Differential expression of crown gall tumor markers in transformants obtained after *in vitro* *Agrobacterium tumefaciens*-induced transformation of cell wall regenerating protoplasts derived from *Nicotiana tabacum*

(tumor-inducing plasmids/opine synthesis/shoot regeneration/DNA-DNA hybridization)

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ABSTRACT To obtain transformation of plant cells, we incubated 3-day-old cell wall-regenerating protoplasts from tobacco with *Agrobacterium tumefaciens* harboring tumor-inducing plasmids. Putative transformed tobacco cells were selected by phytohormone autotrophic growth and were shown to be transformed by the detection of the tumor cell specific enzymes lysopine dehydrogenase or nopaline dehydrogenase. This was substantiated by the detection, in transformed tumor tissues, of DNA sequences homologous to sequences in the tumor-inducing plasmid. Segregation of tumor markers has been observed among the transformants and it is suggested that this happened during the initiation of the transformation. The stable character of the transformed state was shown by the retention of tumor markers in subcloning of primary transformants under nonselective conditions. Suppression of the neoplastic state of transformants could take place, resulting in the development of transformed shoots. Indications were obtained for the inheritance of tumor markers through meiosis from seedlings obtained from seeds of flowering transformed plants that still expressed nopaline synthesis.

There is a natural system for transformation of plant cells which is performed by the soil bacterium *Agrobacterium tumefaciens*, resulting in the appearance of crown gall tumors. Crown galls are characterized by non-self-limiting growth. The transformation of normal cells into tumor cells is due to the transfer of at least part of the tumor-inducing (Ti) plasmid present in virulent strains of *A. tumefaciens* (1). A region of the Ti-plasmid is integrated in the plant genome present in the nucleus (2) where it is transcribed (3, 4). Crown gall cells have acquired a number of new properties that are stably inherited and by which the tissue can be distinguished from normal plant cells. The crown gall cell marker octopine or nopaline synthesis is coded for by Ti-plasmid genes (5) and it is likely that these genes are structural genes on DNA sequences in tumor DNA homologous to Ti-plasmid sequences (T-DNA) (6). The neoplastic state of the tumor tissues and its hormone autotrophy are assumed to be phenotypic traits due to the endogenous phytohormone balance. Recently, it has been shown that the phytohormone balance in tumor cells is controlled by separated loci on the essential part (6) of that region of the Ti-plasmid found in tumor cells (Ti-region) in an octopine Ti-plasmid (7, 8). The size and organization of integrated T-DNA in octopine crown gall tumors have been described in detail (9).

The crown gall system has the potential of being useful for the genetic engineering of plant cells *in vitro*. Used as model

system to study plant cell transformation *in vitro*, the transfer of T-DNA carrying a selectable marker (induction of phytohormone autotrophy) allows the isolation of stable transformants even if they occur at low frequency. The transforming DNA also carries markers (octopine or nopaline synthesis) for the rapid identification of selected cells as transformants which in a later stage can be confirmed by T-DNA analysis. Finally, the transforming DNA is part of a plasmid that can be manipulated *in vivo* and *in vitro*. With the development of plant protoplast isolation and culture techniques, the large-scale transformation of cells *in vitro* can be envisaged.

To obtain transformation, one could use *A. tumefaciens* in which the transforming DNA (the Ti-plasmid) is replicated and well protected. Because the bacteria do not enter the cells during crown gall induction *in vivo*, an *in vitro* transformation system has been developed in which the bacteria are used as a vector to introduce Ti-plasmid DNA into cell wall regenerating plant protoplasts (10). Advantages of this approach are that engineered DNA need not be isolated from the organism in which it is made and, in principle, it can be transferred by the bacterium into plant cells with relatively high frequency and good reproducibility (10).

In the present study we used this plant protoplast transformation system with octopine type and nopaline type bacterial strains as well as a bacterial strain harboring a cointegrate of the octopine and nopaline Ti-plasmid (11) to obtain a range of transformants with different phenotypes.

METHODS

Bacterial Cultures. Bacterial cultures in midlogarithmic phase were obtained by suspending a single colony in TY medium (Difco tryptone, 5 g/liter; Difco yeast extract, 3 g/liter) and cultured for 48 hr at 29°C. This was followed by subculture in TY medium for another 24 hr. The concentration of the bacteria was calculated from OD at 666 nm (1.5 OD unit = 4×10^9 bacteria per ml). Mutant bacterial strains have been isolated and characterized as described earlier (7, 11–13).

