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Transformation of the grain legume *Vigna aconitifolia* Jacq Marechal by *Agrobacterium tumefaciens*, regeneration of shoots

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Abstract. Hypocotyl, stem, leaf and shoot-apex of one-week-old seedlings of *Vigna aconitifolia* were inoculated with the nopaline wild type (T_{37}) of *Agrobacterium tumefaciens* to induce tumours. Hypocotyl segments of 1 cm length from *in vitro* grown plants were also co-cultivated with a bacterial suspension to obtain transformed tissue. Axenic cultures obtained from both the experiments when cultured on basal medium proliferated into callus which differentiated into shoot buds on the same medium after repeated subcultures. The transformed nature of the shoots was confirmed by their failure to produce roots, growth on hormone-free medium and the presence of nopaline and nopaline dehydrogenase.

Keywords. *Agrobacterium tumefaciens*; grain legume; transformation.

1. Introduction

Convincing evidence has accumulated over the last few years indicating that the tumour inducing (Ti) plasmids in *Agrobacterium* are efficient natural gene vectors capable of transferring genes into plant nucleus, where they are stably maintained by covalent integration into the DNA (Nester *et al* 1984). The expression of the T-DNA in the plant cell results in the growth of crowngall tumours, which continue to grow in the absence of exogenous hormones in culture and in the production of the characteristic amino acids, the opines. The Ti plasmid of this natural gene vector has been genetically modified to obtain stable introduction and expression of foreign genes into plant cells. Majority of experiments on transformation so far are restricted to plants belonging to the family Solanaceae (McCormick *et al* 1986; Horsch *et al* 1985).

Among legumes, which comprise important crop species, the majority of experiments on transformation are restricted to the forage legumes such as *Medicago sativa* (Mariotti *et al* 1984; Shahin *et al* 1986), Trifolium species (Webb 1986; White and Greenwood 1987), *Onobrychis viciifolia* (Webb 1986), *Lotus corniculatus* (Webb 1986; Petit *et al* 1987) and *Sesbania rostrata* (Vlachova *et al* 1987). In reports on the grain legumes cowpea (Garcia *et al* 1986) and soybean (Owens and Cress 1985), successful transformation was reported, but no organogenesis could be achieved. Gill and Eapen (1986) and Eapen *et al* (1986) have already established a protocol for plant regeneration in cell, tissue and protoplast cultures of *Vigna aconitifolia*. In the present communication we report the successful transformation of *V. aconitifolia* by wild type Ti plasmid of *Agrobacterium tumefaciens* and its regeneration into shoots.

2. Materials and methods

2.1 *Bacterial strain*

Nopaline strain (T_{37}) of *A. tumefaciens* obtained from National Chemical Laboratory, Poona was maintained on LB medium.

2.2 *Co-cultivation*

Seeds of *V. aconitifolia* were germinated as described before (Eapen *et al* 1986). Hypocotyl pieces of 1 cm length were excised from 6 day old seedlings and incubated for 24 h with 24 h old bacterial suspensions.

2.3 *Tumour induction*

Hypocotyls, stem, leaf and shoot apex of one-week-old seedlings of *V. aconitifolia* growing in pots were inoculated with 24–36 h old cultures of *A. tumefaciens* (T_{37}) after creating a wound with a hypodermic syringe (25 gauge needle). Agar blocks or bacterial suspension in liquid medium were smeared on wounds.

2.4 *In vitro culture of transformed tissue*

Hypocotyl segments along with tumours and co-cultivated hypocotyl segments were rinsed with 70% ethanol for 1 min followed by surface-sterilization with 0·1% mercuric chloride for 5 and 3 min respectively. Aseptic tumours were cut into thin transverse slices and cultured on basal medium containing Murashige and Skoog's (MS, 1962) mineral elements, vitamins after Lin and Staba (1961) along with 3% sucrose. Nutrient medium was solidified with 0·7% agar (SISCO labs, Bombay) after adjusting the pH to 5·8 prior to autoclaving. To prevent the growth of bacteria, carbenicillin (Pyopan, Kosmochem Ltd., Bombay), was added (500 mg/l) to autoclaved medium after Millipore filter-sterilization. For co-cultivated hypocotyls, medium supplemented with 2,4-dichlorophenoxyacetic acid (0·1 mg/l) was used during the first passage and subsequently transferred to MS basal medium. All cultures were grown at $25 \pm 2°C$ under continuous light (1000 lux) and subcultured on fresh medium after 20–30 days.

