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The role of cytokinin in genetic tumorigenesis in *Nicotiana*

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Abstract. *N. glauca, N. langsdorffii,* their tumorous amphidiploid hybrid and the non-tumorous mutant hybrid were used as a model system to identify factors causing genetic tumors. The genetic tumors showed a shooty morphology and Richmond-Lang effect (senescence retarding effect of cytokinin) *in vivo,* suggesting that these tumors may contain an elevated level of cytokinin. This was supported by both cytokinin measurement which was higher cytokinin in tumors than in other tissues and *in vitro* leaf disc culture experiments in which the shooty morphology of tumorous hybrids was not easily altered by treatments with auxin and/or cytokinin. Increases of auxin up to 12 mg/l did not alter the shooty morphology of the callus. This same auxin treatment applied to the parental species only induced root formation. Leaf discs of the non-tumorous mutant developed small shoot-forming teratomas when cultured on 0.8-1.0 mg/l cytokinin. Furthermore, transformation of leaf discs of the non-tumorous mutant with an *Agrobacterium tumefaciens* strain carrying only the cytokinin biosynthetic gene produced similar shooty tumors. Therefore, the mutant can be restored to a shooty tumorous phenotype either by the addition of exogenous cytokinin, or by increasing the endogenous cytokinin level through transformation with a gene encoding cytokinin biosynthesis. These results when taken together support the conclusion that a hormone imbalance is the main cause of genetic tumor formation, and that an increase in the cytokinin level more than a change in auxin level is the cause of the hormone imbalance.

Key words: Cytokinin; Genetic tumors; *Nicotiana*; Tissue culture; Transformation.

Introduction

Genetic tumors have been known to occur in plants for over 100 years (Caspary, 1873). The occurrence of genetic tumors in *Nicotiana* is an excellent example. This genus consists of 64 species (Goodspeed, 1954). About 300 interspecific hybrids have been made and over 10% of those produce spontaneous tumors (Smith, 1972). Genetic tumors in *Nicotiana* were first reported by Kostoff (1930). These tumors may be found in all plant parts or only on the roots of a mature hybrid plant. Näf (1958) divided the *Nicotiana* species capable of producing tumorous hybrids into two groups, plus and minus. A cross between a plus species and a minus species resulted in a tumorous hybrid and crosses within the plus group or minus group resulted in non-tumorous hybrids. Thus, a species-specific interaction is suggested.

Genetic and crown gall tumors share many physiological, morphological, histological and biochemical similarities (Akiyoshi *et al.*, 1983; Bayer, 1984; Kung, 1981; Smith, 1972). Both are caused by complementation or integration of genetic material from different sources. Genetic tumors in *Nicotiana* are induced in the

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interspecific hybrids by genetic complementation from two distant species (Goodspeed, 1954; Smith, 1972), for example, the hybrid between *N. glauca* and *N. langsdorffii* (Smith, 1972) is highly tumorous. Recent evidence shows that an addition of a single chromosome from *N. glauca* is sufficient to produce the tumorous phenotype (Smith, 1988). This indicates that specific genes are responsible for tumor formation. In crown gall tumors certain genes from the Ti plasmid of *Agrobacterium tumefaciens* are transferred to a plant cell and produce the tumorous growth (Schell, 1987).

It is likely that specific genes related to hormone metabolism are involved in genetic tumorigenesis, as well as those known in *Agrobacterium*-induced tumorigenesis. Normal plants must contain not only a proper level but also the proper balance between endogenous auxin and cytokinin for normal development and growth (Skoog and Miller, 1957). It is believed that both types of tumors produce higher than normal regulatory levels of auxin and cytokinin. This, in turn, results in rapid, uncontrolled cell division, and hormonal independence *in vitro* shown by the tumors.

Recent evidence suggests that genetic tumors contain a normal or only slightly elevated level of auxin (Chen, 1987). We present *in vitro* and *in vivo* evidence to indicate that in genetic tumors there is an alteration in the ratio of auxin to cytokinin, which we believe is caused by an increase in the amount of cytokinin. This results in the production of tumorous growth with a shooty morphology, suggesting a cytokinin effect. It is the shift in the hormonal balance, which plays an important role in genetic tumorigenesis.