Crown Gall Tissue Cultures. B6S3. Octopine-type crown gall tissue induced on *Nicotiana tabacum* cv. White Burley by *A. tumefaciens* strain B6S3(LBA2).

4001. Octopine-type crown gall tissue induced on *N. tabacum* cv. Petit Havana SR1 (14) by strain LBA 4001 (wild-type Ach 5) (11).

Abbreviations: Ti, tumor-inducing; Ti-region, part of the Ti-plasmid found in tumor cells; T-DNA, DNA sequences in tumor DNA homologous to Ti-plasmid sequences; Aut, phytohormone autotrophy; Ocs, octopine synthesis in tumor; Nos, nopaline synthesis in tumor; Reg, regeneration of shoots.

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4013. Octopine-type crown gall tissue induced on SR1 plants by strain LBA4013 (13).

C58. Nopaline-type crown gall tissue induced on White Bury plants by wild-type strain C58 (11).

Axenic cultures of all crown gall tumor tissues were grown on standard hormone-free medium according to Linsmaier and Skoog (LS-H) (15). Untransformed normal tobacco tissue was obtained from leaf protoplasts of SR1 plants. This SR1 callus was cultured on a hormone-containing medium [naphthaleneacetic acid, 2 mg/liter; kinetin, 0.2 mg/liter (MS)] according to Murashige and Skoog (16).

Plant Protoplast Isolation. Protoplasts from leaves of sterile cultures of SR1 were obtained as described (17). The protoplasts were cultured in K3 medium (18) with a decreased phytohormone concentration (naphthaleneacetic acid, 0.1 mg/liter; kinetin, 0.2 mg/liter) (K3 + H). After isolation the protoplasts were kept in the dark for 24 hr at 20°C and then at moderate light (500 lux) for 48 hr. After this treatment they were used for transformation.

Transformation of Cell Wall Regenerating Protoplasts. Three-day-old protoplasts, cultured in 10-cm Petri dishes containing 10 ml of K3 + H medium (10^5 protoplasts per ml), were mixed with bacteria (5×10^7 /ml) and incubated at room temperature for 32 hr at 500 lux. Then the mixture was centrifuged and the cells floating at the meniscus were sucked off and washed once with K3 + H medium. The cells were then cultured at $\approx 10^4$ cells per ml in K3 + H medium. When the colonies reached a size of ≈ 0.5 mm they were plated on solidified (with 0.4% agar) K3 medium without phytohormones (K3 - H) and with decreased sucrose (0.3 M). Control plates contained untreated cells or cells incubated with avirulent bacteria. During the washing procedure and the culture of the treated cells, carbenicillin was present at 0.5 mg/ml.

Isolation and Culture of Putative Transformants. Within 1–2 months after cell colonies were transferred onto selective medium, putative transformants were isolated on the basis of their continued proliferation in the absence of phytohormones. They appeared as white to green calli against a background of brown clumps with dying cells. The isolated colonies were subcultured for a second passage on selective medium (K3 - H) with 0.2 M sucrose and 0.8% agar. Finally, the calli that survived the selection on K3 - H medium were transferred to LS - H medium for further propagation and characterization.

Isolation of Subclones. Primary putative transformants were cloned via the isolation and culture of protoplasts. The protoplasts were isolated as described for the SR1 protoplast isolation, cultured for one passage on K3 + H medium, and then cultured on K3 + H medium or K3 - H medium supplemented with agar. Finally, the subclones were transferred to MS medium or to LS - H medium.

Opine Assay in Transformants. Samples of about 10 mg of fresh tissue were homogenized and the lysopine dehydrogenase or nopaline dehydrogenase activity in the extracts was tested as described by Otten and Schilperoort (19).

T-DNA Analysis in Transformants. DNA was isolated from transformants, crown gall tumors, and control tissues as described by Chilton *et al.* (20). *EcoRI* digestion, agarose gel electrophoresis, and Southern blot hybridization were carried out as described (8, 9). The Southern blots were hybridized according to the procedure of Thomashow *et al.* (9) with ^{32}P -labeled probes containing the Ti-region of pTi-Ach5. The labeled probes were a kind gift of E. W. Nester (Seattle) and have been described in detail by Thomashow *et al.* (9). The T-DNA analyses were conducted at the Departments of Biochemistry (M. P. Gordon) and Microbiology (E. W. Nester) of the University of Washington (Seattle).

