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# Morphological and genetic characteristics of *Nicotiana langsdorffii, N. glauca* and its hybrid

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## ABSTRACT

Plant tumors, including genetic tumors, are disorganized and proliferate in an uncontrolled fashion. In this report we describe the morphological, physiological and genetic properties of *Nicotiana. langsdorffii* and *Nicotiana. glauca* and their hybrids (*Nicotiana. langsdorffii* x *Nicotiana. Glauca*). *Nicotiana. langsdorffii* leaves are oblate and pubescent with winged petioles, while *Nicotiana. glauca* leaf-blades are rubbery and oval-to-heart-shaped. The hybrid plants are intermediate in leaf shape, with anisocytic stomata and well-developed trichomes. In addition, they produce tumors in the absence of bacteria and exogenous hormones. Tumor growth in the hybrid plants was not affected by indole-3-acetic acid or kinetin. Genetic polymorphism was analyzed by the randomly amplified polymorphic DNA technique in the parents (*Nicotiana. langsdorffii* and *Nicotiana. glauca*) and in the genetic tumors produced by the hybrids. A total of 128 randomly amplified polymorphic DNA fragments were scored from fifteen random primers, and pronounced differences were found between the genetic tumors and their parents. These observations show that randomly amplified polymorphic DNA markers are might informative about genetic similarities and dissimilarities.

**Keywords**: *Nicotiana langsdorffii*, *Nicotiana glauca*, hybrid plant, genetic tumors, auxin, cytokinin, callus, randomly amplified polymorphic DNA

**Abbreviations:** MS: Murashige and Skoog; *N: Nicotiana*; RAPD: Randomly amplified polymorphic DNA; PCR: Polymerase chain reaction; SDS: Sodium dodecyl sulfate; IAA: Indole-3-acetic acid

## INTRODUCTION

Plant genetic tumors arise as a consequence of spontaneous and differentiated growth dependent on specific combinations of genes in a variety of intraspecific and interspecific hybrids (Smith 1988, Wang 1998). Näf (1954) divided *Nicotiana* species into two groups, namely plus and minus groups. The plus group species are *N. langsdorffii*, *N. sanderae*, *N. alata*, *N. longiflora* and *N. plumbaginifolia,* while the minus group species include *N. tabacum*, *N. suaveolens*, *N. debneyi*, *N. glauca* and *N. rustica*. Genetic tumors are formed only when a plus species is hybridized with a minus species, and it does not matter which parent is male and which parent female. Genetic tumors were produced in any diploid, triploid or tetraploid combination of *N. langsdorffii* and *N. glauca*

that possessed at least one complete genome of each parental species (Smith 1988, Kehr and Smith 1954).

The plant disease, crown gall, is a neoplastic disease induced by bacteria belonging to species of the genus *Agrobacterium*. Crown gall tumors and cells of genetic tumors are able to grow on a basic tissue culture medium while normal plant cells, like callus cultures, require the plant hormones auxin and cytokinin. Crown gall tissue contains higher levels of auxin than normal tissue and also produces cytokinins (Braun 1978). Auxins regulate cell proliferation in plants (Harrar et al. 2003) while cytokinins are involved in physiological and biochemical processes such as leaf senescence and seed germination (D`Agostino and Kieber 1999). High auxin/cytokinin ratios induce root formation, whereas low ratios promote shoot formation (Ross et al. 2001, Vissenberg et al. 2000). Trichome development has been divided into six stages based on specific morphological landmarks (Szymanski et al. 1998). The relationship of morphology and phytohormones to tumorigenesis is unclear. Tumor cells are autonomous and have the ability to direct their own metabolic activities independent of external control (Eklof et al. 2000). A hormone-mediated regulatory system is associated with cell proliferation (Durante et al. 1982).

RAPD (Randomly amplified polymorphic DNA) is a valuable tool for identifying genetic variation because it is economic, quick and simple (Micheli et al. 1994, Willians et al. 1990). It permits identification of DNA polymorphisms and can be used to amplify particular fragments of genomic DNA (Bielawski et al. 1995). DNA fragment profiles have been employed to analyze the genetic relationships of plant species (Ayana et al. 2000). RAPD analysis is based on the presence or absence of polymorphisms in individuals or groups of individuals (Willians et al. 1990). The GT1 protein is related to the protection of tissues due to wounding (Fujita et al. 1993).