2.5 *Detection of nopaline and nopaline dehydrogenase*

Nopaline in tumour, callus and regenerated shoots was extracted by homogenising the tissues in equal weight of 70% ethanol. The solution was centrifuged at 12,000 *g* and aliquots applied to Whatman 3 mm paper. Nopaline was detected after separating it by electrophoresis at 400 V for 60 min using formic acid/acetic acid/water (5:15:80 by volume). The paper was dried and stained with a 1:1 solution of 0·02% (w/w) phenanthraquinone in absolute ethanol and 10% (w/v) NaOH in 60% ethanol. Pure nopaline (Sigma Chemical Co., USA) served as the control.

For detection of nopaline dehydrogenase, the method of Otten and Schilperoort (1978) was used. Tumour, callus and regenerated shoots were ground with extraction buffer (0·1 M Tris HCl, 0·5 M sucrose, 0·1% ascorbic acid, 0·1% cysteine HCl, pH 8) centrifuged at 12,000 *g* and supernatant passed through Sephadex G 25 column. The effluent was used for detection of nopaline dehydrogenase. The extract was incubated with 60 mM α-ketoglutarate, 60 mM L-arginine, 16 mM NADH dissolved in 0·2 M phosphate buffer (pH 6·8) for 60 min and 5 μl was spotted, electrophoresis carried out at 400 V for 45 min and stained with 1 vol phenanthrenequinol/ethanol and 1 vol 10% NaOH/60% ethanol. After drying, the spots were visualised under a long wave ultraviolet lamp.

3. Results

3.1 *Response of tumour explant*

Both *A. tumefaciens* grown on semi-solid as well as liquid medium could induce tumours on plants. However, tumours could be produced only on stem and hypocotyl portions, and never on leaf and shoot apex. Age of the seedlings was a critical factor in tumour induction since only young plants till the age day 10 could be made to induce tumours while older plants remained unaffected. Occasionally secondary tumours were also seen on other parts of stem portions of the seedlings. After 2–3 weeks of wounding, the hypocotyl and stem portions showed small protuberances which gradually developed into tumours. These tumours were round and hard in texture. Transverse sections revealed a hard green core encircled with tightly packed green parenchymatous cells. Within a week, *in vitro* cultures of very thin sections of tumours enlarged and developed into a compact callus on basal medium. Sections of hypocotyl or stem of the same size of untransformed tissue failed to grow under identical conditions on basal medium. The compact tumour callus tissue continued to grow vigorously and gradually became friable after each subculture. After two months, in 80% of the callus culture, small and green patches were observed which eventually developed into shoot buds.

3.2 *Response of co-cultivated hypocotyls*

Within a week of culture, sections from co-cultivated hypocotyls started initiating callus on MS medium supplemented with 2,4-dichlorophenoxyacetic acid (0·1 mg/l). Vigorously growing tissue was transferred during second subculture to hormone free medium where it continued to proliferate and did not require hormone for continued growth. About 70% of the callus differentiated shoot buds after two months of culture. Attempts to induce root formation in shoots derived from tumours or after co-cultivation were met with failure, confirming thereby the transformed nature of regenerants.

3.3 *Detection of nopaline and nopaline dehydrogenase*

Crude extract of callus originating from gall tumours, callus and regenerated shoots when tested for nopaline by paper electrophoresis showed the presence of nopaline

due to its migration to the same position as standard synthetic nopaline. However, nopaline was not found in untransformed tissue (figure 1). Of the callus cultures tested 80% were found to show the presence of nopaline.

