An *Agrobacterium*-transformed cell culture from the monocot *Asparagus officinalis*

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Cultured stem fragments from the monocotyledonous plant *Asparagus officinalis* infected by the oncogenic bacterium *Agrobacterium tumefaciens* developed tumorous proliferations. This tissue was propagated *in vitro* on hormone-free culture medium. The T-DNA-encoded markers nopaline and agrocinopine were unambiguously detected in these tissues. The data demonstrate that stable T-DNA transfer as well as expression of T-DNA genes is possible in at least some monocotyledonous plants. This opens new possibilities for plant genetic engineering using the Ti plasmid as a gene vector.

Key words: asparagus/*Asparagus officinalis*/*Agrobacterium tumefaciens*/monocotyledons/transformation/opines/nopaline/agrocinopine

Introduction

The monocotyledonous plants, including the most important crops, are widely considered insensitive to *Agrobacterium* infection. It is generally thought that the Ti plasmid cannot be used as a gene vector for these crops and that alternative strategies have to be defined. However, we have found that crown gall tumor formation is dependent on the age and physiological state of the plants even in some sensitive dicotyledonous plants, and is also affected by the infection procedure. It is likely that most classical work on the host range of *Agrobacterium* did not consider these factors. Therefore, we re-examined the effect of *Agrobacterium* infection in one species, the monocot *Asparagus officinalis*, a member of the family *Liliaceae*. Asparagus was used as a model plant because the requirements for both *in vitro* culture and for plant regeneration from protoplasts are known in detail for this species (Bui-Dang-Ha and Mackenzie, 1973). Moreover, under similar experimental conditions callus cultures could readily be established from most organs (roots, stems and cladodes). To achieve an easy control of the environment of the infected tissue, cultured organs of asparagus were infected *in vitro* with *A. tumefaciens* C58.

As will be shown, overgrowth can be induced on asparagus stem segments by infection with *Agrobacterium* strains. Such tumors are able to grow on hormone-free medium. In addition, the synthesis of opines is an easily detectable biochemical marker for T-DNA-transformed plant tissue (for review, see Tempé and Goldmann, 1982). For example, nopaline crown galls contain the opines nopaline and agrocinopine. Nopaline is synthesized from arginine, α -ketoglutaric acid and NADPH (Goldmann, 1977; Sutton *et al.*, 1978), and agrocinopine is a phosphorylated oligosac-

charide (Ellis and Murphy, 1981). Both substances were found in the transformed asparagus tissue. These data demonstrate that T-DNA transfer and expression are not limited to dicots.

Results

Infection of Asparagus stem segments

Stem segments of *A. officinalis* were cultured *in vitro* on hormone-free medium and infected with *A. tumefaciens* strain C58 (Hamilton and Fall, 1971). This strain harbors the wild-type nopaline Ti plasmid pTiC58. Uninfected stem fragments or fragments infected by the non-oncogenic Ti plasmid-free strain C58C1 (Van Larebeke *et al.*, 1974) showed no proliferation after 2 months. Proliferation of the infected wound surface became visible after 2 weeks on stem segments infected with strain C58. Large overgrowths were observed 1 month after inoculation (Figure 1A). In some instances, no proliferation was observed at the site of infection itself but proliferating tissue became visible a few millimetres below the infected wound after ~4 weeks. Such tumors arising at sites on the plant distant from the point of inoculation have been previously observed on dicotyledonous plants (for review, see Turgeon, 1982). The formation of tumorous proliferation on asparagus is quite reproducible but it is essential to involve a large number of segments in the infection process since only two to three out of 20 infected segments routinely reacted with clearly detectable proliferations. Most stem fragments that reacted positively were taken from the youngest (apical) part of the spears. This makes us confident that a higher number of tumors can be obtained by careful choice of the plant material.

Fig. 1. (A) Tumorous outgrowth on an asparagus stem segment 1 month after infection by *A. tumefaciens* strain C58. (B) Nopaline-containing asparagus tissue, obtained by infecting stem fragments with *A. tumefaciens* strain C58, cultured on hormone-free medium.

