

Original article:

The effects of exogenous hormones on genetic tumor formation in *Nicotiana* hybrids

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ABSTRACT

The effect of exogenous auxin and cytokinin on cell proliferation and differentiation varies with the tissue system, and depends on the endogenous levels of the two hormones in the tissue. In this report, cytokinin (N⁶-benzyladenine, BA) and auxin (indole-3-acetic acid, IAA) were used to treat young seedlings, leaf explants, and genetic tumors of a *Nicotiana* hybrid. Exogenous BA markedly accelerated genetic tumor formation in young seedlings, while exogenous IAA inhibited it on young seedlings but not on leaf explants. Genetic tumors showed low sensitivity to exogenous BA and IAA. RAPD analysis of the genomes of the genetic tumors and normal tissue suggested that tumor formation is related to genomic instability.

Keywords: auxin, cytokinin, genetic tumors, *Nicotiana* hybrid, Phytohormone, RAPD

INTRODUCTION

Some external agents, including wound tumor virus, *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, can induce tumors in plants. However, tumors also arise without the application of external agents in some interspecific hybrids, such as *Lilium speciosum* “Album” × *L. auratum* (Emsweller et al. 1962), *Gossypium hirsutum* × *G. gossypoides* (Phillips and Merritt, 1972)

and some *Triticum* interspecies hybrids (Joshi, 1972). Of the hybrids that produce spontaneous tumors, those of *Nicotiana* have been most intensively studied. *Nicotiana* species are divided into two groups, “plus” and “minus”; crosses between “plus” and “minus” species give rise to tumorous hybrids, whereas the parents do not produce tumor (Näf, 1958). Since under normal conditions the hybrids produce tumors

spontaneously, they are called genetic tumors. Some types of stress, such as wounding and X-ray irradiation, can enhance the formation of tumors in hybrids (Ahuja and Cameron, 1963). Tumors develop in all parts of the plant, such as leaf and stem, and the tumor tissues grow *in vitro* in the absence of exogenous hormones.

Various studies have been performed to probe the mechanism of tumor formation in tumorous hybrids. The trigger for tumor induction is a reduction in the auxin level in plants, and they showed that GA₃ caused a striking reduction in the rate of tumor formation, whereas cytokinins markedly accelerated it (Ames et al. 1969, 1971, 1972). There is also evidence that IAA conjugates play an important role in tumorigenesis in *Nicotiana* (Liu et al. 1978). Electrophoretic differences between the proteins and enzymes of *Nicotiana glauca* × *N. langsdorffii* and its parent species have been demonstrated (Bhatia et al. 1967). In addition genomic RAPD polymorphism was detected between *N. glauca*, *N. langsdorffii* and a related tumor-prone hybrid; in particular a gene, *GTcyc*, expressed at a high level in tumor tissue relative to non-tumor hybrid tissues was identified (Wang, 2001). Fujita et al (1994) identified some genes involved in the formation of tumors in *N. glauca* × *N. langsdorffii* hybrids.

In the present study we examined the influence of the exogenous hormones, IAA and BA, on tumor formation and growth, in

order to explore the roles of auxin and cytokinin. We also performed a RAPD analysis to determine whether genomic variation is involved in tumorigenesis.

Materials and Methods

The influence of exogenous hormones on tumor formation in seedlings and leaf explants

Surface sterile seeds of the interspecific hybrid *N. glauca* × *N. langsdorffii*, obtained from Dr. H. Smith (Brookhaven National Laboratory, USA), were grown in Petri dish on MS medium (Duchefa, Netherlands) (Murashige and Skoog, 1962) containing 25 g/L sucrose, 8 g/L agar and no exogenous hormones, at 25°C. Hormones (IAA, BA; Duchefa, Netherlands) were added as appropriate: medium I, control containing no exogenous hormones; II, 0.1mg/L IAA; III 1mg/L IAA; IV, 0.1mg/L BA; V, 1mg/L BA.

Uniform 10-day-old seedlings were selected and transferred to MS medium. 15 days later, the incidence of tumor formation was scored. To determine the influence of hormones on tumor formation in leaf explants, axenic leaves were cut into approximately 5 mm sections and transferred to a Petri dish on MS medium with various hormones. Biomass was measured after a month to assay the effects of the hormones. All the data were analyzed by using SPSS 11.5.

RAPD analysis

To determine whether genomic variation is involved in tumorigenesis, RAPD was carried out. Genomic DNA was extracted from normal leaves and tumor tissues of *Nicotiana* hybrids under normal field conditions using the procedure described by Sambrook et al. (1989). Four plants were selected. 13 random primers (Operon, California, USA; Table 1) were used and RAPD was performed. The 50 µl reaction mixtures contained: 20 ng template DNA, 20 mM dNTP mix, 10 pM primer, 2 units of Taq

DNA polymerase and 5 µl 10× PCR buffer.

