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Transformation of *Brassica juncea* by *Agrobacterium tumefaciens* harbouring plasmid pTiT37 and its 'rooty' mutant pTiT37.14a/a

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Abstract. Indian mustard (*Brassica juncea* Linn., Czern and Coss) plants were inoculated with *Agrobacterium tumefaciens* strain A208 harbouring either plasmid pTiT37 or pTiT37·14a/a. The latter carries an insertion at the 'rooty' locus (gene 4 or *cyt*) of the T-DNA governing cytokinin biosynthesis. The tumours induced by pTiT37·14a/a were small and formed many roots. For *in vitro* culture, these transformed cells required supplementation of the basal medium with IAA and kinetin in the initial stages though after six sub-cultures they were also hormone autotrophic. *In vitro* cultures showed profuse rooting like the *in planta* tumours. Unlike transformed tobacco and carrot cells, *Brassica juncea* cells transformed by pTiT37·14a/a could not be induced to differentiate into shoots. In contrast, the cells transformed by the wild type pTiT37 were hormone autotrophic and occasionally differentiated into shoots but could not be induced to produce roots. These results demonstrate how a single gene mutation affecting cytokinin biosynthesis can alter the *in planta* as well as the *in vitro* response of a transformed cell of the same genotype.

Keywords. *Agrobacterium tumefaciens*; rooty mutant; *Brassica juncea*; regeneration; Ti plasmid.

1. Introduction

Transformation of plant cells by the Ti plasmids of *Agrobacterium tumefaciens* is associated with transfer of a part of the plasmid DNA called T-DNA into the host cell where it gets covalently integrated with chromosomal DNA (Chilton *et al* 1977; Lemmers *et al* 1980; Thomashow *et al* 1980; Yadav *et al* 1980; Zambryski *et al* 1980) and the T-DNA genes are expressed in the plant cells. This natural transformation system also enables introduction of specific mutations in the T-DNA into plant cells and study of the comparative responses elicited in the host cells.

Regeneration of shoots from Indian mustard (*Brassica juncea*) cells transformed by *Agrobacterium tumefaciens* strain A208 having wild type plasmid pTiT37 was reported previously (Mathews *et al* 1985). The regenerated shoots did not produce roots under any culture conditions and were raised to maturity by micrografting on normal regenerated plants (Mathews and Bhatia, unpublished results). Regeneration of plants from tobacco and carrot tumour cells transformed by disarmed 'rooty' mutants of pTiT37 has been reported (Barton *et al* 1983; Chilton 1983). By inserting foreign DNA at the Hpa1 site in pTiT37, Bam H1 fragment 14a, they could overcome the problem of

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plant regeneration. The 'rooty' mutants of both nopaline and octopine type Ti plasmids have been mapped in the Bam H1 fragment 14a (Ooms *et al* 1981; Garfinkel *et al* 1981; Barton *et al* 1983; Nester *et al* 1984). The Hpa I site in this fragment falls in the region of gene 4 (also designated as *cyt*) governing cytokinin biosynthesis (Bevan and Chilton 1982). The tumours induced by such insertional mutations of gene 4 are not cytokinin autotrophs. pTiT37·14a/a used in the present investigations is a complex engineered plasmid developed by Matzke and Chilton (1981). In this, the Bam H1 14a fragment of pTiT37 carries two kanamycin^R genes from Tn5 with pBR322 between them. In this paper, we report the *in vitro* growth characteristics of *Brassica juncea* cells transformed with pTiT37 and pTiT37·14a/a.

2. Materials and methods

2.1 *Tumour induction*

Two-week old seedlings of *Brassica juncea* grown in pots were inoculated by wounding the stem and applying a drop of a 48-hr old suspension culture of *Agrobacterium tumefaciens* strain A208. Wild type strain harbouring pTiT37 and its 'rooty' mutant strain having pTiT37·14a/a were used. The original bacterial strains were obtained from Dr Mary-Dell Chilton, CIBA-GEIGY Corporation, Research Triangle Park.

