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# Studies on the recovery of crown gall tumor cells

(teratoma/meiosis/vegetative propagation/nopaline)

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ABSTRACT **Our previous studies have shown that, while a persistent but potentially reversible suppression of the tumorous state appears to be a characteristic feature of the vegetative phase of teratoma shoot growth in the crown gall disease of plants, a recovery from that state occurs during the reproductive phase. An analysis has now been made of the reproductive process in an attempt to define the precise stage at which recovery occurs. The results of this analysis have shown that diploid somatic cells of teratoma-derived flower parts such as those found in petals and filaments are inherently neoplastic. On the other hand, haploid cells of plants grown from anthers of the same flowers and diploid cells of $F_1$ generation plants grown from teratoma-derived seed have, by generally accepted criteria, recovered from the tumorous state. These findings have been interpreted to mean that the loss of neoplastic properties occurs in crown gall teratoma cells during meiosis rather than during fertilization or later stages of the reproductive process.**

**An analysis of more than 2000 teratoma-derived tumor shoots has shown, moreover, that a recovery from the tumorous state may also occur, although apparently as a very rare event, during the vegetative phase of teratoma shoot growth.**

Cloned lines of crown gall teratoma tissues of tobacco (*Nicotiana tabacum* L.) are characterized by a capacity to organize tumor shoots when grown on a basic culture medium. When such teratoma-derived shoots are isolated and grafted at cambial level into appropriate morphologically distinct stock plants a broad spectrum of responses may be obtained (1). These responses range at the one extreme to a breakdown of the implanted shoot with a resulting development of typical disorganized teratomatous growths, while at the other extreme in this spectrum of examples are found shoots that develop quite normally, some of which ultimately flower and set viable seed. The leaves that develop from such teratoma shoots are composed of all of the specialized cell types found in normal leaves and they appear by all generally accepted criteria to be normal. It has been found, however, that when tissues are isolated from the teratoma-derived leaves and planted on a basic culture medium they again assume their neoplastic properties. The cells of these tissues are therefore inherently neoplastic and need only to be released from the morphogenetic restraints that govern so precisely the growth of all normal cells in an intact organism to again exhibit the tumor phenotype. While a persistent but potentially reversible suppression of the tumorous state appears to be a characteristic feature of the vegetative phase of teratoma shoot growth, a recovery from that state results during the reproductive phase and may involve either meiosis or fertilization, or it may occur during seed formation. It is the purpose of the present paper to characterize the precise stage in the sexual reproductive process in which a recovery from the tumorous state occurs in the crown gall disease as well as to show that a recovery may also occur, although apparently very rarely, during the vegetative phase of teratoma shoot growth.

## MATERIALS AND METHODS

Studies were conducted with teratoma shoots derived from a clone of cultured tobacco cells (*N. tabacum* cv Havana) transformed by the T37 strain of the crown gall bacterium *Agrobacterium tumefaciens*. Procedures used in these studies for grafting teratoma shoots and for culturing cells have previously been described elsewhere (1). Briefly, shoots produced by teratoma tissue grown in liquid culture were grafted at cambial level to detopped stems of healthy tobacco plants of the morphologically distinguishable Turkish cultivar. Pieces of leaf, flower, and pith tissue derived from grafted teratoma and normal shoots were cultured on the agar-containing basic medium of Linsmaier and Skoog (2). The growth hormones naphthalene acetic acid (NAA) (0.5 mg/liter) and kinetin (6-furfurylaminopurine) (0.5 mg/liter) were added to the medium in certain cases as described in the *Experimental Results*.

Haploid plants were obtained by the anther-culture technique (3) using the agar-containing medium of Murashige and Skoog (4) without hormone supplement. When plants reached appropriate size they were transferred to soil in pots and grown in a greenhouse. The haploid condition was confirmed by chromosome counts made on Feulgen-stained nuclei of root tips.

Susceptibility to tumor induction was analyzed by applying suspensions of either the T37 or B6 strain of *A. tumefaciens* to the cut surface of detopped stems of test plants. The T37 strain of *A. tumefaciens* is moderately virulent and when inoculated into tobacco plants induces tumors (teratomas) with a pronounced capacity for organizing abnormal though recognizable shoots. The B6 strain is highly virulent and induces unorganized tumors on tobacco plants. Tumor cells transformed by the T37 and B6 strains synthesize the tumor-specific amino acids nopaline [$N^2$-(1,3-dicarboxypropyl)-L-arginine] and octopine [$N^2$-(D-1-carboxyethyl)-L-arginine], respectively (5).

Procedures used in these studies for the extraction and identification of nopaline have been described elsewhere (1, 5).

