BIOLOGIA PLANTARUM (PRAHA)

33 (1) : 40–48, 1991

Transgenic Tobacco Plants with T-DNA Phytohormone Synthesis Genes

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Abstract. *Agrobacterium tumefaciens* binary vectors carrying kanamycin resistance gene and either C58 T-DNA gene 4 for cytokinin synthesis or genes 1 and 2 for auxin synthesis were constructed and used for transformation of a short-day tobacco Maryland Mammoth. Kanamycin resistant plants were regenerated from a small fraction of transformed tissue and the presence of T-DNA in their genome was verified by Southern blotting. The level of endogenous cytokinin in plants transgenic for gene 4 and the level of endogenous IAA in those transgenic for genes 1 and 2 increased by more than 100 %. A number of morphological characteristics distinguish them from untransformed controls.

Agrobacterium tumefaciens transformation offers a new approach to the study of the role of auxins and cytokinins in plant development, because the expression of T-DNA genes leads to increased level of both phytohormones. *A. tumefaciens* T-DNA gene 1 codes for tryptophan monooxygenase, which converts tryptophan to indolylacetamide (Follin *et al.* 1985) and gene 2 codes for indolylacetamide hydrolase which converts this promotor to IAA (Thomashow *et al.* 1984). Gene 4 codes for isopentenyltransferase (Barry *et al.* 1984), which is the key enzyme for the synthesis of cytokinins. In T-DNA of the C58 Ti plasmid there are a further 11 genes (Willmitzer *et al.* 1983) and the products of some of them most probably interact with those of genes 1, 2 and 4.

Received December 7, 1989; accepted April 5, 1990
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We constructed special *A. tumefaciens* vectors as tools for transfer to the plant genome of genes 1 and 2 or gene 4 without the other T-DNA genes (Ondřej *et al.* 1989b). The aim of this study was transformation and selection of transgenic tissues with capacity of plant regeneration but with some activity of the T-DNA phytohormone synthesizing genes. A short-day tobacco Maryland Mammonth widely used in flowering studies was transformed. It will be further used to investigate the hormonal control of flowering of transgenic plants.

MATERIAL AND METHODS

Nicotiana tabacum cv. Maryland Mammoth was used throughout this study. For construction of vectors, *A. tumefaciens* strain LBA4404, which carries the helper plasmid pAL4404 deprived of T-DNA (Hoekema *et al.* 1983) was obtained by courtesy of Dr. P. Hooykaas, Department of Biochemistry, University of Leiden. plasmid pGVO354 which carries pTi C58 T-DNA segments (Depicker *et al.* 1980), was the gift of prof. J. Schell, Max-Planck-Institut für Züchtungsforschung, Köln. *E. coli* DH1 strain was used.

Plasmid DNA of *A. tumefaciens* and *E. coli* was isolated and electrophoretically identified by the method of Casse *et al.* (1979). This partly was also used for physical mapping and cloning. *Hind* III fragments of pGVO354 were cloned into a single *Hind* III site of pGA472, the vector plasmid for binary vectors which carries chimeric in plants expressed kanamycin resistance gene. This plasmid was constructed by AN *et al.* (1985). Recombinant plasmids were selected according to their size and their structure was determined by restriction endonuclease mapping. The cloning methods described in Maniatis *et al.* (1982) were used. *A. tumefaciens* strain LBA 4404 was transformed by the plasmids constructed according to Holsters *et al.* (1978).

For tobacco transformation, the modification of leaf disc method of Horsch *et al.* (1985) according to Simpson *et al.* (1985) was used. Transformed tissues were cultivated on the Murashige and Skoog (1962) medium with phytohormones, 1 mg l^{-1} benzylaminopurine (Lachema, Czechoslovakia) and 0.1 mg l^{-1} α-naphthaleneacetic acid. A similar medium without growth regulators was used for subsequent subcultivations. Control non-transformed tissues were cultivated in the same way. Antibiotics (used only in transformed plants) were ommited after six subcultivations, each lasting one month. After this period, cloned plants were used for Southern hybridization. Plant DNA was isolated by the method of Roger and Bendich (1985) and Southern hybridization performed according to Maniatis *et al.* (1982).

Transformed and control plants were then transferred to soil and cultivated further in a greenhouse at temperatures of 25–27 °C (day)/20–22 °C (night), illuminance about 20 000 lx (xenon lamps) and daylight of 18 h (LD) or 8 h (SD).

Leaves of three to four months old plants were used for the determination of phytohormone levels. Leaves from the middle part of the stem were cut off, frozen immediately on liquid nitrogen and kept frozen until analyzed.