Fig. 2. (A) Detection of nopaline by paper electrophoresis at pH 6.5. **Lane 1**, synthetic nopaline; **lane 2**, extract from tumor tissue induced by *A. tumefaciens* strain C58 on asparagus and cultured on hormone-free medium; **lane 3**, extract from tissue from the same tumor cultured on hormone-containing medium; **lane 4**, extract from untransformed asparagus tissue. N, nopaline. (B) Confirmation of the identity of nopaline by electrophoresis at pH 1.8. **Lane 1**, synthetic nopaline; **lane 2**, nopaline obtained by paper electrophoresis at pH 6.5 from tumor tissue induced by *A. tumefaciens* C58 on asparagus; **lane 3**, synthetic nopaline after paper electrophoresis at pH 6.5; **lane 4**, nopaline obtained from paper electrophoresis at pH 6.5 from a tobacco crown gall tumor. N, nopaline; PN, pyronopaline (formed by slow spontaneous conversion of nopaline). (C) Confirmation of the identity of nopaline by conversion into pyronopaline and electrophoretic separation at pH 1.8. **Lane 1**, pyronopaline obtained by heating synthetic nopaline to 100°C in 2 M acetic acid for 1 h; **lane 2**, synthetic nopaline containing pyronopaline by slow spontaneous conversion; **lane 3**, nopaline from tumor tissue induced by *A. tumefaciens* C58 on asparagus; **lane 4**, the same after heating to 100°C in 2 M acetic acid for 1 h. N, nopaline; PN, pyronopaline. (D) Confirmation of the identity of nopaline by its Ti plasmid-mediated catabolism. **Lane 1**, synthetic nopaline; **lane 2**, nopaline from tumor tissue induced by *A. tumefaciens* C58 on asparagus; **lane 3**, the same after incubation for 24 h with *A. tumefaciens* strain C58C1 which does not degrade nopaline; **lane 4**, the same after incubation for 24 h with the nopaline-catabolizing *Agrobacterium* strain C58. N, nopaline; PN, pyronopaline.

In vitro culture of the tumor tissue and opine detection

An *in vitro* culture was initiated from one of the asparagus tumors. The proliferating tissue was separated from the stem and placed on medium containing 1 mg/l of both benzylaminopurine and naphthalene acetic acid and also on hormone-free culture medium. On hormone-free medium, the tissue started developing into a compact, slow-growing, white callus after 4–5 weeks (Figure 1B).

To confirm the transformation of the tissue it was analyzed for T-DNA-specified opines, nopaline and agrocinopine. A substance with the same electrophoretic mobility as nopaline both at pH 6.5 (Figure 2A) and 1.8 (Figure 2B) was detected in this tissue and was absent from untransformed asparagus callus. This product was confirmed to be nopaline since it is converted into pyronopaline (Tempé, 1983) by heating at low pH (Figure 2C) and since it could be labelled with ^3H by feeding [^3H]arginine to the tumor culture (data not shown). A specific biological identification test for nopaline was also used (Petit *et al.*, 1970; Bomhoff *et al.*, 1976) to show that the putative nopaline was specifically degraded by *Agrobacterium* carrying the Ti-linked nopaline catabolism genes (Figure 2D). Furthermore, after incubating in $\text{H}_3^{32}\text{PO}_4$ -containing medium, a compound with the same electrophoretic mobility as agrocinopine could be detected in the transformed asparagus culture. This substance was not found in untransformed *Asparagus* cells (data not shown).

It proved essential to grow the transformed tumor tissue on hormone-free media to select for the transformed cells. Nopaline and agrocinopine were not present in the culture grown on hormone-containing medium (Figure 2A).

Discussion

Morphological evidence exists that a number of monocots, all belonging to the orders *Liliales* and *Arales* produce swellings when infected with virulent agrobacteria (De Cleene and De Ley, 1976, 1984; De Cleene, 1984). However, formal proof that these swellings resulted from T-DNA-induced cell proliferation was not given. Tissue cultures of transformed cell lines were not available and T-DNA-specific markers such as opines were not identified. Recently, the detection of opines in such swellings on *Narcissus* has been reported (Hooykaas *et al.*, 1984).

However, the presence of nopaline in swellings could be due to a transient expression of the nopaline synthase and would not be indicative for stable T-DNA transformation. Thus, it was essential to demonstrate the presence of opines in established cell cultures derived from the inoculated plant tissues. A further problem was the fact that we found compounds interfering with the standard nopaline detection, using electrophoresis at pH 1.8 and phenanthrenequinone staining (Otten and Schilperoort, 1978) both in untransformed tissue of *Asparagus* and in some other *Liliaceae*. Therefore, we took particular precautions to demonstrate the presence of nopaline. The asparagus cell cultures reported here retained constant levels of nopaline and agrocinopine comparable with the concentration found in dicot crown gall cultures. We conclude that T-DNA transfer to at least some of the monocots is possible, and that the opine-specific T-DNA promoters are active in these cells with an efficiency similar to that found in dicots. Also genes for hormone-independent growth are most likely expressed since transfer

of T-DNA to asparagus cells leads to proliferation on hormone-free culture medium, which does not occur with untransformed tissue explants. Whether this finding can be used as a general method to select transformed monocot cell lines is presently under investigation.

The development of non-oncogenic Ti plasmid vectors with chimeric antibiotic resistance genes as dominant selectable markers for plant cells allowed the routine use of the system to transform and regenerate dicotyledonous plants (De Block *et al.*, 1984; Horsch *et al.*, 1984; Zambryski *et al.*, 1984). The present data give us confidence that the same vectors will be of crucial importance to obtain engineered asparagus plants, hence, further validating the use of asparagus as a model system. Finally, we hope to stimulate attempts for the use of *A. tumefaciens* as a gene delivery system for economically important *Gramineae*.