The present study focuses on the properties of hybrids of *N. langsdorffii* and *N. glauca*. We examined the morphological and physiological responses of the hybrid plants to various concentrations of auxin and cytokinin, and also analyzed the genetic polymorphism of the parental and hybrid plants with the help of RAPD.

## MATERIALS AND METHODS

*Plant sources and growth conditions*

*N. langsdorffii*, *N. glauca* and its interspecific hybrid were used in this study. Seeds were surface sterilized in 1.2% (w/v) sodium hypochlorite and rinsed three times in sterile distilled water. The seeds of *N. langsdorffii* and *N. glauca* were obtained from Dr. Verne A. Sisson (North Carolina State University) and grown in pots at 25°C with a 16 h light/8 h dark cycle. Alternatively, seeds were germinated on agar-solidified 0.8% (w/v) MS medium (Murashige and Skoog 1962) at 25°C, after sterilization. To investigate morphological structures, leaves from each plant were cut into approximately 5 mm sections and transferred to culture vessels containing 30 ml of agar-solidified medium, containing auxin and cytokinin as plant regulators.

*Morphological examination*

Fresh leaf tissues from 4 week-old parental and hybrid plants were examined under a bright-field microscope and photomicrographs were taken.

*DNA isolation*

Genomic DNA was isolated from leaf tissues as follows: small pieces of leaf tissues (1 g) were incubated with 2 ml extraction buffer [10 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% sodium dodecyl sulfate (SDS) solution, 10 mM NaCl] in a Falcon tube for 30 min. Thereafter, they were extracted three times with equal volumes of phenol:chloroform (1:1) then precipitated with ethanol and the DNA was resuspended in 100 µl sterilized water. After treatment with RNase, DNA concentration was measured with a UV-VIS spectrometer (Hitachi, Japan).

*RAPD analyses*

Fifteen random 10-mer oligonucleotide primers (Operon Technologies, Alameda, CA, USA) were used to generate RAPD fragments (Table 1). PCR reactions were performed using a programmable thermal cycler (DNA Thermal Cycler, Germany) in a volume of 50 µl containing 10 mM buffer Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 450 nM primer, 5-10 ng of genomic DNA and 1 unit of *Taq* DNA polymerase. Cycling conditions were as following: 45 cycles of 94ºC for 1 min, 35ºC for 1 min, and 72ºC for 2 min followed by one cycle of 94ºC for 5 min, then 94ºC for 1 min, 35ºC for 1 min, and 72ºC for 2 min. PCR products were separated on 1% agarose gels and stained with ethidium bromide. Fragment sizes were estimated by reference to a 1 kb ladder marker. All reactions were repeated at least three times, and only consistently reproduced bands were considered.

## RESULTS AND DISCUSSION

*Morphological analysis*

Hybrid plants from crosses between *N. langsdorffii* and *N. glauca* produce genetic tumors (Näf 1954). As shown in figure 1A, *N. langsdorffii* leaves are oblate and pubescent with a short winged petiole. *N. glauca* leaves are greenish or bluish-purple in color and the leaf-blades are rubbery and oval- or heart-shaped. Hybrid plants had an intermediate phenotype. They did not produce genetic tumors early in development under our experimental conditions; the first signs of tumors were only apparent 3-4 weeks after germination. Occasionally, hybrid plants produced normal leaves early in development and these gradually formed a compact cluster of curly leaves at the apex: this is clear evidence of tumorigenesis.