Materials and Methods

Plant Materials

Nicotiana glauca, N. langsdorffii, their tumorous amphidiploid hybrid, and the X-ray-induced non-tumorous mutant (Izard, 1957) were used in this study.

In vitro Leaf Disc Culture and Transformation Experiments

Leaves were excised from greenhouse-grown plants, soaked in 25% commerical laundry bleach for 10 min and washed three times with sterile deionized water. Discs (6 mm in diameter) were punched from leaves and immediately placed on an MS medium (Murashige and Skoog, 1962) supplemented with various combinations of auxin and cytokinin. Auxin (IAA) was added to the MS medium at 0, 3, 6, 12 or 20 mg/l. At each auxin concentration, cytokinin was applied at 0, 0.2, 0.4 or 0.8 mg/l. There were a total of 20 treatment combinations. All cultures were incubated at 26°C, with one duplicate in the dark and another in the light with a 16-hour photoperiod. All treatments were repeated at least twice.

The detailed procedure of leaf disc transformation using mpGV2462 (a derivative of pGV2462 carrying T-DNA gene 4) was described in Feng *et al.* (1990).

Immunoassay of Cytokinins in Plant Tissues

Extraction of crude samples was performed as described by Weiler *et al.* (1986). Fresh plant tissues were homogenized in a buffer containing 80% methanol and 10 mg/l butylated hydroxytoluene (BHT; Sigma). The homogenate was stirred at 4°C for 4 hours and then centrifuged at full speed in an IEC benchtop centrifuge. The supernatant was adjusted to a final methanol concentration of 70% with deionized water. This was then passed through a SepPac C-18 cartridge column (Waters Associates) and the eluent was dried down in a Speed Vac rotary evaporator and dissolved in 25 mM Tris-buffered saline, pH 7.5. The quantitation of cytokinins in sample extract was carried out with Phytodetek immunoassay kits (Idetek).

Results

Genetic Tumors Produce Shoot-Forming Teratomas

The genetic tumors of the highly tumorous hybrid *N. glauca* × *N. langsdorffii* occur on the leaf, flower, stem and root, and are all shoot-forming teratomas (Fig. 1, A-D) developing clusters of plantlets (Fig. 2). This shooty morphology suggests that genetic tumors may have an altered ratio of auxin to cytokinin, possibly due to the production of excess amounts of cytokinin. To test this the following experiments were performed. Leaf discs were prepared from the highly tumorous hybrid *N. glauca* × *N. langsdorffii* and grown on hormone-free MS medium. The experiments were performed both in the light and in the dark. As illustrated by Fig. 3A, this callus exhibited a shooty morphology similar to the intact plants (compare Fig. 2 and 3A) as if excess amounts of cytokinin had been added to the medium. Under the same culture conditions no detectable growth occurred on leaf discs prepared from

the normal parental species *N. glauca* or *N. langsdorffii*. This shooty morphology further suggests that there is a higher than normal level of cytokinin in the genetic tumors.

Genetic Tumors Show a Cytokinin Effect in vivo

To further demonstrate the cytokinin effect, a genetic tumor was induced on a leaf from the tumorous hybrid of *N. glauca* × *N. langsdorffii* by making a cut at the midrib. A large tumor was formed at the cut site (Fig. 1A). The induction of tumors by wounding on young hybrid plants is a typical observation with genetic tumors (Smith, 1972). At the end of the growing season, the tumor-bearing leaf had wilted and died, leaving only the intact tumor which was green as if it had been treated with cytokinin (Fig. 2C). This senescence retarding effect of cytokinin has previously been reported (Richmond and Lang, 1957). This is a clear demonstration that genetic tumors exhibit a strong cytokinin effect and therefore must contain greater than normal amounts of cytokinin.