RESULTS

Conditions for Transformation. To achieve transformation of tobacco cells *in vitro*, the protoplasts had to have initiated the regeneration of a new cell wall without having undergone cell division. These conditions were present 3 days after isolation of the protoplasts. The minimal incubation period of these cells with the bacteria was 32 hr. Neither freshly isolated protoplasts nor cells that had completed the cell wall synthesis could be transformed by this treatment. This suggests that, for transformation, the protoplasts must have a condition that is comparable with the condition created by the wound reaction of a wounded plant stem (21). During the incubation, aggregation of protoplast-derived cells occurred, presumably due to attachment of bacteria to the partly synthesized cell wall. Detailed observations during this early transformation period will be described elsewhere.

Selection of Putative Transformants. Selection of hormone autotrophic calli was started by plating the cell suspension that was growing in hormone-containing medium onto solidified medium without hormones. Controls consisting of normal SR1 cells or SR1 cells incubated with avirulent bacteria were treated similarly. After about 4 weeks, proliferating colonies were observed in those selective plates that contained cells treated with virulent bacteria. The cells of such colonies showed a high division rate and a small size, compared with the cells of nonproliferating SR1 cells in control selective plates. The normal SR1 cells present on selective medium were large and mostly plasmolyzed. Because of these differences, presumed transformants were identified and subcultured for prolonged periods on selective medium. During these subcultures, a further distinction could be made between real phytohormone autotrophic colonies and colonies that finally appeared to be hormone auxotrophic and ceased its proliferation on the selective hormone free medium.

To estimate the transformation frequency, the cells left after the washing procedure were counted and plated at $\approx 10^3$ cells per ml in 10 ml of medium. The definite plating efficiency of these washed cells was between 70% and 90% when they were cultured continuously in hormone containing medium. Ten plates (10^4 cells per plate) were analyzed per treatment. The estimated frequencies (Table 1) were based on a total of about 100–200 hormone autotrophic colonies per treatment. From the most efficient experiments, about 100 colonies per plate could be isolated.

The different bacterial strains used for transformation, their Ti-plasmids, and the most relevant characters of the plasmids are also presented in Table 1. The transformation frequency of the different strains was more or less the same except for mutant octopine strain LBA4210, which gave a lower frequency. No differences in transformation frequency were observed between the wild-type octopine strain LBA4001 and the nopaline strain LBA4058 carrying the nopaline Ti-plasmid of strain T37 in the genetic background of LBA4001, which was cured of its octopine Ti-plasmid.

Phenotypes of Transformants. Although different types of bacterial strains were similar in their transformation efficiency, they had induced primary transformants with a wide variation in phenotypes. The characters of representative transformants are presented in Table 2. Transformants were screened for the tumor markers phytohormone autotrophy (Aut), octopine synthesis (Ocs), and nopaline synthesis (Nos) and for regeneration of shoots (Reg). With octopine-type bacteria, transformants were obtained with an Aut⁺, Ocs⁺; Aut⁺, Ocs⁻; or Aut⁻, Ocs⁺ phenotype. This last phenotype was observed in one transformant (SR1-4001-18). Although it was selected as being a hormone autotroph originally, the tissue ceased its proliferation

Table 1. Bacterial strains used in transformation of tobacco protoplasts

Strain	Ti-plasmid	Character (ref.)	Frequency of trans-formation*	Opine in trans-formants
LBA4001	pTi-Ach5	Wild type (13)	10^{-2} – 10^{-3}	Octopine
LBA4011	—	Plasmid cured, avirulent (12)	—	—
LBA4013	pAL105	Derivative of Ach5 (13)	10^{-2} – 10^{-3}	Octopine
LBA4060	pAL108	IS-insertion in <i>Sma</i> I fragment, 10C (8, 12)	10^{-2} – 10^{-4}	Octopine
LBA4210	pAL288	Tn904 insertion in <i>Sma</i> I fragment, 10C; <i>Hpa</i> I, 13; <i>Kpn</i> I, 10 (7, 13)	$<10^{-4}$	Octopine
LBA4229	pAL246	Tn904 insertion in <i>Sma</i> I fragment, 3b; <i>Hpa</i> I, 1; <i>Kpn</i> I, 6 (7)	10^{-2} – 10^{-3}	Octopine
LBA4058	pAL8374	pTi-T37 in Ach5 background (11)	10^{-2} – 10^{-3}	Nopaline
LBA4057	pAL672	oct::nop cointegrate (11)	10^{-2} – 10^{-3}	Octopine + nopaline

