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Growth Requirements of *Arabidopsis thaliana* Crown Galls

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Abstract. Crown galls induced on *Arabidopsis thaliana* plants by octopine or nopaline strains of *Agrobacterium tumefaciens* were grown *in vitro* on different media. Dark growth of all tumor tissues was strictly hormone-dependent. In contrast, hormonal autonomy was observed in the light where crown gall calli readily differentiated into teratomas and (sometimes fertile) plants. Differentiating tissues always grew more vigorously than subtended calli. The growth of transformed calli was stimulated by vitamins and partly inhibited by growth regulators in concentrations used for the maintenance of untransformed calli. Crown gall calli, teratomas and sometimes regenerated plants were shown to express lysopine or nopaline dehydrogenase activities.

Agrobacterium tumefaciens produces tumor transformation of plant cells by integrating a part of the bacterial Ti plasmid, so called transferred DNA or T-DNA, into the plant genome (Chilton *et al.* 1977). Ti plasmid is used as the vector for the transfer of exogenous DNA into the plant genome (Leemans *et al.* 1981, Matzke and Chilton 1981). There are two main types of Ti plasmids. The octopine Ti plasmids bring into the plant genotype the information for the synthesis of the octopine group of opines and code for their utilization in the bacteria. The second group of Ti plasmids is the nopaline group. Crown galls produced by the octopine and nopaline Ti plasmids differ in their morphology and regeneration ability.

Aerts *et al.* (1979) have found that, *in vitro*, crown galls of *A. thaliana* readily differentiate into teratomas and plants. Here we studied the differentiation of *A. thaliana* crown galls cultivated *in vitro* in different conditions with the perspective of establishing a system for the study of the genetic transmission of T-DNA.

MATERIAL AND METHODS

The crucifer plant, *Arabidopsis thaliana* Heyn, race Dijon was used throughout the experiments. The following strains of *A. tumefaciens* were utilized: octopine strains Ach5, B6-806 and 374 00, nopaline strains C58 and T37. Seeds of *A. thaliana* were surface sterilized in the mixture (1 : 1) of 3%

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hydrogen peroxide and ethanol for 5 min, than in 5% sodium hypochlorite for other 5 min and thoroughly washed with sterile water. Seeds were sown in 1% agar medium with mineral salts of B5 medium (GAMBORG *et al.* 1968) and 2% glucose (minimal medium). The plants were grown for about three weeks (25 °C, 3 000 lx, 12 h photoperiod). Desired suspension of *A. tumefaciens*, incubated for 20 h at 28 °C in the liquid medium of LANGLEY and KADO (1972) (approximately 10^9 cells.ml^{-1}) was applied to the middle of the leaf rosette by puncturing with a glass capillary needle.

After 2—3 weeks, the crown gall tumors measuring about 1 mm were isolated and grown on minimal medium, to which the following antibiotics were added: (μg ml^{-1}) carbenicilin 100, vancomycin 100, tetracyclin 2. Tumor tissues cultivated in the light rapidly proliferated and were subcultivated in the presence of antibiotics for three months before media without antibiotics were used. The lysopine and nopaline dehydrogenase activities were examined by the method after OTTEN and SCHILPEROORT (1978).

For the quantitative evaluation of the growth *in vitro*, crown gall tumors were cultured in the continuous light (5 000 lx) or in the dark, at 25 °C. Four types of media were tested: a) minimal medium (see above), b) B5 medium containing vitamins and inositol according to the original prescription (vitamin medium), c) vitamin medium supplemented with 2,4-D and kinetin 1.0 and 0.2 mg l^{-1} according to XUAN and MENCZEL (1980), and d) PG_0 medium of NEGRUTIU *et al.* (1975) (*i.e.* without growth regulators). For other purposes, also the media PG_2 (developed for maintenance of calli) and PG_1 (for induction of callogenesis) were used. In all cases, the cultures were established with about 10 mg pieces of crown gall calli, which were put into vials with 6 ml of media. Four weeks later, fresh mass of 30 calli was evaluated in each experiment.