2.2 *In vitro aseptic culture of tumour tissue*

Stem segments with tumours were surface sterilised with 0·1 % $HgCl_2$ for 10 minutes. Segments of tumours induced by pTiT37 were cultured on the basal medium of Murashige and Skoog (1962) with streptomycin 100 μg/ml. pTiT37·14a/a induced tumours were cultured on an MS medium supplemented with indole acetic acid (IAA) and kinetin(kn). The nutrient medium was adjusted to pH 5·8 and solidified with 0·8 % Difco agar. Millipore filter sterilised streptomycin sulphate was added to the sterilised medium. After 3–4 passages, streptomycin was not required to control *Agrobacterium* growth. Cultures were grown at 25 ± 2°C under continuous light.

2.3 *Liquid cultures*

After the initiation axenic tissue could also be maintained in agitated liquid flasks containing either MS alone or MS + IAA + kn.

2.4 *Growth rate*

The growth rate of tissue was calculated from fresh weights.

2.5 *Attempts to induce organogenesis*

To induce differentiation of shoots and roots, axenic callus transformed with pTiT37 and pTiT37·14a/a were cultured on various hormonal combinations including IAA, NAA, IBA, 2,4-D, TIBA, BA, kn and various levels of sucrose.

2.6 *Detection of nopaline*

Axenic, cultured tumour tissue was homogenised in an equal weight of 70% ethanol. After centrifugation at 12,000 g, aliquots of the supernatant were applied to Whatman 3 MM paper and electrophoresed at 400 V for 70 minutes in formic acid/acetic acid/water (5:15:80 by volume) following the method described by Otten and Schilperoort (1978).

3. Results

3.1 *In planta tumour*

Tumours produced on mustard plants following inoculation with the wild type pTiT37 (figure 1) were similar to those described previously (Mathews *et al* 1985). The tumours produced by the mutant pTiT37·14 a/a (figure 2) were morphologically different from those produced by pTiT37. Slight callus with plenty of small roots were produced at the inoculation site after 20–25 days (figure 2).

3.2 *In vitro culture*

pTiT37·14a/a tumours when cultured on a basal medium devoid of hormones did not grow, and hence, combinations of auxins and cytokinins were used. Remarkable growth of the tissue was obtained on an MS medium fortified with IAA (0·1 mg/l) and kinetin (1 mg/l). Therefore in further experiments a wide range of IAA and kinetin concentrations (0·2–10·0 mg/l) were tried. However, no significant difference in the rate of growth was observed with different treatments. In a period of 50 days, the average fresh weight growth of the tissue was $5{\cdot}82 \pm 0{\cdot}83$. Like the *in planta* tumours, the *in vitro* cultured tumours were characterized by profuse rooting (figure 3). Shoot regeneration has not been observed in these cultures during the past 18 months. Though the callus was initiated on a hormone-containing medium, after a period of 6 sub-cultures it could be maintained on basal media without IAA and kinetin. The liquid cultures also showed characteristic rooting of the transformed tissue independent of the presence or absence of hormones (figure 4).

3.3 *Opines*

Axenic cultures of pTiT37 and pTiT37·14a/a transformed tissues were nopaline positive (figure 5).