In vitro Culture of the Interspecific Tumorous Hybrid

Leaf discs from the interspecific hybrid of *N. glauca* × *N. langsdorffii* were prepared and tested for their response to various levels and combinations of auxin and cytokinin in the same manner as for their parental species. The leaf discs from this hybrid produced callus vigorously on MS medium without added auxin and/or cytokinin in the light and dark. Addition of auxin or cytokinin alone or in combination at a wide range of concentrations altered very little the pattern of growth and the shooty morphology of the

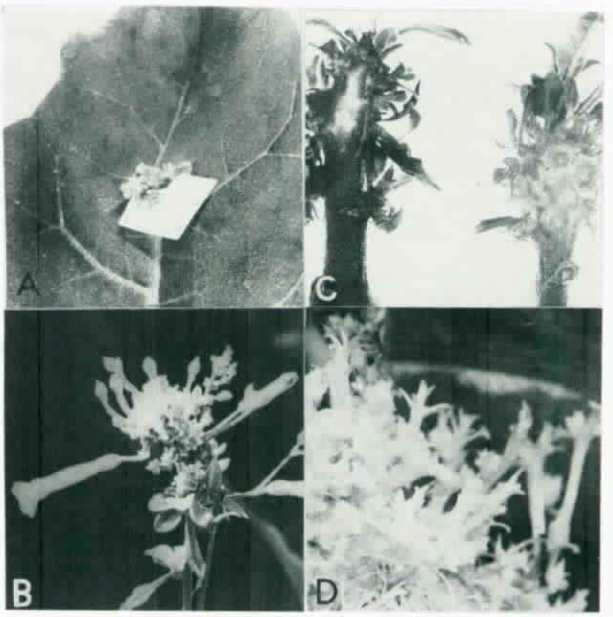

Fig. 1. Formation of genetic tumors on GGLL hybrid leaves (A), flowers (B), stems (C) and roots (D).

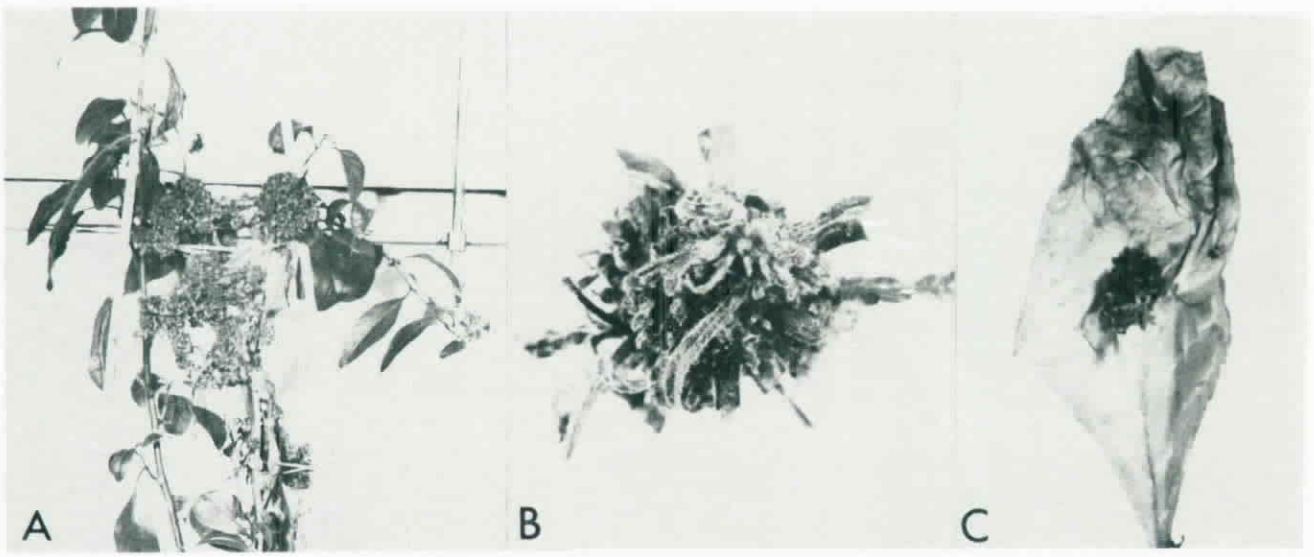

Fig. 2. Genetic tumors on GGLL hybrids showing the shooty morphology of the tumors on whole plants (A), close up of shooty tumor (B) and shooty tumor remaining after senescence of the leaf (C).