RESULTS

One to two weeks after inoculation of plants, undifferentiated crown gall tumors appeared at 20—50% of the sites inoculated. It is generally believed that the excessive production of growth regulators by the transformed dividing cell population also induces the cell division in the neighbouring untransformed cells. Therefore we assume that the original tumor is a mixture of proliferating tumorous and non-tumorous cells. Several tens of crown galls of independent origin were derived from each inciting *A. tumefaciens* strain. In the light, the original undifferentiated crown gall tumors were gradually overgrown by single leaves, then buds, teratomas and plants. Teratoma and fully differentiated plants often occurred together with undifferentiated callus. The character of growth differed with the subcultivation interval. When the subcultivation interval was very short (1—2 weeks), it was easily possible to isolate stable undifferentiated subclones, subclones with teratoma growth and fully differentiated plants. When the undifferentiated tissues were detached from differentiated ones, they often stopped their growth and died. The character of growth of individual subclones becomes stabilized within a few (usually four) subcultivations. If the subcultivation interval was long (four weeks), the differentiated tissues tended to over-

Abbreviation used: 2,4-D = 2,4-dichlorphenoxyacetic acid.

grow the undifferentiated ones and mostly plants and teratomas were recovered. There were, however, considerable differences between octopine and nopaline crown galls.

Teratomas and differentiated tumors of both the octopine and nopaline types, derived directly from the tumors, incited by *Agrobacterium* on the plants, never form roots. Despite that, the plants flowered and some of them were partly fertile.

Octopine Crown Galls

In octopine crown galls, individual leaves appeared very soon after detachment of the tumor from the original plant. The crown gall calli were gradually overgrown by minute modified leaves (teratomas) and differentiated plants. The tissue fraction of the original crown gall which gave rise to teratomas and whole plants was larger than the fraction of the undifferentiated tissue. The differentiated tissues grew much more rapidly than the undifferentiated ones. The undifferentiated tumor calli always showed lysopine dehydrogenase activity. In teratomas and plants, lysopine dehydrogenase activity was found in some, usually older tissues.

Nopaline Crown Galls

After detachment of the nopaline crown gall tumors from plants and transfer to the agar medium, differentiated organs appeared much less frequently than in octopine tumors. The tissue fraction which gave rise to the teratomas and whole differentiated plants was smaller than the fraction of undifferentiated crown gall calli. A majority of the subcultures grew as undifferentiated tissues. The teratomas could be easily separated from fully differentiated plants and they retained permanently their teratomous type of growth. They consisted of dense clusters of modified, pulpy leaves, from which sometimes grew a pulpy flower stem. The teratomas, like the undifferentiated tumors exhibited nopaline dehydrogenase activity. In fully differentiated flowering plants the nopaline dehydrogenase activity was detected only in some cases.

In the long term cultures (over six months) the undifferentiated transformed calli of the nopaline type tended to revert to teratomas; dense clusters of modified leaves appeared on the whole surface of the callus. The appearing teratomas were quite stable in their type of growth; the different clones of teratomas thus obtained represent a whole scale of transitions between the undifferentiated tumor tissue and the regenerated plant. Some of them have now been in culture for two years.

Quantitative Evaluation of the Growth of Crown Gall Calli on Different Media

Crown galls can be grown permanently on culture media without growth regulators. The autonomy for the growth regulators is often connected with the autonomy for vitamins. Not only growth regulators, but also vitamins were omitted from the minimal medium.

As representatives of the octopine crown galls, five crown gall tumors induced by the *A. tumefaciens* strain B6-806 were chosen. In the nopaline group, five crown gall tumors induced by the strain C58 were used. All of them exhibited lysopine or nopaline dehydrogenase activity. Crown galls were grown on four different media given in Methods. Calli derived from

the leaves of the untransformed *Arabidopsis* plants and maintained for a long time on the B5 medium with growth regulators and vitamins were used as controls. Before the establishment of the experiment, crown galls were cultivated in two-week subcultivations on the minimal medium with continuous light. In all variants, the cultures were grown in both continuous