* The frequency was calculated by counting the number of hormone autotrophic colonies in relation to the number of cells plated after the incubation.

after two passages on LS – H medium. It was rescued by transfer to MS medium containing phytohormones. After four passages on this medium, the well-growing tissue was rechecked for hormone autotrophy on LS – H medium. Although it failed to grow in the absence of hormones, it had retained the Ocs⁺ character.

Contrary to the crown gall octopine tumors induced by LBA4103 and LBA4001 on tobacco shoots, which did not develop teratomata on the plants nor in tissue culture, about 50% of the *in vitro* transformants did regenerate shoots. Ocs⁺ callus

Table 2. Phenotypes of representative transformants obtained after *in vitro* transformation of tobacco protoplasts by *A. tumefaciens*

Type of bacteria	Transformant line*	Phenotype				Total representatives, no.†
		Aut	Ocs	Nos	Reg	
Octopine	SR1-4013-3	+	+		+	147
	SR1-4001-5	+	+		—	
	SR1-4229-19	+	—		+	
	SR1-4013-28	+	—		—	65
	SR1-4001-18	—	+		—	
Nopaline	SR1-4058-38	+		+	+	89
	SR1-4058-22	+		—	+	26
Cointegrate	SR1-4057-11	+	+	+	+	3
	SR1-4057-4	+	+	+	—	
	SR1-4057-10	+	+	—	+	
	SR1-4057-5	+	+	—	—	8
	SR1-4057-14	+	—	+	+	
	SR1-4057-8	+	—	+	—	8
	SR1-4057-17	+	—	—	+	
	SR1-4057-7	+	—	—	—	4
	SR1-4057-12	—	+	—	+	
	SR1-4057-12	—	+	—	+	1

* The last numbers of the notation indicate individual primary transformants.

† Regeneration of the transformants is not taken into account.

tissues gave Ocs⁺ shoots, whereas Ocs[—] callus tissues gave Ocs[—] shoots.

Transformants with different phenotypes also were obtained by using the nopaline-type strain LBA4058. Although they could be Nos⁺ or Nos[—], they all were Aut⁺ and demonstrated a strong capacity for shoot formation.

Table 2 also presents the phenotypes of transformants obtained with strain LBA4057 (SR1-4057). This strain carries pAL672, a cointegrate plasmid consisting of octopine-type pTi-Ach5 and nopaline-type pTi-C58, in the Ach5 background (11). The cointegrate plasmid was stable and did not dissociate into its component plasmids. Tumor induction on *kalanchoë* stems has shown that this strain induces smooth tumors (typical for nopaline-type strains) in which octopine as well as nopaline was produced, demonstrating that genes of both of the component Ti-plasmids were expressed (13). This strain, with the two markers Ocs and Nos, allowed the study of the behavior of an extra large Ti-plasmid in *in vitro* transformation experiments. From Table 1 it can be seen that no significant difference in transformation frequency existed between this cointegrate strain and the wild-type octopine- or nopaline-type strains.

Contrary to the SR1-4058 transformants, not all SR1-4057 transformants developed shoots (Table 2). In general, all possible combinations of tumor markers were found among the various primary SR1-4057 transformants.

Although primary transformants were isolated as single colonies, it cannot be excluded that they were composed of mixed populations of normal cells and tumor cells or of mixtures of cells resulting from different transformation events. Therefore, the cointegrate transformant SR1-4057-4 (Aut⁺, Ocs⁺, Nos⁺, Reg[—]) was subcloned by protoplast isolation and subculture. The protoplasts were cultured under nonselective conditions to be sure that all types of individual cells could proliferate and form subclones. From this experiment, 105 individual subclones were taken at random from the plates and transferred to selective medium. All subclones continued their proliferation, demonstrating that they were all still Aut⁺. In addition, analysis of opine synthesis showed that they were Ocs⁺ and Nos⁺ without exception. The data demonstrated that the 105 subclones had the same phenotype (Aut⁺, Ocs⁺, Nos⁺) as the primary transformant SR1-4057-4, including the inability to regenerate shoots (Reg[—]).