PCR conditions were: 92°C for 1 min, 35°C for 1 min, 72°C for 1.5 min, followed by 44 cycles with initial denaturation at 92°C for 3 min and final extension at 72°C for 7 min. The products were separated electrophoretically on 1% agarose gels using TAE buffer, and the experiments were repeated three times.

Table 1: The sequence of primers for the assessment between wild type and transgenic plants

Primer	Sequence
OPAG-02	CTGAGGTCCT
OPAG-03	TGCGGGAGTG
OPAG-04	GGAGCGTACT
OPAG-05	CACAGACCTG
OPAG-06	GGTGGCCAAG
OPAG-13	GGCTTGGCGA
OPAG-14	CTCTCGGCGA
OPAG-15	CCCACACGCA
OPAG-16	CCTGCGACAG
OPAG-17	AGCGCAAGTG
OPAG-18	GTGCGCATAAC
OPAG-19	AGCCTCGGTT
OPAG-20	AGCCATACTG

RESULTS AND DISCUSSION

The influence of phytohormones on tumor formation in seedlings and leaf explants

To determine the influence of exogenous hormones on tumor formation, exogenous

hormones were applied to an interspecific hybrid of *Nicotiana glauca* x *Nicotiana langsdorffii*. BA markedly accelerated the formation of tumors in seedlings (Fig. 1). Tumors formed within 10 days on all the

seedlings treated with BA. 1mg/L BA inhibited seedling growth, but strikingly enhanced tumor formation within 5 days; the hypocotyls of the seedlings swelled rapidly and formed tumors. In contrast, IAA inhibited tumorigenesis; young seedlings

dwarfed after exposure to 1 mg/L IAA and no tumors formed (Fig. 2). These two hormones also had only very slight effects on the growth of tumors, indicating that the tumors lose their sensitivity to auxin and cytokinin (Fig. 3).

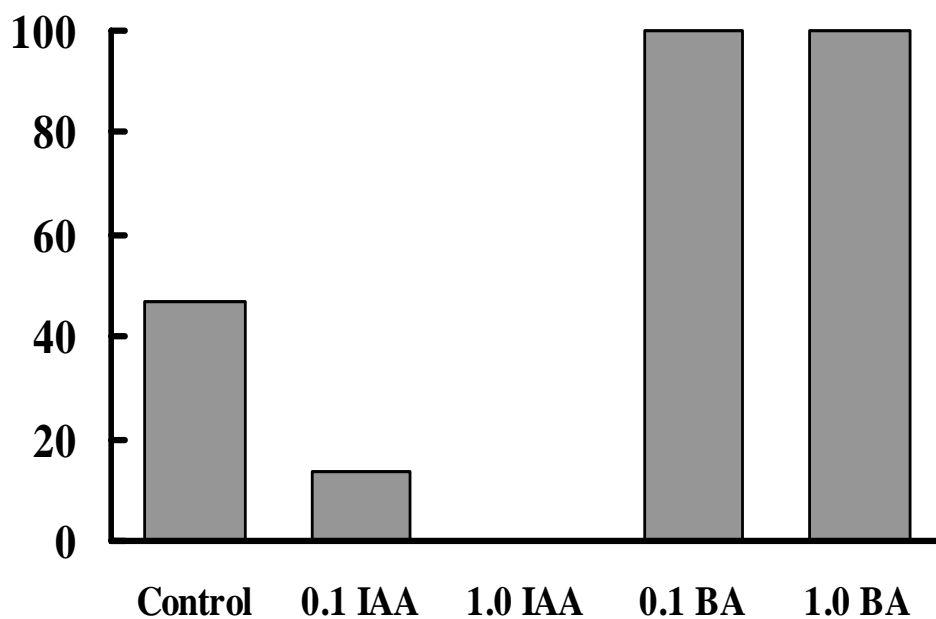


Figure 1: Young seedlings were grown in MS medium containing various levels of IAA or BA. 15 days later, the incidence of tumor formation was scored (n=10).