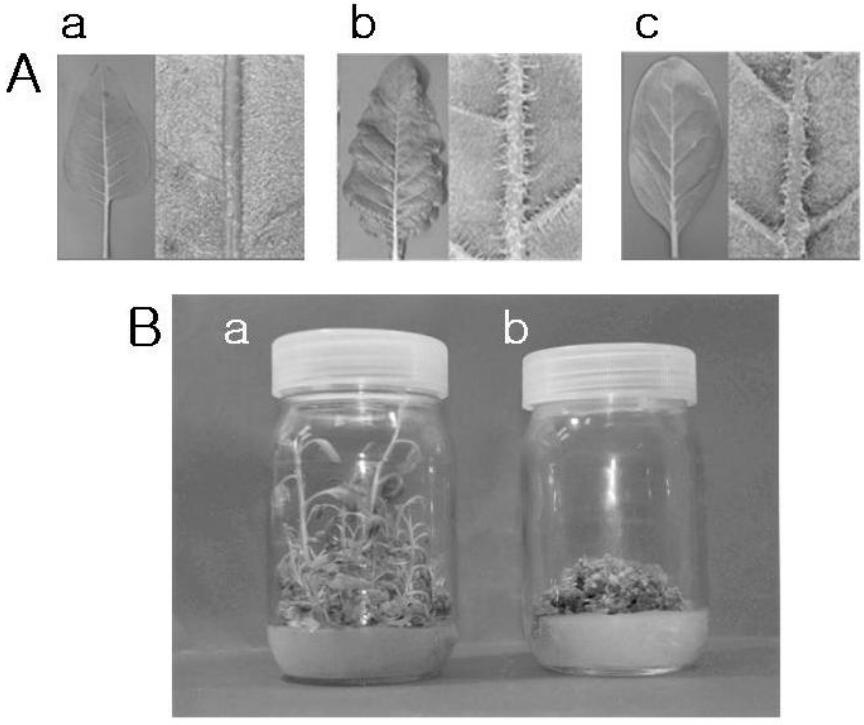



**Figure 1:** Phenotypes of *Nicotiana. langsdorffii* (a), *Nicotiana. glauca* (b) and its hybrid (c). A. Morphology and anatomy of leaves (x 35). B. *In vitro* cultures of *Nicotiana. glauca* (a) and the hybrid plants (b) were grown for 40 days from leaf discs in MS medium enriched with 0.1 mg $l^{-1}$ indole-3-acetic acid and 0.1 mg $l^{-1}$ kinetin, respectively.

We also compared the growth response of the three types of plant in tissue culture. Our analysis revealed striking morphological differences between explants of the hybrid plants and *N. glauca* in tissue culture. As shown in figure 1B, *N. glauca* grew normally whereas the hybrid plant did not produce shoots in response to cytokinin; *N. glauca* produced stems and leaves along with roots, while the hybrid plants formed a cluster of leaves without developing distinct shoots or roots though the hybrid plants did form shoots at low frequencies. It is possible that different genes are responsible for (a) disorganized tissue formation, and (b) tumor formation. On the other hand the same gene or group of genes could be responsible for both, tissue disorganisation and tumor formation.

It would be of interest to know why the hybrid does not produce normal shoots and roots. Genetic tumors produced spontaneously from hybrids between certain *Nicotiana* species (Ichikawa et al. 1990), have also been shown to develop shooty growth that does not develop into normal shoots (Figure 1B). Tobacco plants harboring the *CHRK1* gene resemble genetic tumors in forming partially differentiated shoots in hormone-free medium (Lee et al. 2004). Genetic tumors are known to overexpress the cytokinin biosynthetic gene for isopentenyltransferase, suggesting that changes in growth conditions of the hybrids may influence the tendency to form tumors (Fujita et al. 1994).

Observation by light microscopy revealed that stomata were present over the entire surface of the leaves of both of the parents and of the hybrids (Figure 2), but trichomes were observed only in *N. langsdorffii* and hybrid plants. Trichomes are unicellular structures derived from protodermal cells and may be involved in cell cycle control during leaf development (Szymanski and Marks 1998). Trichome development in Arabidopsis is related to the fundamental biological processes of cell fate regulation and pattern formation (Marks 1997).