4. Discussion

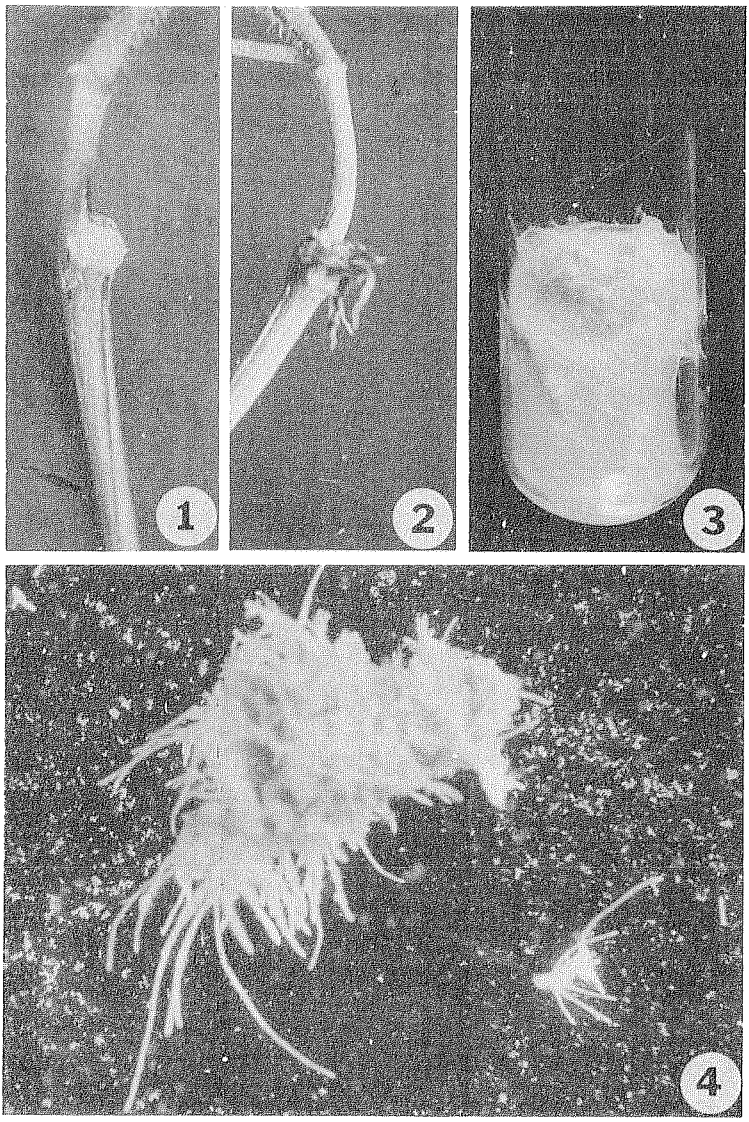
Tumour cells transformed by T-DNA are characterized by over-production of phytohormones and opine synthesis. Recent evidences indicate that the so called *onc* genes of T-DNA, which are responsible for the tumourous growth, govern auxin and cytokinin biosynthesis (Akiyoshi *et al* 1984; Inze *et al* 1984; Schroder *et al* 1984; Thomashow *et al* 1984, 1986). T-DNA carries a conserved set of three genes 1, 2 and 4 previously described as *tms* (1 and 2) and *tmr* (4) responsible for hormone independence (Garfinkel *et al* 1981; Leemans *et al* 1982; Joos *et al* 1983; Ream *et al* 1983; Klee *et al* 1984; van Onckelen *et al* 1985). In tobacco, *tmr* mutants induce tumours having a low cytokinin/auxin ratio, favouring root differentiation (Akiyoshi *et al* 1984). It has been shown that the *tmr* gene encodes for the enzyme dimethylallyl transferase involved in cytokinin biosynthesis (Akiyoshi *et al* 1984; Barry *et al* 1984). Genes 1 and 2 encode for enzymes involved in auxin indole-3-acetic acid synthesis (Inze *et al* 1984; Thomashow *et al* 1984) and hence *tms* mutants induce shoot formation and inhibit rooting.

pTiT37·14a/a used in our investigations and other mutations at the *tmr* loci induce attenuated tumours in tobacco and *Kalanchoe tubiflorae*, *K. daigremontiana* and *Helianthus annus* which are not cytokinin autotrophic (Barton *et al* 1983; Chilton 1983; Ream *et al* 1983). In a comparative study on *Vitis*, *Agrobacterium* strain A208 carrying pTiT37·14a/a produced larger tumours than the same strain having wild type pTiT37, only one tumour induced by 14 a/a showed root formation (Hemstad and Reisch 1985). In *Brassica juncea*, both *in planta* and *in vitro* tumours induced by rooty mutant strains were distinctly characterised by profuse rooting. Table 1 gives a comparative account of the tumour characteristics in *B. juncea* by wild and mutant strains. Crown galls with roots were also reported in soybean after injection with a 'rooty' mutant of *Agrobacterium tumefaciens* strain 338::Tn5 (Owens 1985).

