

ON THE INABILITY OF *AGROBACTERIUM TUMEFACIENS* Ti-PLASMID TO TRANSFORM POLLEN CELLS OF *PETUNIA HYBRIDA*

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SUMMARY

The interaction of crown gall provoking *Agrobacterium tumefaciens* strains and Ti plasmids isolated from them, with pollen grains from *Petunia hybrida* as well as other haploid cells from this plant have been investigated. Octopine and nopaline synthesis were used as indication of transformation. Tumours could be induced in all ploidy levels (2n, 3n, 4n) of vegetative *P. hybrida* tissue including haploid (n), but not in pollen grains.

1. INTRODUCTION

It has been reported that *Petunia hybrida* pollen can take up bacterial or other DNA, and that this DNA can effect the phenotype of plants produced when the treated pollen is used for pollination (HESS et al. 1974, 1976). Furthermore, it has been claimed that lac transducing phages have genetic effects in *Petunia* when phage-treated pollen is used in fertilization (HESS 1978). Although some of these results on DNA-mediated transformation of *Petunia* have met with criticism (e.g. LURQUIN 1977), it was thought worthwhile to consider the crown gall Ti-plasmid as a transforming agent for pollen, since, if new genetic information can be introduced into pollen prior to fertilization, it may be possible to incorporate the new and perhaps desirable information including incompatibility factors into the zygote during fertilization. The extrachromosomal DNA plasmid of *Agrobacterium tumefaciens* is transferred from bacterial cells and stably maintained and expressed in dicotyledonous plant stem and leaf cells, including *Petunia* (SCHELL et al. 1979). On this basis, taken together with the reports of HESS et al. on pollen transformation by DNA, one would expect a facile transfer to *Petunia hybrida* pollen and subsequent transformation of the plant cell by the Ti-plasmid. We have therefore studied and report here on the interaction between *Agrobacterium tumefaciens*, Ti-plasmid and *Petunia hybrida* pollen and other *P. hybrida* haploid cells.

2. MATERIALS AND METHODS

Agrobacterium tumefaciens strains C58 (nopaline-producing) and Ach5 (octopine-producing) were obtained from Prof. Dr. J. Schell and Prof. Dr. J. van

Montagu of the Laboratory of Genetics, Gent University, Belgium. The Ti-plasmid was isolated by a method similar to that described by LIN & KADO (1978). The *Petunia hybrida* mutants used are described by LINSKENS & STRAUB (1973). *Petunia hybrida* was infected either by removing a slice from the stem of a whole plant and adding to the cut surface of the stem a suspension of *A. tumefaciens*, or, for quantitative work, an *in vitro* test was devised, similar to that described for potato discs by AREND & HEBERLEIN (1977). For the latter, 3.5 cm long *P. hybrida* stem segments were cultured, partly immersed in MURASHIGUE & SKOOG (1962) medium. Tumour induction was scored after 7 days. Pollen was incubated in 10% sucrose - 0.01% H_3BO_3 medium with or without *A. tumefaciens*, Ti-plasmid and/or the carcinogen 4-nitroquinoline-1-oxide. Treated pollen was incubated on the agar-containing medium of MURASHIGE & SKOOG (1962) or in the liquid medium of SANGWAN & NORREEL (1975), both with and without added hormones, to test for any induced tumours. Growth without hormone would indicate transformation by the Ti-plasmid. Treated W43 pollen was used to pollinate W166H flowers and the resulting seeds were collected. These were used to produce young seedlings, 4 weeks old, which were then examined for octopine and nopaline; after extraction (SCHRAUWEN & LINSKENS 1974) and H.P.L.C. purification the amino acids have been determined by biacetyl-naphthol reagent (PETIT & TEMPÉ 1978). A positive result in this case would indicate transformation by the Ti-plasmid.