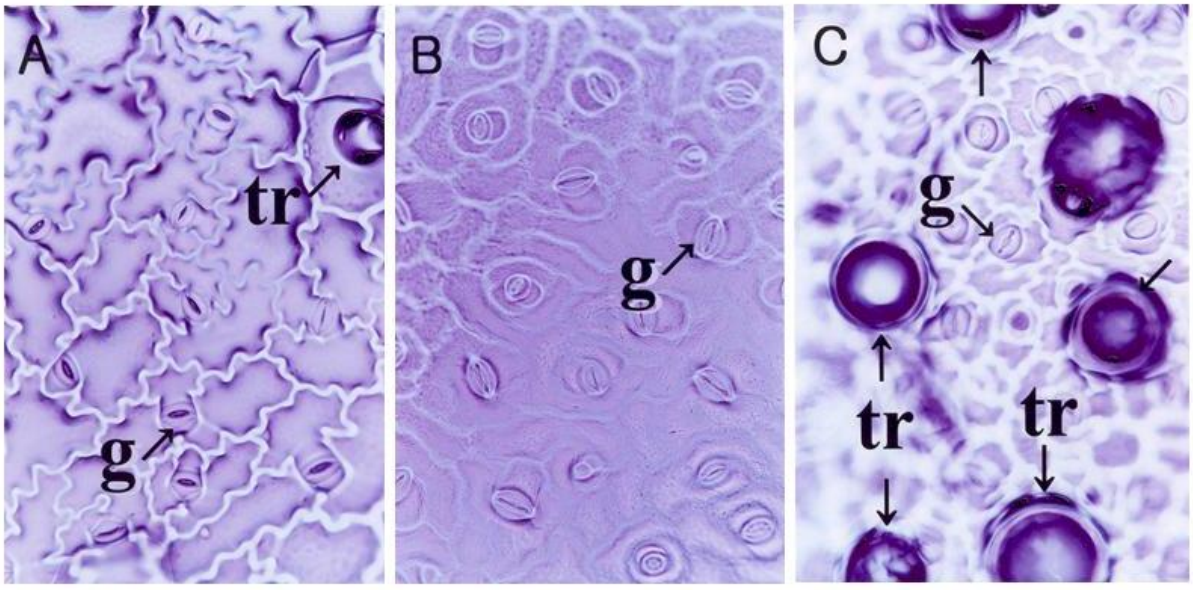
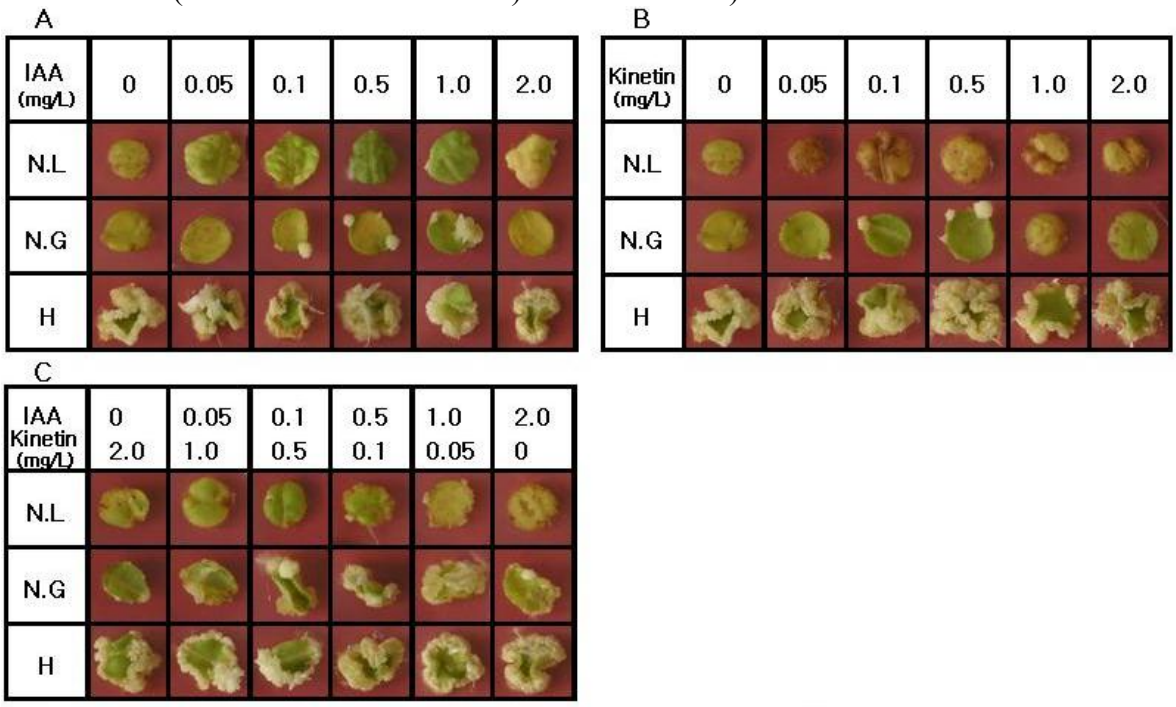