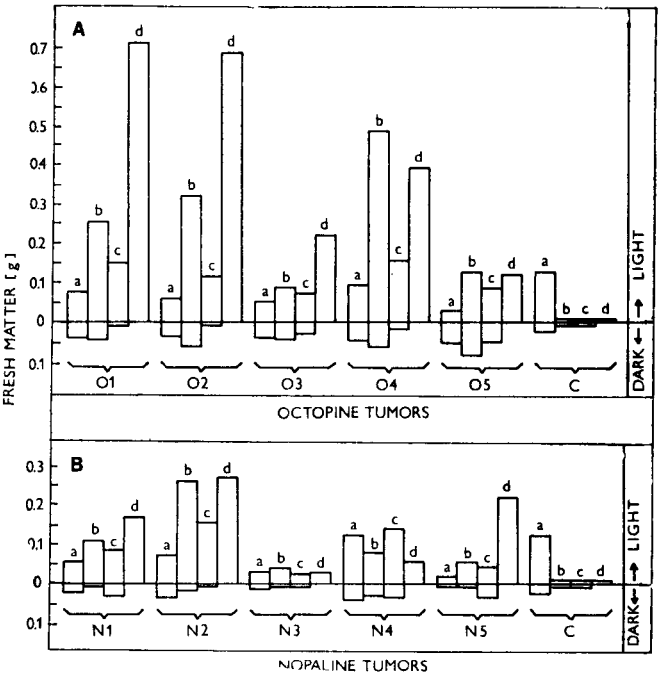

Fig. 1. Evaluation of the fresh matter of crown gall tumors cultured on different media in the light and in the dark. — a = minimal medium; b = vitamin medium; c = medium with growth regulators and vitamins; d = PG_0 medium. — 01—05 = individual octopine crown gall tumors of independent origin; N1—N5 = individual nopaline crown gall clones; C = control untransformed callus clone.

light and the dark. In the dark, the tumors soon became brown and invariably died. An exception were tumors cultivated in the dark on the media with both growth regulators and vitamins. The intensities of growth measured as the fresh matter increase during four weeks of cultivation are shown in Fig. 1. Clones 4N and 5N in the nopaline group became teratomous during cultivation.

The figure shows considerable differences in the growth intensity of individual tumors. The growth of different tumor clones was influenced by the composition of the media in a similar way. The most intensely growing octopine tumors showed larger proliferation capacity than the most intensely growing nopaline tumors. The growth of tumors was highest on the media supplemented with vitamins, then followed the minimal medium. The medium with growth regulators was the least suitable for tumor growth. There were also differences in the appearance of the crown gall tumors

cultivated on different media. The crown gall tumors cultivated on the minimal and vitamin media were compact and hard and they consisted of 1—3 mm long large globules. They were of a green colour of various tints. The tumors grown on media with growth substances were yellowish and consisted of small globules with diameter 1—3 mm. These calli were of soft consistency resembling untransformed calli, nevertheless, even after several subcultivations on the medium with growth regulators the crown gall tumors still exhibited lysopine and nopaline dehydrogenase activities. The appearance of teratomas on the medium with growth regulators did not change during four weeks and no induction of callus was observed, but callogenesis was induced on PG_1 medium.

The fourth columns on the histograms show the growth of crown gall calli on the PG_0 medium. Some authors use B5 medium for the culture of *Arabidopsis* (GLEBA and HOFFMANN 1978) while others prefer PG media (AERTS *et al.* 1979). In our experiments, the growth of crown gall tumors was higher on PG_0 than on the B5 medium.

DISCUSSION

We found that *Arabidopsis thaliana* crown galls are capable of hormonally independent growth only in the light. *A. thaliana* crown galls fail to grow also in low illuminance and most probably some processes related to photosynthesis are involved.

Concerning the detection of opines or opine synthase activities in different states of differentiation, we come to a somewhat different conclusion than AERTS *et al.* (1979). They never found octopine or nopaline in fully differentiated plants but we found lysopine dehydrogenase and nopaline dehydrogenase activities in a part of them. Both activities were expressed only in old plants. We checked lysopine and nopaline dehydrogenase activities repeatedly. The first checking of the plants was done usually at the beginning of flowering and the second near the end of the plant life. In the first checking, the assay gave always negative results while in the second altogether 25 out of 51 repeatedly checked plants were positive. It is very probable that only those plants which were positive in the repeated test were transformed and the others were derived from untransformed tissue.

When calli were derived on PG_1 from the differentiated plants and then maintained either on PG_0 in the light or on PG_2 in the dark, about half of the callus clones (derived from crown galls incited by *A. tumefaciens* B6-806) showed lysopine dehydrogenase activity. The same was true of calli derived from plants originated from crown gall tumors incited by *A. tumefaciens* C58 cultured on PG_0; 9 calli showed nopaline dehydrogenase activity and 9 did not. All 18 calli, cultivated in PG_2, however, did not show any detectable nopaline dehydrogenase activity. This demonstrates that regardless of the differentiation state and unlike in tobacco, opine-synthesizing activities are not always present in the transformed *Arabidopsis* tissues.

WILLMITZER *et al.* (1982) determined the T-DNA genes in tobacco octopine crown gall, responsible for the suppression of the shoot and root differentiation. In *Arabidopsis*, the shoot differentiation inhibiting genes are not fully suppressed. It demonstrates how strongly the expression of genes, located on T-DNA is modified by the plant genotype. Up to date, most of the results

were obtained on tobacco and the variability of the expression of the same T-DNA sequence in different plant genotypes is not yet well studied; comparative studies of this kind on the transcriptional level will be of great interest.

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