There is other evidence that phytohormones play an important role in the formation and development of tumors (Bayer, 1982). The application of auxin (IAA) to the cut surfaces of excised plant buds or to the nutrient medium inhibited tumor formation in *Nicotiana* hybrids, whereas application of cytokinins to the plant apices accelerated it,

and they put forward the hypothesis that the trigger for tumor induction in *Nicotiana* hybrids is a relative reduction in the level of endogenous auxin (Ames et al. 1969, 1975). Our results with hormone treatment of seedlings support this hypothesis. However it is unclear why IAA and BA had no significant effect on tumor formation in leaf

explants. Tumor can be induced from almost any tissue of tumor-prone *Nicotiana* hybrids. We propose that dedifferentiation of some cells are a crucial step in tumor formation, and that once cells begin to dedifferentiate they acquire the characteristics of tumorous cells, such as rapid division and loss of sensitivity to hormone. Many agents influence dedifferentiation. When seedlings are treated with exogenous cytokinin, some are induced to dedifferentiate. On the other hand, exogenous auxin inhibits dedifferentiation by interfering with cytokinin transport, and so inhibits tumor

formation in seedlings. Harrison and Kaufman (1984) reported that exogenous IAA inhibits the transport of cytokinin and promotes cytokinin breakdown in oats. Moreover, exogenous IAA can have toxic effects on seedling growth. For example, in the present study exogenous IAA had an inhibitory effect on the growth of seedlings. In the case of leaf explants, the inhibitory effect of exogenous IAA was eliminated because they had direct contact with the medium in order to obtain nutrients and so begin to dedifferentiate.

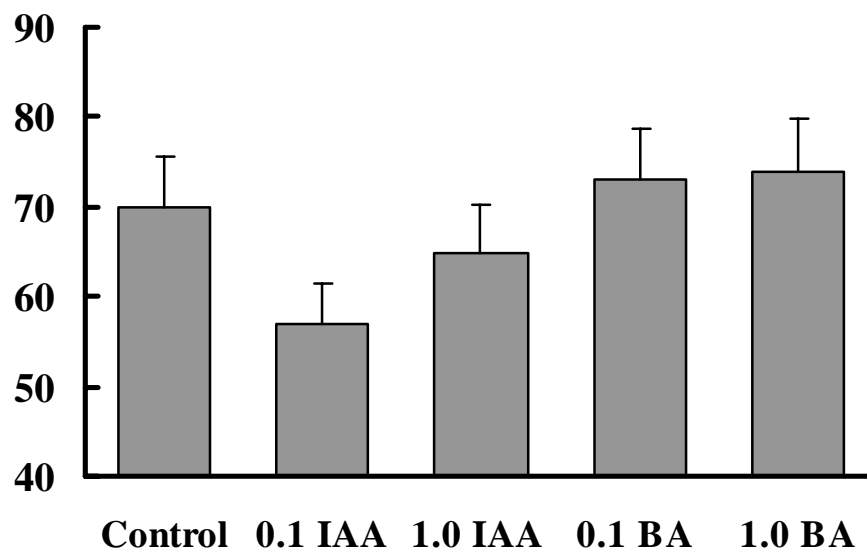


Figure 2: Leaf explants of seedlings were grown in MS medium containing various levels of IAA or BA and weighed on the first day and after thirty days. W, weight at thirty days; W0, weight on first day. (Errors bars show the s.d for each experiment. n=5 Petri dishes for each experiment). * $P < 0.05$ (ONE-WAY ANOVA Duncan multiply test, not significantly different among these hormone treatments)