Pollen Treatments: Prior to its use in pollinating *P. hybrida* W166H flowers or to culture in solution or on agar medium, pollen from *P. hybrida* W43 was treated with *A. tumefaciens* (ACh5 or C58) or Ti-plasmid. At a pollen concentration of 7 mg/ml, and at 28 °C, incubation mixtures also contained 10% sucrose-0.01% H_3BO_3 (pH 5.2) and *A. tumefaciens* (48 hour culture containing 10^6 – 10^7 bacteria) or several μ g of Ti-plasmid. Pollen was first treated with UV light (JACKSON & LINSKENS 1978), then hydrated for 1 hour at 100% humidity. Incubation times were varied from a few seconds to 14 hours, mostly 3 hours, some shaken, others not shaken. UV light was used to induce DNA repair, since it is known that mature *P. hybrida* pollen has no replicative DNA synthesis, and it is possible that DNA synthesis is necessary for successful crown gall infection. In some cases DNA repair synthesis was induced by 4-nitroquinoline (0.05–0.005 mM) (JACKSON & LINSKENS 1979). Occasionally octopine or nopaline (1 mM) was added to the incubation mixture, since these compounds may aid in plasmid transfer (LIPINCOTT 1977). Another variation was to pretreat pollen before incubation with pectinase/cellulase to aid in *A. tumefaciens* binding to pollen (LIPINCOTT et al. 1978). The *A. tumefaciens* culture was tested on whole plants for successful tumour infection capability.

3. RESULTS

3.1. Effect of treatments on seed-set and on derived seedlings

None of the treatments gave rise to plants bearing tumours; morphologically the

plants were normal, and the flower colour of these first generation seedlings was intermediate between W43 and W166H. When several of the seedlings were subjected to nopaline and octopine analysis (PETIT & TEMPE 1978), no unusually high concentrations of these compounds were found, indicating that the Ti-plasmid had not been carried through to derived seedlings.

Seedset after pollination with treated pollen appeared normal, except that long pectinase/cellulase (0.1–0.3 mg/l) manipulation completely abolished all seedset, and incubation times as long as 14 hrs. in still-culture before pollination gave a poor degree of seed set.

3.2. Effect of treatment on pollen cultures

When Ti-plasmid is transferred to stem and leaf cells, these cells are transformed into autonomously growing tumour cells. This implies growth on a simple media devoid of the usual hormones (SCHELL et al. 1979). Transformation of treated pollen by *A. tumefaciens* was therefore investigated by culture of the pollen either in a liquid medium as described by SANGWAN & NORREEL (1975) or on agar plates (10% agar with half concentration of the Murashige & Skoog medium, and with boric acid 0.01% and sucrose 10% added for better pollen germination). Although over 200 liquid cultures and agar plates (each containing approximately 50,000 pollen grains) were prepared in this way, we did not find any evidence for autonomous growth of pollen cells (growth in the absence of hormones) as a result of any of the treatments tried. Use of immature pollen (or microspores) did not yield colonies either.

3.3. Ploidy of *Petunia hybrida* and tumour induction

One interpretation of previous work is that haploid plant cells may not be transformed by the Ti-plasmid (e.g. see GORDON et al. 1978). Although in the case of *Nicotiana tabacum*, haploid cells have been successfully infected with *Agrobacterium tumefaciens*-derived plasmid (TURGEON et al. 1976), it was thought desirable to check this out for *Petunia hybrida* since pollen from this plant is itself haploid. Table 1 shows tumour formation following infection with

Table 1. Ploidy of *P. hybrida* and tumour induction.

<i>P. hybrida</i> mutant ploidy	Tumour Induction	
	Whole plant test (stem incision)	Stem culture test
Haploid G86a	---	++-
Haploid W42	---	+++
Diploid W166H	+++	++-
Triploid W75	+++	+-
Triploid A226	+-	---
Tetraploid Q96	+++	
Tetraploid T ₃ AF		+-

plasmid-bearing *A. tumefaciens* (AcH₅) on both whole plants and stem cultures. Overall, it would seem that tumours can be induced in all ploidy levels of *P. hybrida* tested, including the haploid level. However, it is apparent that tumour can be induced more easily on whole plants showing tetraploid, triploid and diploid states, while haploid and diploid derived tissue is infected more easily in stem cultures. Reasons for these differences are obscure, although it should be noted here that the haploid whole plants used were extremely poor 'growers' (G86 and W42). That haploidy in itself is not a reason for non-infection is further strengthened by our observation that both haploid and diploid potato plants could be infected successfully with crown gall tumours (following stem incision).