**Figure 2:** Leaf epidermis. A) Nicotiana. langsdorffi; B) Nicotiana. glauca which did not have trichomes, but C) trichomes in the hybrid (x 200). G and Tr refer to guard cells and trichome, respectively.

*Hormone treatments*

We examined the effect of exogenous phytohormones on explants of *N. langsdorffii*, *N glauca* and its hybrid in vitro (Figure 3). A high auxin to cytokinin ratio promotes the formation of roots, whereas a low auxin to cytokinin ratio results in the regeneration of shoots (Sieberer et al. 2003). Kehr and Smith (1954) reported that auxin metabolism is associated with spontaneous tumor formation. The hybrid plants produced genetic tumors in the presence of IAA (Indole-3-acetic acid), while *N. glauca* formed calluses in 0.1~0.5 mg $l^{-1}$ IAA, and roots in 1 mg $l^{-1}$ IAA. *N. langsdorffii* was largely unresponsive to hormone treatment. In response to cytokinin, *N. glauca* produced calluses in 0.1~0.5 mg $l^{-1}$ kinetin and shoots in 2.0 mg $l^{-1}$ kinetin, whereas the hybrid produced genetic tumors even at 0.05 mg $l^{-1}$ kinetin. Cytokinins promote cell division in plants and influence numerous aspects of plant development and physiology such as seed germination, chloroplast differentiation and leaf senescence (Smigocki et al. 1993, Griffaut et al. 2004), and the levels of cytokinin in crown gall and genetic tumors are not same (Palni and Summons 1987). The fact that the hybrid plants produce genetic tumors even in the absence of IAA or kinetin could be due to abnormal responses associated with some kinds of stress. This is consistent with the suggestion that phytohormone imbalance is intimately involved in the induction and maintenance of genetic tumors (Smith 1988). We cultured the genetic tumor tissue in hormone-free medium in the same manner as crown gall tissues (Näf 1954, Kehr and Smith 1954), and, as shown in figures 3 and 4, neither *N. langsdorffii* nor *N. glauca* formed calluses to a significant extent even in the presence of kinetin plus IAA. The best callus or tumorous growth in explants of the parental and hybrid plants was observed on medium supplemented with 1 mg $l^{-1}$ IAA while the hybrid plants showed a better response in medium supplemented with 0.5 mg $l^{-1}$ kinetin (Figure 4). Feng et al. (1990) reported that the hormone-requiring character of a non-tumorous hybrid mutant was attributable to its lack of cytokinin-biosynthetic activity. In a similar way, the effect of *rolC*-transgenic carnation plants is related to altered levels of endogenous auxin or cytokinins (Casanova et al. 2004).




*N.L, *Nicotiana langsdorffii* ; N.G, *Nicotiana glauca* ; H, Hybrid plants

**Figure 3:** Effects of different concentrations of exogenous phytohormones on tumor and callus growth. Leaf discs of parents and hybrid were cultured on MS medium with different concentrations of indole-3-acetic acid alone (A), kinetin alone (B), and various combinations of indole-3-acetic acid and kinetin (C). Tissues were cultured at 25°C in the dark for 25 days.