The oncogenic properties as well as the host range of Ti plasmids is partly governed by the genes on the T-DNA (Knauf *et al* 1983; Buchholz and Thomashow 1984). Inactivation or introduction of the *cyt* gene of T-DNA altered the host range of

Table 1. The tumour characteristics of wild and rooty mutant strains.

	pTiT37	pTiT37·14a/a
Morphology of *in planta* tumour	White/yellowish smooth tumour	Whitish mass with roots
Culture amenability and *in vitro* growth characteristics	*In vitro* growth was initiated in hormone free media	Initiation of aseptic tissues required the presence of phytohormones
	Growth of callus was 21·5 ± 0·96 (culture period: 30 days)	Growth rate of callus was 5·38 ± 0·833 (culture period: 50 days)
	Callus could be grown only on solid media. The tissue necrosed in agitated liquid media	Callus could be cultivated either in solid or agitated liquid media
	Yellowish, homogenous callus mass	Brownish callus with plenty of roots
	Optimum period of sub-culture: 25–30 days	Optimum period of sub-culture: 50–60 days
	Spontaneous regeneration of shoots	No shoot regeneration



Figures 1 and 2. Tumours induced on *B. juncea* plants after inoculation with *Agrobacterium tumefaciens* A208 having wild type plasmid pTiT37 (**1**) or pTiT37·14a/a (**2**).

Figures 3 and 4. Axenic culture of tumour tissue induced by pTiT37·14a/a (**3**) on solid medium and (**4**) in liquid culture.

Agrobacterium tumefaciens strain LBA649 with pTiAg57 (Hoekema *et al* 1984). The differential *in planta* response of pTiT37·14a/a on tobacco, carrot, *Vitis* and *Brassica juncea* as well as the characteristics of *in vitro* transformed cells of *B. juncea* in the present report are of interest. They point to the fact that the alteration of gene affecting cytokinin production elicits different responses in different host species. It would be of interest to investigate the response on different genotypes within the same host species.

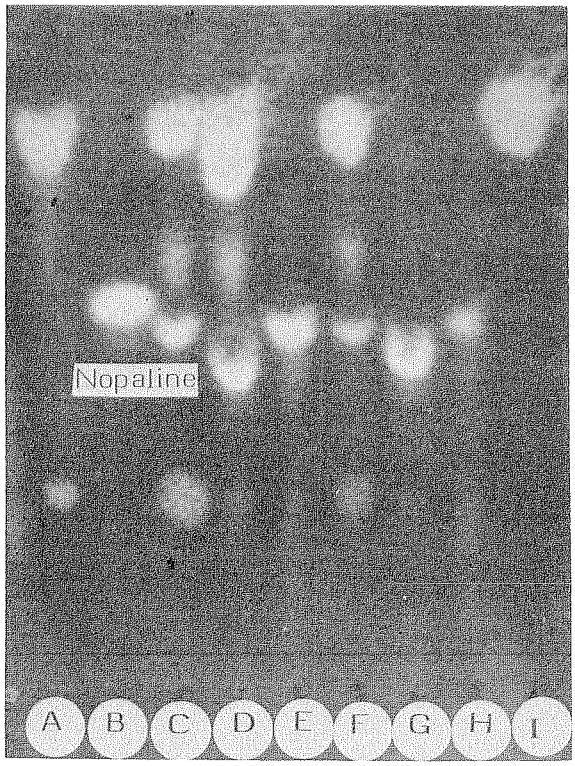


Figure 5. Nopaline in tumour tissue: A. *B. juncea* callus; B. nopaline standard; C. pTiT37 induced *in vitro* cultured tumour tissue; D. shoot regenerated from C; E. pTiT37·14a/a induced *in vitro* cultured tumour tissue with roots; F. roots alone from E; G. callus alone from E; H. *B. juncea* shoot. Tissues were extracted with equal weight/volume of 70% ethanol. 20 µl aliquots of extract were spotted on paper and subjected to electrophoresis.

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