Results of octopine and nopaline assays with extracts of SR1-4057 transformants representing the different phenotypes are shown in Fig. 1 (lanes 1–6). Extracts of axenic crown galls B6S3 (lane I), C58 (lane II), and normal SR1 callus tissue (lane III) were assayed as controls. No endogenous nopaline (lanes 1a and 1b) or nopaline synthesis (lane 1c) and no endogenous octopine (lanes 1a and 1c) or octopine synthesis (lane 1b) were detected in SR1-4057-7 (Ocs[—], Nos[—]). SR1-4057-8 (channel 2) shows the Ocs[—], Nos⁺ phenotype. This extract contained a high endogenous concentration of nopaline (lanes 2a and 2b) as well as a high nopaline dehydrogenase activity. SR1-4057-9 (Ocs⁺, Nos⁺) (lane 3) did not show detectable endogenous octopine (lanes 3a and 3c) or nopaline (lanes 3a and 3b) levels in the extract, but enzyme activity for both types of opines was apparent (lanes 3b and 3c).

SR1-4057-11 had the same phenotype and, in addition, a high level of endogenous nopaline (lanes 5a and 5b). The Ocs⁺, Nos[—] phenotype was presented by lines SR1-4047-10 and SR1-4057-12; in these transformants, only the synthesis of octopine was demonstrated (lanes 4b and 6b).

T-DNA Analysis. The results of the *in vitro* transformation experiments strongly suggested that the obtained tissues are real transformants and therefore one could expect the presence of T-DNA in these tissues. In order to confirm this, we per-

FIG. 1. Electropherogram showing the results of the assay for the detection of endogenous and newly synthesized opines in extracts of SR1-4057 transformants. Lanes: a, reaction mixture at $t = 0$, endogenous octopine or nopaline; b, $t = 60$ min, octopine synthesis and endogenous nopaline; c, $t = 60$ min, nopaline synthesis and endogenous octopine; I, B6S3 crown gall callus; II, C58 crown gall callus; III, normal SR1 tissue; 1, SR1-4057-7; 2, SR1-4057-8; 3, SR1-4057-9; 4, SR1-4057-10; 5, SR1-4057-11; 6, SR1-4057-12.

formed hybridizations between cloned Ti-region fragments and DNA from the *in vitro* transformed tissue lines SR1-4013-3 and SR1-4001-5. The Ti-region clones used—pNW31C-8, 29-1 and pNW31C-2, 19-1—were identical to those used by Thomashow *et al.* (9) when they analyzed T-DNA sequences in four subcloned crown gall tissues induced *in vivo*. *Eco*RI Ti-plasmid fragments 1, 2, 13, 7, and 22/24 hybridized with the cloned Ti-region DNA (Fig. 2 *Left*, lane 4). The relative positions of these *Eco*RI fragments on a restriction endonuclease map and the extent of the *Bam*HI clones used are shown in Fig. 2 *Right*. In the reconstruction experiment, one molecule of Ti-plasmid DNA was mixed per diploid genome tobacco DNA (one-copy reconstruction).

We did not detect hybridization with tobacco DNA isolated from SR1 tissue. However, DNA from SR1-4013-3 and from SR1-4001-5 did show hybridization. In both tissues, bands comigrating with *Eco*RI fragment 7 were present. This indicates that *Eco*RI fragment 7 is part of a noninterrupted linear piece of DNA present in the transformed plant cells, from which it

could be recovered after restriction endonuclease treatment. The intensities of the bands that result from hybridizations with Ti-plasmid *Eco*RI fragment 7 and the fragments in transformant DNA that comigrated with the *Eco*RI fragment 7 could be directly compared. The intensities were similar, suggesting the presence of about 1 copy of DNA per diploid tobacco genome. Also, other fragments from transformant DNA hybridized with the Ti-region clones. These fragments did not comigrate with Ti-plasmid fragments in a reconstruction experiment and did not comigrate with each other (lanes 1 and 3).

