENSTRA, W.J. (1989). Efficient transformation of potato *(Solanum tuberosum* L.) using a binary vector in *Agrobacterium rhizogenes. Theor. Appl. Genet. 78:* 594-600.

WHITE, F.F. and NESTER, E.W. (1980). Hairy root: plasmid encodes virulence traits in *Agrobacterium rhizogenes. J. Bacteriol. 141:* 1134-1141.