callus. On hormone-free MS medium, this hybrid still produced vigorous shoot-forming teratomas, suggesting again that this tumorous hybrid contained a lower than normal ratio of auxin to cytokinin. In order to test this, a series of biological titrations on the inferred amount of cytokinin with a known added amount of auxin was carried out. This was based on the evidence that in tissue culture, increases in the exogenous auxin would shift the hormonal balance in favor of root development (Skoog and Miller, 1957). If the tissue contains excess amounts of cytokinin a much higher amount of auxin would be needed to shift the balance to induce roots. The result of this biological titration demonstrated that the inferred level of cytokinin in the tumorous hybrids is indeed high. When a relatively high concentration (3 mg/l) of auxin (IAA) was added to the MS medium, the tumorous hybrid still produced shoots indicating that the hybrid contains a high level of endogeneous cytokinin.

The non-tumorous mutant hybrid of *N. glauca* × *N. langsdorffii* induced by x-ray treatment (Izard, 1957) was included for comparative studies. In contrast to the highly tumorous wild type, leaf discs from the mutant did not grow on MS medium in the absence of hormones. Callus was formed only when both auxin and cytokinin were added to the MS medium. In many ways, the mutant behaved similarly to the parental species. When auxin (6 mg/l) alone was added to the MS medium, the leaf discs from the tumorous hybrid still developed shoot-forming teratomas whereas the non-tumorous mutant developed only small callus.

When the auxin concentration was increased to 12 mg/l, leaf discs from the tumorous hybrid developed more than two times as many shoots as roots, while the non-tumorous mutant initiated only roots (Table 1). It was also observed that when the same amount of auxin alone was applied to the leaf discs from both parental species only roots were produced (Table 1). The biological titration experiment demonstrated very clearly that there is a high concentration of cytokinin in the tumorous hybrids. *N. langsdorffii* exhibited a much greater ability to initiate roots than *N. glauca* (Table 1). It was observed that leaf discs from *N. langsdorffii*

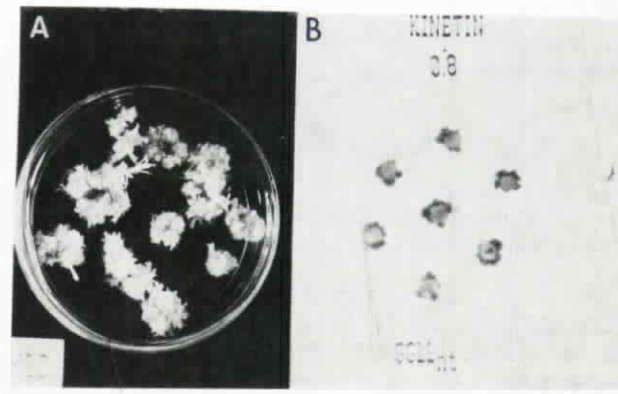

Fig. 3. (A) Shoot forming teratomas on leaf discs of the GGLL hybrid cultured in the dark on hormone-free MS medium. (B) Shoot-forming teratomas on leaf discs of the non-tumorous GGLL mutant on MS medium containing 0.8 mg/l kinetin.

Table 1. *Numbers of shoots and roots on leaf discs from tumorous hybrid, non-tumorous mutant and their parental species (N. glauca, N. langsdorffii) treated with IAA (12 mg/l) or NAA (3 mg/l)*

The numbers of shoots and roots shown in this table are the average of each plate containing 7 leaf discs.

Species	IAA				NAA	
	Shoots		Roots		Shoots	Roots
	4*	6*	4	6	6	6
Tumorous hybrid (GGLL)	5	16	2	7	21	1
Non-tumorous mutant (GGLL)	0	0	10	18	0	16
N. glauca (GG)	0	0	30	32		
N. langsdorffii (LL)	0	0	58	207		

*Weeks

could initiate roots without apparent callus growth. Similar results were also obtained when NAA was used in place of IAA. However, NAA at high concentration (12 mg/l) has negative effects on root growth in tissue culture.