DISCUSSION

The *in vitro* transformation by *A. tumefaciens* of cell-wall regenerating plant protoplasts offers a number of advantages compared with crown gall induction *in vivo*. Millions of individual cells can be accessed by the bacteria, and thousands of independent tumor tissues can be isolated, of which in principle each is obtained from the proliferation of a single transformed cell. Starting with protoplasts, a homogeneous population (e.g., leaf mesophyll cells) can be used, whereas a crown gall tumor that arises on a plant stem upon infection might be composed of cells that originated from different plant tissues (e.g., cortex, cambium, pith). For practical reasons, the number of different tumors induced *in vivo* by a certain bacterial strain will be relatively low.

A prerequisite for an efficient *in vitro* transformation system is the availability of an effective selection system. The hormone-free selective culture medium used in our studies allowed transformants to grow but prevented survival of control SR1 cells. The data show that the frequency of cells derived from SR1 leaf protoplasts that switched from hormone auxotrophy to hormone autotrophy is negligible.

The homogeneous composition of transformants was demonstrated by the subcloning experiment performed with the cointegrate type transformant SR1-4057-4 (*Aut*⁺, *Ocs*⁺, *Nos*⁺). All subclones isolated under nonselective conditions had the same phenotype as the primary tissue. In general, the phenotypes of the various transformants remained stable during prolonged periods in tissue culture. Exceptions to this observation

FIG. 2. (*Left*) Autoradiograph of Southern blots obtained with *Eco*RI-digested DNA from *in vitro* transformants hybridized to ³²P-labeled probes containing the Ti-region of pTi-Ach5. The blots were hybridized with a mixture of ³²P-labeled pNW31C-8,29-1 and pNW31C-2,19-1 (9). Lanes: 1, SR1-4013-3 DNA; 2, normal SR1 callus DNA; 3, SR1-4001-5 DNA; 4, one-copy Ti-plasmid per diploid tobacco genome reconstruction. The positions of *Eco*RI fragments 1, 2, 7, 13, and 22/24 are indicated. (*Right*) Physical map of a portion of octopine Ti-plasmids and a representation of the cloned Ti-plasmid sequences used in this study. [Redrawn from Thomashow *et al.* (9).]

are two transformants (SR1-4001-18 and SR1-4057-12) that, although isolated on hormone-free medium, had lost their hormone autotrophic phenotype against the selective pressure. That this loss of autotrophy was not due to complete reversion to the normal phenotype is shown by the retention of octopine synthesis. It is not clear yet whether the observed phenotypic differences are related to differences in the T-DNA content of these transformants.

It can be concluded that the results obtained from our initial T-DNA analysis of the transformants SR1-4013-3 and SR1-4001-5 seem to be fully comparable to the results of T-DNA analysis of cloned crown gall tumors obtained by Tomashow *et al.* (9), such as those with the cloned crown gall tumor A6S/2. Part of the octopine Ti-plasmid is present in the transformant DNA, which does not differ to a large extent from T-DNA in crown gall tumor tissue. This was concluded from the fact that a fragment comigrating with the reconstruction experiment fragment 7 was present in the transformants, whereas boundary fragments were present that did not comigrate with the reconstruction experiment fragments 2 and 24. The boundary fragments of both the transformants also did not comigrate with each other. This is comparable to the observed variability of T-DNA oligonucleotide patterns in different cloned crown gall tumor tissues (9).

That shoots that arose on transformed calli consisted of transformed tissue has been shown by the detection of opine synthesis and by obtaining transformed subclones from protoplasts isolated from these shoots (22). In this context it is relevant to mention that transformed shoots of both nopaline and octopine type flowered and set seed. Analysis of F₁ seedlings has detected nopaline in a number of the seedlings (22). With regard to the possible application of transferable Ti-plasmid DNA for introducing desirable DNA into higher plants, the *in vitro* transformation system described in this paper offers a number of favorable properties.

(i) Millions of single cells can be exposed to the transforming bacteria from which individual transformants can be selected with relatively high frequency.

(ii) Regeneration into shoots occurs in an early stage of tissue culture, avoiding as much as possible genomic changes due to tissue culture conditions.

(iii) Integrated T-DNA is maintained and at least partly expressed (opine synthesis) during morphogenesis of transformants into flowering plants.

(iv) T-DNA can be transmitted to the F₁ progeny of transformed plants in which it is still expressed (nopaline synthesis).

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