Cytokinins were extracted by cold 80 % methanol (5 ml g^{-1} f. m.), the extraction being repeated three times. After evaporation of methanol and centrifugation, the remaining water phase was repeatedly partitioned against *n*-hexan until the hexan phase remained colourless. The pH of the water phase was adjusted to 8.0 by 25 % KOH and it was partitioned three times against *n*-butanol. Combined butanol fractions were evaporated to dryness, the residue dissolved in a small volume of 96 % ethanol and chromatographed on a thin layer of silicagel (Silufol UV-254, Lachema, Czechoslovakia) in *n*-butanol : NH_4OH : water (86 : 5 : 9 v.v.). The R_f zones corresponding to the individual cytokinins were scraped off, eluted with 96 % ethanol and evaporated. The content of cytokinins was determined by the *Amaranthus* bioassay (Mazin *et al.* 1976). Two plants were analyzed in each determination: one transformed plant and one untransformed control plant. Such comparisons were repeated three times with plants of different age and developmental stage.

IAA determination was performed by the method described by Eder *et al.* (1988) and Ondřej *et al.* (1989a), using HPLC with fluorimetric detection.

Determination of the content of chlorophyll and carotenoids was performed as described earlier (Wettstein 1957). Leaf samples were taken from the middle part of the stem.

RESULTS AND DISCUSSION

Derivation of Transformation Vectors

Vector plasmids for binary vectors pCB1334 and pCB1349 were constructed with the use of pGVO354 plasmid (Depicker *et al.* 1980) as the source of pC58 T-DNA. The 7.5 kb continuous part of T-DNA containing genes 5, 2, 1 and 4 (*Hind* III fragments 19.4 and 22) was cloned into the unique *Hind* III site of pGA472 plant vector (AN *et al.* 1985). from the resulting vector pCB1341, vectors pCB1334 and pCB1349 were obtained. The pCB1334 vector carries active gene 4 only (deletion of *Hind* III fragment 19) and vector pCB1349 contains the genes 1,2 and 5 (deletion of Pst I fragment 0.85kb) (Fig. 1). The vector plasmids were introduced into *A. tumefaciens* by transformation.

Plant Transformation and Regeneration

Tobacco leaf discs were transformed by *A. tumefaciens (LA4404) (pCB1334) or A. tumefaciens* (LA4404) (pCB1349) vectors. The vector plasmid pCB1334 brings into the plant genome chimeric in plants expressed kanamycin resistance gene and cytokinin synthesis gene 4. Mostly teratoma tissues appeared on the cut surface of

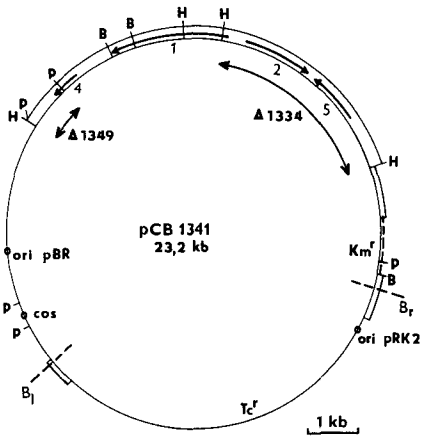

Fig. 1: Newly constructed vector plasmids pCB1341, pCB1334 and pCB1349 for *A. tumefaciens* binary vectors. The plasmids carry pTiC58 T-DNA fragments. B_r – right T-DNA border, B_L = left T-DNA border. Generally accepted T-DNA gene transcript numbers (Willmitzer *et al.* 1983) are given, together with the orientation of T-DNA genes. H – Hind II sites, B – Bam HI sites, P – Pst I sites, ori RK2 – origin of replication of the wide spectrum of the generally used vector plasmid pRK2, ori pBR – origin of replication of *E. coli* pBR322 vector plasmid, cos – cos site, Tc – in bacteria expressed tetracyclin resistance gene, Km^r – in plants expressed kanamycin resistance gene.

the leaf discs on the medium with growth regulators and antibiotics. During their further cultivation the shoots with altered morphology occasionally differentiated, and some of them rooted on the medium with kanamycin. One of the transformed rooted shoots with shortened internodes was selected and cloned for this study. The presence of pCB1334 T-DNA was confirmed by Southern blotting (Fig. 2).