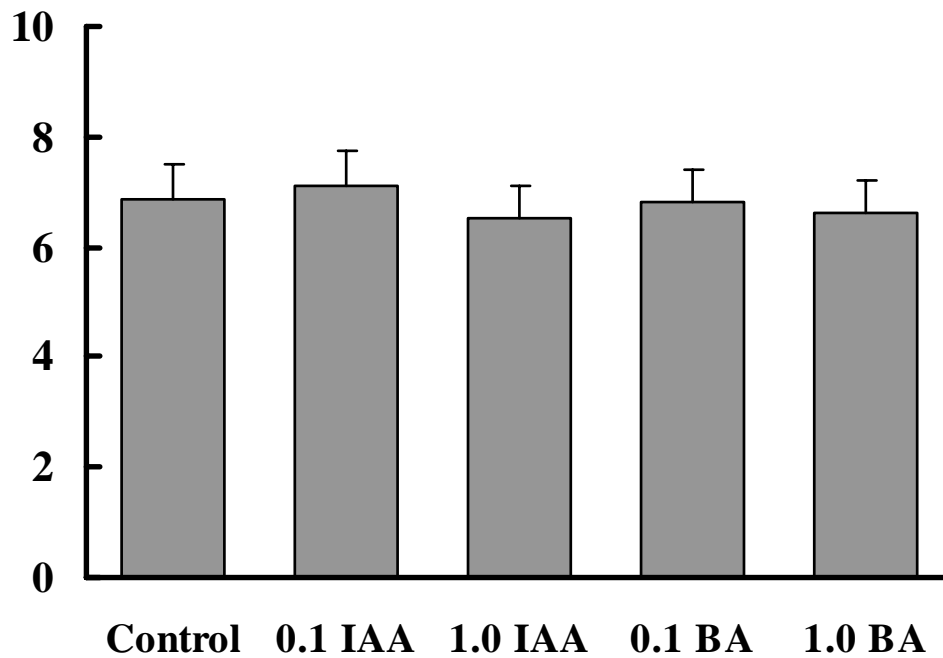


Figure 3: Tumors were grown in MS medium containing various levels of IAA or BA and weighed on the first day and after ten days. W, weight at ten days; W0, weight on first day. (Errors bars show the s.d for each experiment. n=5 Petri dishes for each experiment. * $P < 0.05$ (ONE-WAY ANOVA Duncan multiply test, not significantly different among these hormone treatments))

RAPD analysis

To determine whether genomic alteration was involved in tumorigenesis, we carried out a RAPD analysis of genomic polymorphism in tumorous and normal leaves of the same plant. As shown in Table 1, we used 13 primers. Two primers, OPAG-2 and OPAG-5 revealed genomic differences between tumor and leaf (Fig. 4), indicating that some of the tumor cells had undergone mutation. Cells of tumors of *Picea glauca* displayed cytological instability, with aberrant mitoses involving 3 to more than 70 chromosomes (De Torok and

White, 1960). In the present study, we investigated the mitotic chromosome number of the amphiploid hybrid (n=42) and its tumor, and found that their mitotic behavior was very similar, with chromosome numbers ranging from 32 to 42 (data not shown). This suggests that the genomic instability of tumors is probably due to small changes, such as deletion of some loci, rather than loss of whole chromosomes. Although spontaneous tumors of tobacco are thought to be caused by aberrant gene regulation rather than by gene mutation (Bayer, 1982), no

direct molecular biological evidence has so far been presented to support this hypothesis. Some plant tumors induced by agents such as viruses or bacteria are associated with genomic alterations. This occurs most commonly in tumors induced by *Agrobacterium tumefaciens*. In mammals, tumors frequently result from gene mutations or genomic alterations. For example, some ovarian and colorectal tumors are linked to

K-ras mutations (Vogelstein et al, 1988; Ichikawa 1994). In the present study, we detected alterations in the genomes of only two of the four plants examined. We suggest two possible explanations: one is that the random primers we used were not able to detect every alteration, and the other is that the genomic changes occur after tumor formation and do not involve all the tumor cells.

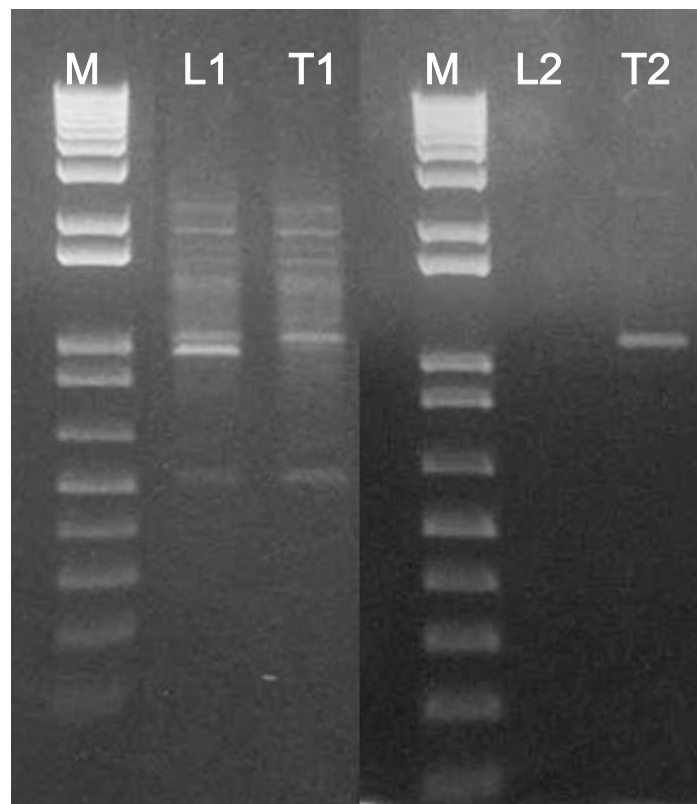


Figure 4: RAPD polymorphisms in the genomic DNA of tumors and normal leaves on plants 1 and 2, using random primers OPAG-02, OPAG-05, respectively. Arrows indicate the RAPD polymorphisms. M, 1 Kb marker; L, normal leaves; T, tumor.

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