Materials and methods

Inoculation of *Asparagus*

A. officinalis cv. 'Roem van Brunswijk' plants were obtained from seeds. The plants were cultured in vermiculite and watered daily with mineral fertilizer solution (Bayfolan, Bayer, 0.3 ml/l) supplemented with the micro-elements of the Murashige and Skoog (MS) medium (Murashige and Skoog, 1962).

Young spears, 20 cm long, from 10-month-old seedlings were surface-sterilized with (i) 70% ethanol for 2 min, (ii) 0.5% HgCl₂ for 5 min, and (iii) 10% commercial bleach (15° Chl) and 1% laboratory detergent (Decontamin®) for 30 min, followed by five rinses in sterile tap water.

The sterile spears, ~2–3 mm in diameter, were cut into 3 cm long segments and incubated vertically in jars containing half-strength hormone-free MS medium, solidified with 0.7% Difco Bacto agar. *A. tumefaciens* C58 was cultured for 48 h on an LB plate (Miller, 1972) and applied directly onto the upper end of the segments with a spatula.

Tissue culture

Asparagus calli were induced and cultured on medium containing the mineral components, iron chelate, inositol, thiamine and sucrose of the Linsmaier and Skoog medium (Linsmaier and Skoog, 1965), supplemented with 1 mg/l benzylaminopurine (BAP), 1 mg/l naphthalene acetic acid (NAA) and 200 mg/l glutamine. To culture asparagus tissue after infection by *Agrobacterium*, 500 mg/l of cefotaximum (Claforan®, Hoechst) was added to the medium. The same culture medium without BAP and NAA was used for the selection of hormone-independent cell cultures.

Opine detection

Tissue samples of ~2–3 mm in diameter were used for the detection of nopaline. The samples were incubated overnight in 20 µl MS medium supplemented with 10 mM arginine and 10 mM α-ketoglutaric acid and adjusted to pH 5.7. Then, the tissue was ground in the incubation medium with a plastic rod, and the mixture was spun in an Eppendorf centrifuge for 2 min.

5 µl samples of the supernatant were applied to Whatman 3MM paper 15 cm long for electrophoretic separation of nopaline. After electrophoresis towards the anode for 90 min at 350 V using pH 6.5 buffer (5% pyridine and 0.25% acetic acid in H₂O) the papers were dried and phenanthrenequinone-positive spots were visualized as described by Otten and Schilperoort (1978). Pure synthetic nopaline was used as a control. To confirm the identity of the putative nopaline, strips (2 cm wide) were cut after electrophoresis from an unstained paper at the position where nopaline migrates. These strips were placed in a plastic micro test tube, with a small hole pierced near its bottom, which was suspended into a larger Eppendorf tube. The paper was saturated with 20 µl of H₂O and both tubes were spun for 1 min in an Eppendorf centrifuge. This procedure was repeated five times, and the liquid collected was concentrated under vacuum. These samples were used for paper electrophoresis at pH 1.8 and the phenanthrenequinone-positive spots were detected as described by Otten and Schilperoort (1978).

The incorporation of [³H]arginine into nopaline was detected essentially as described (Messens *et al.*, in preparation), but electrophoresis at pH 1.8 was used instead of thin-layer chromatography.

The assay system for detecting agropinopine synthase activity will be published later (Messens *et al.*, in preparation). It entails incubating tumor tissue in medium containing H₃³²PO₄, electrophoresing tumor extracts and identifying radioactive agropinopine on the electrophoretogram by autoradiography.

Conversion of nopaline into pyronopaline

Samples (10 µl) of the putative nopaline, obtained by elution from an elec-

trophoretogram run at pH 6.5 were mixed with an equal volume of 4 M acetic acid and treated at 100°C for 1 h. The conversion of nopaline into pyronopaline, a compound migrating slightly slower in an electrophoresis at pH 1.8, is detected by phenanthrenequinone staining.

Ti plasmid-mediated catabolism of nopaline

Overnight cultures of *Agrobacterium* strains C58 and C58C1 were grown in LB (Miller, 1972) medium. The cultures were washed twice in a medium consisting of twice concentrated mineral components of *Agrobacterium* minimal medium (Bomhoff *et al.*, 1976) and resuspended in half of the initial volume in this medium. Fractions of 10 µl of these bacterial cultures were mixed with an equal volume of the putative nopaline, eluted from an electrophoretogram run at pH 6.5. After incubation for 24 h at 28°C the presence of nopaline was tested; the sample was centrifuged for 2 min and the supernatant was submitted to electrophoresis at pH 1.8 and phenanthrenequinone staining.

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