Regeneration of complete plants from transformed tissue is rather exceptional. It is not possible to give an exact frequency of their incidence, but it may be approximated that one out of twenty to fifty pCB1334 carrying leaf discs gave rise to kanamycin-resistant rooted shoots. In leaf discs transformed with kanamycin resistance gene only, usually more than ten rooted shoots per disc differentiate. The capacity for regeneration of fully differentiated plants is thus reduced to 1/200–1/500 in plants carrying the gene for cytokinin synthesis. In the present experiment selection of rooted plants had to be repeated during the first 2–4 subcultivations as the plants often produced teratoma again. On the other hand, cloning of already established and several times subcultivated rooted plants by axillary buds resulted mostly in the formation of rooted plants. It should be stressed that the whole process of obtaining fully differentiated rooted plants from the original transformed teratomas is slow and takes several subcultivations.

Transformation of leaf discs by *A. tumefaciens* carrying pCB1349 vector plasmid, which brings into the plant genome chimeric in plants expressed kanamycin resistance gene and auxin synthesis genes 1, 2 leads mostly to the formation of

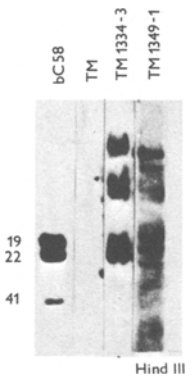

Fig. 2: Detection of pCB1334 and pCB1349 T–DNA fragments in transgenic plants and controls. The pCB1341 plasmid was used as probe. 15 μg of plant DNA of each sample was digested with *Hind* III, electrophoresized, blotted and hybridized with total plasmic DNA of pCB1341 labelled with $^{3}2P$ by nick translation to specific activity of 2.4×10^{8} cpm . μg^{-1} . bC58 – bacterial DNA of *A. tumefaciens* C58 strain, TM – untransformed tobacco DNA, TM1334–3 – tobacco transformed by pCB1334 T–DNA, TM1349–1 – tobacco transformed by pCB1349 vector plasmid.

undifferentiated tumor tissues. This may be due to interaction of cytokinins in the medium and IAA synthesized by transformed plant tissues. Rarely, however, buds appear and develop into kanamycin resistant plants. One of these plants was cloned for further studies. The presence of T–DNA of pCB1349 was confirmed by Southern blotting (Fig. 2).

Transgenic Regenerated Plants

Transgenic plants carrying gene 4 showed an increase in cytokinin content, never being more than the double of the initial content. The results of a representative

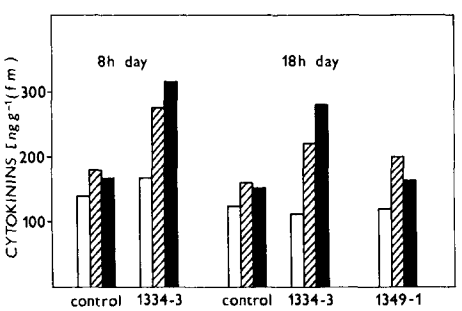

Fig. 3: Level of cytokinins in leaves of transformed tobacco Maryland Mammoth carrying gene 4 for cytokinin synthesis (1334–3) or genes 1, 2 for auxin synthesis (1349–1) compared with non-transformed control.

empty column – zeatin riboside;
full column – isopentenyladenine;
hatched column – zeatin.

TABLE 1

IAA level in the leaves of 4 months old tobacco Maryland Mammoth

Plant	IAA [ng g^{-1} (f.m.)]
Control (non transformed)	24.4
1349–1 (genes 1, 2 for auxin synthesis)	50.6
1334–3 (gene 4 for cytokinin synthesis)	20.6

experiment are demonstrated in Fig. 3. Usually, the rise was most pronounced with zeatin and isopentenyladenosine and was rather slight with zeatin riboside. The cytokinin level in plants carrying genes 1,2 and 5 (pCB1349 transformants) remained unchanged.

The levels of IAA in transgenic plants carrying T–DNA genes 1, 2 and 5 were approximately the double of those in untransformed controls (Table 1).

A strong shortening of internodes was obtained in plants with gene 4 (Table 2, Figs. 4, 5). Leaf number was only slightly reduced. Leaf size and morphology was not altered. There was no change in chlorophyll content (Table 3). Transformed plants with genes 1,2 and 5 showed also some inhibition of stem growth (Table 2, Fig. 5).

Transformation of plant tissues by *A. tumefaciens* strains with inactive genes 1 and 2 but with an active gene 4 usually leads to teratoma formation (see Ondřej *et al.* 1989b). The same was obtained with another cultivar of tobacco. Teratomas consist of clumps of differentiated tissue of small modified leaves, numerous abnormal bud primordia and some very short shoots. Roots are absent. The levels of cytokinins in teratoma tissues are increased up to three orders of magnitude in comparison to untransformed leaves. The ratio of cytokinins to auxin is, thus, extremely shifted in favour of cytokinins. On the contrary transgenic plants selected in the present experiments have relatively very slight elevated levels of phytohormones and show normal plant differentiation with some quantitative deviations in growth.