Relative Levels of Cytokinins in Genetic Tumor Cells

Three forms of cytokinins, isopentenyl adenosine (IPA), dihydrozeatin riboside (DHZR), and *trans*-zeatin riboside (*t*-ZR), were measured in the tumors, stems and leaves of *N. glauca, N. langsdorffii*, their tumorous amphidiploid hybrid and the non-tumorous mutant. The individual and total cytokinin contents are shown in Figure 4. The most obvious difference in the measurements is the high amount of cytokinin present in tumors of the tumorous hybrid (A). There is a higher cytokinin content present in the hybrid leaves (F) than stems (C), but in general the hybrid tissues had higher overall levels of cytokinin than *N. glauca* (D and H) and *N. langsdorffii* (E and I) tissues. It also appears that the real differences in total cytokinin level in tumors and plant tissues was due to IPA. This was always followed by DHZR, and then *t*-ZR always being the lowest of the three measured. It would seem that the IPA level could have been used as the indicator of total cytokinin level in all cases. The observed increase in the level of cytokinins in the tumor lends support to the conclusion that cytokinin plays an important role in genetic tumorigenesis in *Nicotiana*.

Can Cytokinin Induce Tumorous Growth both in vitro and in vivo?

If the tumorous growth is induced solely by the hormonal imbalance caused by the elevation of cytokinin, then the application of cytokinin alone should be able to induce tumorous growth both *in vitro* or *in vivo*. Leaf discs from *N. glauca* and *N. langsdorffii* did not develop shoot-forming teratomas resembling the genetic tumors under various treatments with cytokinin. However, the leaf discs from the non-tumorous mutant did develop small shoot-forming teratomas resembling the tumorous hybrid (Fig. 3B), when cytokinin (0.8–1.0 mg/l) was added to the MS medium. This result can be demonstrated in the light or in the dark. This observation suggests that factor(s) in addition to cytokinin may also be involved (Kung, 1989).

Young plants of *N. glauca, N. langsdorffii*, and both tumorous and non-tumorous hybrids were wounded or decapitated and treated with or without zeatin (400 mg/g) in a lanolin paste. No induction of the shooty tumorous growth by zeatin treatment on *N. glauca, N. langsdorffii* or the non-tumorous hybrid plants was observed. The tumorous hybrid plants developed tumors from all treatments whether they were wounded, cut back, or treated with or without zeatin. It is quite clear that it is the wounding which induces tumors on young hybrid plants since no tumors developed on the young hybrid plants without wounding.

Transformation of the Non-tumorous Mutant Cells by the Cytokinin Gene from T-DNA

Since the non-tumorous hybrid can be induced to mimic tumorous growth *in vitro* by cytokinin treatment, the mutation seems likely to affect cytokinin synthesis. If this is the case, transformation of the mutant with a cytokinin gene of T-DNA from *Agrobacterium tumefaciens* should restore the tumorous growth. To test this, leaf discs from the tumorous and non-tumorous hybrids as well as from the parental species, *N. glauca* and *N. langsdorffii* were cocultivated with an *Agrobacterium* strain carrying either gene 4 for cytokinin biosynthesis or genes 1 and 2 for IAA biosynthesis described by Feng *et al.* (1990). Transformation was achieved, for gene 4 shown by the ability of