3.4. Effect of mutagen inducing unscheduled DNA synthesis on tumour development in plants or stems

It was found that 4-nitroquinoline-1-oxide, which is known to induce unscheduled DNA synthesis in pollen of *P. hybrida* (JACKSON & LINSKENS 1979), does not induce tumours when applied alone to whole plants or stem cultures, neither does it have any significant effect (at 0.001–0.01 mM) on tumour induction in



Fig. 1. Comparison of mechanical and ether wounding of *P. hybrida* stems in tumour induction. (a) Photograph taken 18 days after slicing stem of *P. hybrida* W166H with scalpel, followed 4 hrs. later by introduction of *A. tumefaciens* Ach5. (b) Photograph taken 18 days after one ml of ether had been allowed to drip onto *P. hybrida* W166H stem followed 4 hrs. later by introduction of *A. tumefaciens* Ach5.

either type of test when applied together with plasmid-bearing *A. tumefaciens*. DNA replication, perhaps a result of wounding, does take place in these cells, so that it may not be surprising that a mutagen inducing DNA repair synthesis has no further significant effect on tumour induction.

3.5. Ether wounding of *P. hybrida* and transformation by *A. tumefaciens*

In general, successful transformation of dicotyledonous cells by *A. tumefaciens* requires prior wounding of the plant, usually by a mechanical means ('rubbing' or an incision). Since this is not realistic in dealing with pollen grains, another wounding method was developed. As shown in *fig. 1*, wounding of *P. hybrida* stems by diethyl ether leads to tumour induction following application of *A. tumefaciens*. Furthermore, these tumours do not appear to be any smaller than those appearing after stem incision, nor do they develop any more slowly.

Nevertheless, when pollen grains were first treated with diethyl ether, then incubated with *A. tumefaciens*, no autonomously growing colonies developed on agar plates (10 tried) or liquid cultures (10 attempted) in the absence of added hormones. It was established beforehand that diethylether does not significantly effect the germination of *P. hybrida* pollen.

4. DISCUSSION

We have shown that *P. hybrida* haploid cells, like the polyploid counterparts (diploid, triploid, tetraploid), can be successfully infected with *A. tumefaciens* to yield tumours. In looking for an explanation for the lack of activity of the Ti-plasmid with pollen, we need then to look elsewhere than at the ploidy of the pollen cells involved. Perhaps the explanation lies in the fact that pollen are very specialized, highly differentiated cells, which, as suggested by GALAU *et al.* (1976) for other highly differentiated cells, could lack many biochemical activities. Some of these may well be essential for the transfer and integration of the Ti-plasmid. The biochemistry of the plant-Ti-plasmid interaction is not at all well understood, so we can merely speculate on these matters. However, it should be mentioned here that in *Petunia* – as in many other plant species – the pollen grains lack chloroplasts and precursors. This could be related to our findings in two ways – either we could postulate that they confer on the cell an activity essential for Ti-plasmid transformation, or perhaps the very mode of destruction of chloroplasts in pollen microspores or even of the vegetative nucleus in mature pollen, could prevent the establishment of Ti-plasmids in these cells. DNA replication, which could well be important in the integration of Ti-plasmid is not present in mature pollen (JACKSON & LINSKENS 1978), and could therefore also be a reason for the lack of activity of Ti-plasmid in pollen cells. However, we have also attempted the transformation of microspores, which do undergo DNA replication, again with no success. In addition, since it is possible that DNA repair synthesis could aid in Ti-plasmid integration in the absence of DNA replication, we have induced repair synthesis in our pollen cells with UV irradiation (JACKSON &

LINSKENS 1978) or by means of a chemical mutagen (JACKSON & LINSKENS 1979). These procedures did not aid in the transformation of pollen cells.

It could be pointed out that the mechanical wounding step, an essential part of successful infection in stem and leaf cells, was missing from our procedures. We recognised this fact, and devised a wounding step involving the organic solvent ether, which we found to be quite effective for inducing tumours in stems and leaves of *P. hybrida*. However, ether pretreatment of pollen, while not harmful to subsequent germination, did not aid at all in transformation by *A. tumefaciens* or Ti-plasmid. The wounding reaction, insofar as it is important for transformation by Ti-plasmid, is not at all well understood biochemically and until it is, the lack of transformation of pollen cells by the Ti-plasmid will be difficult to explain. The barrier effect of the pollen walls should also be taken into consideration.

However, notwithstanding the above conclusions, it is extremely difficult to reconcile our findings with those of HESS and collaborators (1974, 1976, 1978), who report the transformation of *P. hybrida* with either exogenous DNA or bacteriophages. Perhaps these reports need further investigation to explain the differences.

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