TABLE 2

Growth of 4 months old regenerated transgenic plants of tobacco Maryland Mammoth ($\bar{x} \pm SE$)

Plant	Leaf number	Leaf length	Leaf width	Stem length
Control	9.0 ± 1.00	23.7 ± 0.48	8.0 ± 0.41	11.5 ± 0.50
1334–3	7.6 ± 0.33	22.0 ± 0.97	10.0 ± 1.00	4.8 ± 0.44
1349–1	7.00 ± 0.00	23.5 ± 1.94	9.1 ± 0.83	7.0 ± 1.00

The basic feature of the expression of transgenes is the variability of the degree of expression in different transgenic clones. There are differences of about two orders of magnitude (Odeil *et al.* 1987) and there is not always correlation with the degree of expression of the signal gene in different transgenic clones. The rare transgenic shoots, which show the presence of gene 4 by Southern blotting and can root on media with a high concentration of kanamycin, are obvious examples of a strong expression of the kanamycin transgene and a low expression of the cointroduced gene 4.

TABLE 3

Pigment content in the leaves on the middle stem of tobacco Maryland Mammoth

Plant	Chlorophyll a + b [mg g^{-1} (f.m.)]	Carotenoids [mg g^{-1} (f.m.)]	Chlorophyll [ng mm^{-2}]	Carotenoids [ng mm^{-2}]
Control	1.52	0.61	38.0	17.0
1334–3	1.53	0.58	33.0	17.0

As the roots usually appear on the transgenic pCB1334 vector T–DNA transformed shoots during later (second, third or even fourth) subcultivations, DNA methylation of the promotor region of gene 4 may be involved. The T–DNA sequences, introduced into the plant genome are often subject to intensive hypermethylations (Gelvin *et al.* 1983).

Regenerated rooted plants may occur as a consequence of recombination between the signal gene (for kanamycin resistance) and gene 4 (for cytokinin synthesis) during the integration of the vector into the plant genome. Such recombinations may appear. In our experiments establishing the presence of genes 1,2 and 5 or gene 4 by Southern blotting, they were found in a fraction of kanamycin resistant regenerated plants to present less than 30 % of cases.

Vectors which bring into the plant genome only the T–DNA gene 4 have already been constructed (An *et al.* 1985), but they were not used for regeneration of whole rooted plants. The results of this study show that *A. tumefaciens* T–DNA vectors of the types pCB1334 and pCB1349 described here can be used for the induction of transgenic plants with increased endogenous level of either IAA or cytokinins. These plants are useful for a number of physiological studies. Experiments on flowering are in progress.

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Fig. 4–5 at the end of the issue.

Atkin, R. K., Clifford, R. D. (ed.): Mechanism and Regulation of Transport Processes. Brit. Plant Growth Regul. Group, Monograph No. 18. – Parchments Ltd., Oxford 1989. 128 pp.

The latest booklet published by the British Plant Growth regulation Group is a record of the proceedings of a meeting organized jointly by this Group and the Physicochemical and Biophysical Panel of the Society of Chemical Industry's Pesticides Group in November 1988 in London. Most attention is paid to the role of plant hormones, especially of auxin in the regulation of transport processes, both the events at the mebranes and the long-distance transport and source-sink relationship. (D. A. Morris: Auxin-promoted assimilate transport; T. Rausch: Effects of plant growth regulators on glucose transport; A. Parsons, D. Sanders: Cytokinin-stimulation of the plasmamembrane proton pump – its role in hormonal stimulus transduction). Mechanisms of phloem loading and unloading and phloem translocation of foliage-applied herbicides are also discussed. (D. A. Baker: Regulation of long-distance transport of assimilates; D. Coupland: Factors affecting the phloem translocation of foliage-applied herbicides).

One contribution (F. K. Bangerth, J. D. Gruber, S. Shehata: Auxin transport in relation to dominance and development of reproductive structures) is devoted to the importance of polar auxin transport in determining dominance of fruit and other reproductive sinks.

The last chapter (R. H. Bromilow, K. Chamberlain: Designing molecules for systemicity) deals with the criteria of systematic design of pesticides and growth regulators, taking into account especially their lipophilicity and acid strength, which are decisive for their phloem mobility.

The content of the booklet is a bit heterogeneous, but it brings reasonable information on some new aspects of the regulation of transport processes in plants.

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