## EXPERIMENTAL RESULTS

Experiments were carried out to determine the precise stage in the sexual reproductive cycle in which a recovery from the tumorous state occurs in the crown gall disease of plants. Three generally accepted criteria for recovery were applied to the experimental test system. These were (*i*) an inability of cells to grow continuously on a basic culture medium, (*ii*) the loss of a capacity by the cells to synthesize the teratoma-specific amino acid nopaline, and (*iii*) susceptibility of cells to transformation with resulting tumor formation when inoculated with virulent strains of the crown gall bacterium.

**Recovery during Sexual Reproduction.** Our earlier studies had shown that some of the more normal-appearing teratoma shoots were composed largely, if not entirely, of tumor cells during the vegetative phase of growth (1). The flowers that developed from some of these shoots contained all of the component floral parts found in normal tobacco flowers. The teratoma-derived flowers appeared morphologically normal, the petals were characteristically colored, functionally reproductive pollen was present, and viable seeds were produced. In the

present study it was found that when fragments of tissue were isolated from petals or from filaments of individual teratoma-derived flowers and planted on a basic culture medium the cells at the cut edges of the tissue fragments proliferated actively and developed into typical teratomatous growths. The teratoma-derived petal and filament tissues have now been carried continuously on a basic culture medium for more than one year without obvious reduction in their growth rates. These tissues synthesize large amounts of nopaline. Thus the very normal-appearing teratoma-derived flower petals and filaments, like the leaves that develop on the same teratoma shoots, appear to be composed largely if not entirely of cells that are inherently neoplastic. These specialized cells need only to be released from the morphogenetic restraints that govern so precisely the growth of all normal cells in an intact organism to again assume their neoplastic properties. In contrast, cells at the cut edges of comparable tissue fragments isolated from petals and filaments of normal Havana tobacco flowers did not proliferate in a non-self-limiting manner when planted on the basic culture medium.

Haploid plants derived from the culture of both teratoma-derived and normal anthers grew vigorously, and when planted in soil and allowed to mature in a greenhouse showed no evidence in either case of gross morphological abnormalities. Chromosome counts made of cells present in root tips showed these plants to be haploids. Pith and leaf tissues from 15 teratoma-derived haploid plants were tested for growth potential in culture. In every instance the growth response of these tissues was identical to that of the haploid tissues isolated from normal control haploid plants. Continued proliferation of pith tissues of both occurred only when the basic culture medium was supplemented with an auxin and kinetin. When auxin alone was added to the culture medium growth in both instances was due largely, if not entirely, to cell expansion. There was, moreover, no evidence of cell expansion or of cell division in the teratoma-derived or the normal haploid pith tissues in the absence of the two growth-regulating hormones or in the presence of kinetin alone. The teratoma-derived haploid pith tissues have therefore lost the capacity to grow continuously on a basic culture medium and behave in this respect as do normal tobacco pith tissues.

Fragments of both teratoma-derived and normal haploid leaf tissues displayed greater growth potential in culture than did cells from pith, possibly due to the presence in those tissues of small amounts of endogenous hormones. In the presence of kinetin alone shoots were initiated in both tissues. These shoots appeared morphologically normal, in contrast to the often more abnormal-appearing shoots that develop in typical teratoma cultures. In the presence of auxin alone or occasionally in the absence of hormones, roots, but not shoots, were initiated in both the teratoma-derived and normal haploid leaf tissue fragments. This again is an indication that the haploid leaf tissues were no longer neoplastic, since we have never observed root initiation in typical teratoma cultures. Callus tissues derived from leaf fragments of the haploid plants, like the haploid pith tissues, required the continued presence in the culture medium of both an auxin and a cell-division-promoting factor for the continued proliferation of those tissues. The two essential growth-substance-synthesizing systems had therefore again become persistently repressed in the haploid cells of teratoma origin and those cells behaved in culture as do normal tobacco cells.

Suitably prepared extracts of leaves and of callus initiated from leaf and pith fragments of teratoma-derived haploid plants did not show detectable amounts of nopaline. Since nopaline was readily detectable when typical teratoma tissue extracts were diluted by a factor of 100, the haploid tissues, if they synthesize nopaline at all, must have produced it in less than one-hundredth the amount present in typical teratoma tissues. Haploid plants of teratoma origin were also found to be susceptible and formed tumors when inoculated with either the B6 or the T37 strain of the crown gall bacterium. Thus the three criteria required for a demonstration of the reversion of a crown gall tumor cell to a normal cell have been satisfied and the results of this study are interpreted to mean that recovery from the tumorous state occurs during meiosis rather than in later stages of the sexual reproductive cycle. If that interpretation is correct, then plants derived from seed of flowers that developed from a teratoma shoot should also have recovered from the tumorous state. An analysis of 15 $F_1$ progeny of such plants in the present study showed that they had in all instances recovered from the tumorous state according to the three criteria used in this study. Seed from these recovered plants was again sown and again produced plants that were, as far as could be determined, normal. These results are in accord with earlier studies from this laboratory, which showed that a recovery from the tumorous state in the crown gall disease occurs during the reproductive phase (1, 6, 7).