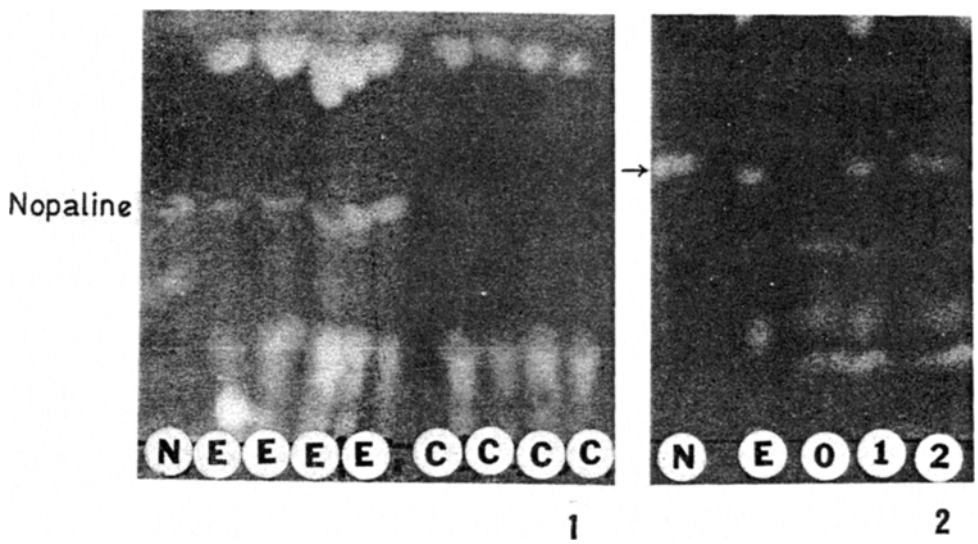
Activity of nopaline dehydrogenase was also tested for the synthesis of nopaline. No traces of endogenous nopaline could be detected after passing through Sephadex G-25 but could be found again after 1 h when effluent from Sephadex column was incubated with incubation mixture (figure 2). The tumour, callus and regenerated shoots showed the presence of nopaline dehydrogenase.

4. Discussion

Since the earlier work on transformation by Lippincott and Lippincott (1967) in legumes, considerable work has followed. Mariotti *et al* (1984) reported successful transformation of cells of a forage legume, *Medicago sativa* to callus and Deak *et al* (1986) obtained shoots from transformed tissue in *M. varia*. Recently Garcia *et al* (1986) have reported transformation of cowpea (*Vigna unguiculata*) cells with an antibiotic resistance gene using a Ti plasmid derived vector. However, they were unable to regenerate whole plants from the transformed callus tissue. Similar results have been obtained in *Glycine max* and *G. soja* (Owens and Cress 1985). We have been able to extend cell transformation studies using an oncogenic type wild strain (T_{37}) of *A. tumefaciens* to the grain legume *V. aconitifolia* using simpler techniques of *in planta* inoculation and co-cultivation with explants. These methods have been used in legumes by earlier workers (Deak *et al* 1986; Garcia *et al* 1986; Mariotti *et al* 1984; Owens and Cress 1985) and also in a few other members of *Solanaceae* namely tobacco, petunia and tomato (Horsch *et al* 1985). Age of the plant was found to be a critical factor for susceptibility to bacterial strain for tumour induction. Similar observation has been made in another grain legume *Glycine max* and *G. soja* (Owens and Cress 1985).

In the present study no difficulty was encountered in culturing tumours *in vitro* whereas in *Medicago sativa*, nopaline tumours could not be successfuly cultured due to necrosis (Mariotti *et al* 1984). Cultures whether derived from tumour explants or co-cultivated hypocotyl segments showed two characteristic features of transformed tissue i.e. hormone-independent growth and the presence of nopaline and nopaline dehydrogenase.

Transformed tissue readily differentiated into shoot buds on basal medium in 80% of cultures. However, shoots did not develop roots; which is a characteristic of tumours induced by wild type *Agrobacterium* strains. Wild type plasmid is known to introduce hormone imbalance and cause problems in regeneration (Akiyoshi *et al* 1983; Schroder *et al* 1983; Nester *et al* 1984; An *et al* 1985). In an earlier study on a forage legume *Medicago sativa*, similar results were observed using the same plasmid (Mariotti *et al* 1984). In mothbean, due to the very small diameter of shootlets, grafting to healthy plants proved difficult. Further attempts are underway in this direction. The relative ease with which cells of *V. aconitifolia* could be transformed by wild type strain of *A. tumefaciens* and its ability to regenerate shoots readily points to the possibilities of using this system for incorporating foreign genes in this species through the use of genetically modified strains. Recently, however chimaeric genes for kanamycin resistance has been transferred to mothbean by direct (Kohler *et al* 1987) and *Agrobacterium* mediated gene transfer



Figures 1 and 2. **1.** Presence of nopaline in transformed tissue. (N), Standard nopaline; (E), different samples; (C), control samples. **2.** Presence of nopaline dehydrogenase activity. (N), Standard nopaline; (E), extract before passing through Sephadex showing the presence of indigenous nopaline; (O), extract at zero hour showing absence of nopaline after passing through Sephadex; (1–2), shows reoccurrence of nopaline after incubating for 1–2 h in incubation mixture.

(Eapen *et al* 1987) and plants regenerated. Southern blot hybridization had demonstrated the successful integration of bacterial gene into the mothbean genome in these experiments.

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