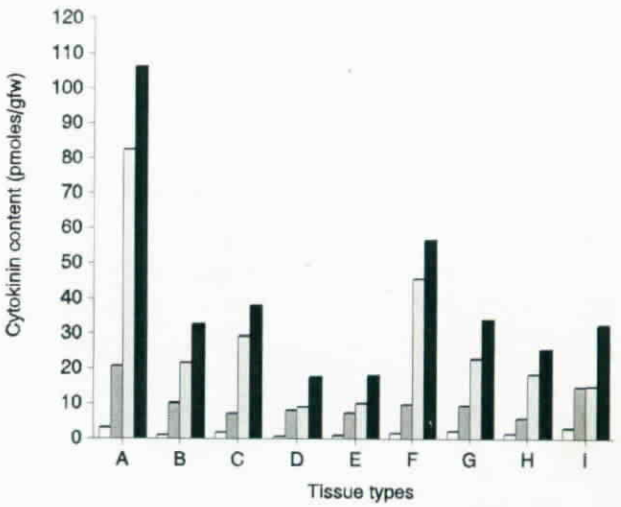

Fig. 4. Gytokinin content of tumor tissues derived from GGLL tumorous hybrid (A); stem tissues from GGLL tumorous hybrid (B), GGLL mutant (C), *N. glauca* (D), and *N. langsdorffii* (E); and leaf tissues from GGLL tumorous hybrid (F), GGLL mutant (G), *N. glauca* (H), and *N. langsdorffii* (I). □ *t*-ZR; ▨ DHZR; ▩ IPA; ■ TOTAL.

cells of the tumorous and non-tumorous hybrids and *N. glauca* but not *N. langsdorffii* to grow on medium containing 200 mg/l kanamycin without hormones.

The transformed non-tumorous (mutant) hybrid grew as vigorously as the tumorous hybrid and developed small clusters of shoot-forming teratomas, only when transformed with the cytokinin gene and not with the auxin genes. Morphologically the shooty tumors were indistinguishable from those of the tumorous hybrid (Fig. 5). It is evident that the non-tumorous mutant, when transformed with only the cytokinin gene, mimics the *in vitro* shooty growth shown by the genetic tumorous plants. This result complements the results obtained by treating the mutant directly with cytokinin resulting in shoot-forming callus. We have recently published more details of this finding (Feng *et al.*, 1990).

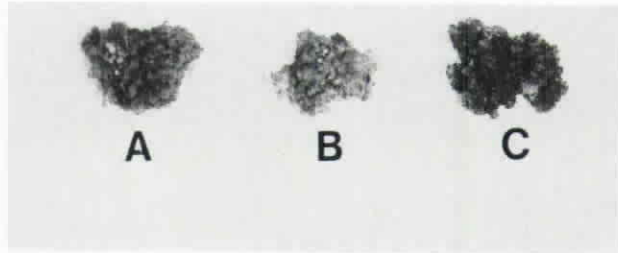

Fig. 5. Similarity of shoot morphology of untransformed wild type GGLL tumors (A), GGLL transformed with mpGV2462 carrying the cytokinin gene only (B), and GGLL non-tumorous mutant also transformed with mpGV2462 (C) cultured on hormone-free MS medium.

Discussion

We have observed shoot-forming teratomas on three different tumorous hybrids systems (*N. glauca* × *N. langsdorffii*, *N. suaveolens* × *N. plumbaginifolia*, *N. gossei* × *N. longiflora*). The highly tumorous hybrid of *N. glauca* × *N. langsdorffii* produced the most vigorous shoot-forming teratomas both on the intact plant and on leaf discs in culture. On intact plants, the shoot-forming teratomas on roots, stems or floral parts all developed into clusters of plantlets. The simplest explanation for such a morphological response is that tumorous hybrids contain excess amounts of cytokinin. This alters the normal regulatory ratio of auxin to cytokinin and triggers the formation of shoot-forming teratomas. In fact, there is a link between genetic tumors and cytokinin (Ames *et al.*, 1979). Since the internal level of cytokinin is elevated, high concentrations of auxin (1-6 mg/l), when added to the culture medium, still failed to normalize the cellular ratio of auxin to cytokinin and consequently shoot-forming teratomas result. Only when excess amounts of auxin (12 mg/l) were added to the MS medium were some roots initiated. Even under such conditions, more shoots formed than roots (Table 1) still suggesting an excess amount of internal cytokinin in the tumorous hybrid. These biological titration experiments clearly demonstrate that there is hormonal imbalance in the tumorous hybrids. This is supported by cytokinin measurement (Fig. 4) and the shooty morphology of tumorous hybrids observed both *in vivo* and *in vitro*. On the other hand, the internal cytokinin level was close to normal in the non-tumorous mutant so roots were initiated under similar auxin treatments. Shifts in the normal hormone balance by either treating the non-tumorous mutant with cytokinin or transforming it with a cytokinin gene from T-DNA can mimic the shooty tumorous growth in the mutant (Feng *et al.*, 1990). Similar transformation experiments with the same results were reported by Nacmias *et al.* (1987). This provides strong evidence that cytokinin plays an important role in genetic tumorigenesis.