**Recovery during Vegetative Propagation.** Of a total of somewhat more than 2000 teratoma shoots that have been analyzed by grafting techniques over the past five years, two shoots grew in a highly regular manner and appeared normal from the beginning. These two shoots were derived not directly from cultures of stock teratoma tissues but from the culture of leaf tissue obtained from grafted teratoma-derived shoots. The two shoots in question grew normally from the beginning, did not synthesize nopaline, and ultimately flowered and set viable seed. This behavior is highly abnormal for grafted teratoma shoots; even those shoots that eventually grow to maturity and enter the reproductive phase commonly produce teratomatous growths at the graft union or show other abnormal growth patterns at the base of the shoot. Since the teratoma tissue from which the two shoots were derived was initially cloned and since characteristic morphological markers were available to distinguish the implanted shoot from the stock plant, vegetative recovery in these two instances appears to be real, although it apparently occurs as a rare event.

## DISCUSSION

Experimental evidence is presented here to show that a recovery from the tumorous state occurs during the meiotic process rather than during later stages of sexual reproduction. The flowers that develop from certain of the teratoma-derived shoots are perfectly organized, contain all of the components found in normal tobacco flowers, and appear normal in every respect. An analysis of individual flower parts has shown, however, that diploid cells present in petals or filaments of the teratoma-derived flowers, like those found in the well-organized teratoma leaves, are inherently neoplastic. Such cells grow profusely and indefinitely as typical teratomatous growths on a basic culture medium that does not support the continued growth of normal tobacco cells. Tissues derived from petals and filaments synthesize nopaline, which is a specific biochemical marker for crown gall teratoma tissue. The cells of haploid plants produced by anthers of these same teratoma-derived flowers do not grow on a basic culture medium and, like normal tissues, require both an auxin and a cell-division-promoting factor for their continued growth in culture. The haploid tissues do not synthesize nopaline, at least not in amounts 100 times less than that found in typical teratoma tissues. When haploid plants are inoculated with virulent strains of the crown gall

bacterium they produce tumors. Diploid plants that develop from seed formed by the teratoma-derived flowers appeared normal and the resulting plants again set viable seed. Cells from the $F_1$ generation of seed grown plants did not grow on a basic culture medium and did not synthesize detectable amounts of nopaline, and when such plants were inoculated with virulent crown gall bacteria, tumors were initiated. The results of this study are interpreted by us to mean that a recovery from the tumorous state in this experimental test system occurs during meiosis rather than during fertilization or seed formation. Evidence is presented to show that a recovery from the tumorous state may also occur, although apparently very rarely, during the vegetative phase of teratoma shoot growth.

If, as indirect evidence suggests (8–10), the crown gall disease is caused by a self-replicating entity transmitted by the inciting bacterium, the loss of such a factor would result in the recovery of cells from the tumorous state. The elimination of a significant number of different viruses from systemically infected plants during sexual reproduction is well documented in the literature (11). Recovery could also occur as a rare event during vegetative growth and the elimination of plant viruses by vegetative propagation of shoots in culture has been described (12). That viral genetic information may be lost even in those instances in which that information has become integrated into the host cell genome and replicates with the genome has now been demonstrated in the case of cloned lines of cat cells transformed by the murine sarcoma virus (13).

It is, of course, possible that the heritable cellular change that underlies the tumorous state in the crown gall disease may not depend on the continued presence of a self-replicating entity of viral nature. Persistent but potentially reversible changes in the phenotype without corresponding changes in the integrity of the cellular genome occur commonly in normal plant development and are dramatically illustrated in the case of topophysis or phase change involving juvenile and adult forms of certain higher plant species. These striking phenotypic changes, like the crown gall transformation, do not persist through sexual reproduction and are occasionally reversed during vegetative propagation (14). Habituation (15, 16) and the Kostoff genetic tumors (17) appear to represent other examples of this type of heritable cellular change.

While a distinction has been made above between heritable cellular changes that depend on the addition of new genetic information, on the one hand, and those of the phase change, habituation, and Kostoff genetic tumor type, on the other, it might be suggested that the underlying heritable cellular change in all appears to be fundamentally the same and ultimately depends for its expression on a persistent but potentially reversible activation or repression of select biosynthetic systems of host cell origin.

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