These results are significant in elucidating the mechanisms of spontaneous genetic tumors in *Nicotiana*. They suggest that a hormone imbalance is the main cause of genetic tumor formation and an increase in cytokinin level more than a change in auxin may be responsible for the shift in hormone balance. This also explains why genetic tumors exhibit a shooty morphology (Kung, 1989). It also suggests that the mutation to the non-tumorous state may involve a defect in cytokinin biosynthesis or accumulation since the mutant phenotype can be overcome by adding cytokinin or by transformation with a cytokinin gene from T-DNA.

The available evidence supports this interpretation. Reduction of auxin or elevation of cytokinin level promotes tumor growth (Ames, 1979), indicating that it is the imbalance in the normal auxin to cytokinin ratio and not the high level of both hormones that is important in tumorigenesis. This is further supported by the fact that in crown gall tumors the cytokinin level is increased by at least 50-fold whereas the change in

auxin level is within sampling variation (Akiyoshi *et al.*, 1983). Furthermore, in tobacco cells, the expression of both auxin and cytokinin genes from the T-DNA produced undifferentiated tumors whereas the expression of the cytokinin gene alone is sufficient to induce shoot-forming teratomas (Budar *et al.*, 1986) which are indistinguishable from genetic tumors. For genetic tumors, no significant difference in auxin level could be detected between the tumorous and non-tumorous hybrids or between the tumorous hybrids and the non-tumorous parents (Chen, 1987). This suggests, again, that genetic tumors are not due to an auxin effect. This conclusion represents a major shift in the concept in elucidating the possible mechanisms of genetic tumors. Currently, the generally accepted concept is that tumorous hybrids produce greater than normal amounts of both auxin and cytokinin. In contrast, we propose that in genetic tumors only the level of cytokinin is elevated. Furthermore, it has been shown that in the T - DNA transformed tissue, the cytokinin - producing cells provide the signal inducing auxin autonomy (Binns *et al.*, 1987). A similar mechanism may operate in genetic tumors.

There is a distinct difference between *N. glauca* and *N. langsdorffii* in response to the various hormonal treatments. *N. glauca* formed callus in almost all treatments except the control where no hormones were added. In contrast, *N. langsdorffii* developed very little callus in all treatments. However, *N. glauca* is less sensitive to auxin treatment in initiating root development than *N. langsdorffii*. This difference may also play an important role in producing tumors in their interspecific hybrids.

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Cytokinin 在煙草屬植物的遺傳腫瘤形成過程中之作用

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煙草屬植物 *N. glauca*, *N. langsdorffii* 及其野生型(能產生腫瘤的)和突變型(不能產生腫瘤的)雜交後代是研究植物腫瘤形成因子的好材料。爲研究 cytokinin 在腫瘤形成過程中的作用，本文特進行有關腫瘤的發生形態、cytokinin 含量以及遺傳學的研究。實驗結果顯示：(1)植物遺傳腫瘤具有多芽的形態特徵和 Richmond-Lang 效應；(2)腫瘤組織含有較其他組織爲高的 cytokinin 量；(3)超量的 auxin (濃度爲 12 mg/l)不能夠改變腫瘤組織的多芽形態。相反地，同樣的處理卻只能在培養過程中誘導親本組織和突變型組織產生多根；(4)外源的 cytokinin 的加入能夠促使突變型恢復多芽形態；(5)利用 *Agrobacterium* 作爲運載工具將 T-DNA 上合成 cytokinin 的基因轉移至突變型細胞體內，亦能將其轉化成多芽的腫瘤細胞。因此我們認爲，植物激素的不平衡(尤其是 cytokinin 含量的增加)是煙草屬遺傳腫瘤形成的主要原因。