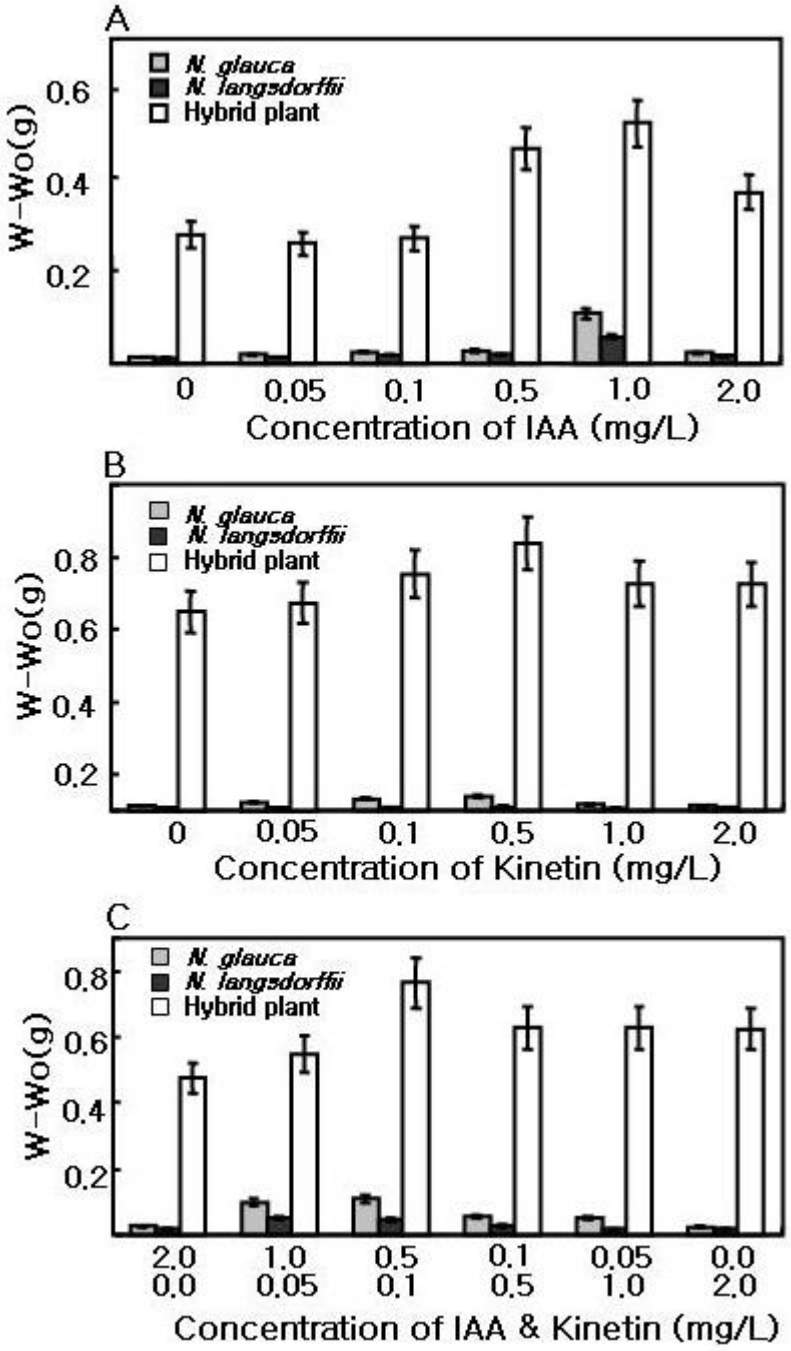



**Figure 4:** Growth rates of parental and hybrid *Nicotiana* plants supplemented with different concentrations of exogenous hormones. W refers to fresh weight and Wo to dry weight. Calluses were grown on various concentrations of indole-3-acetic acid alone (A), kinetin alone (B), and indole-3-acetic acid and kinetin (C). Plotted values are means (±SE) of five independent trials.

*Identification and analysis of genetic variation*

We used fifteen oligonucleotide primers for the RAPD analysis. Each primer amplified between one and five fragments, ranging from 0.4 to 7 kb (Figure 5). A total of 128 RAPD fragments were scored from the fifteen random primers (Table 1). Each amplification product was scored as present or absent in each individual, and faint or indistinct bands were ignored. During tumorization, *Nicotiana glauca* (Ng)ORF13 was induced at an early stage (Nagata et al. 1996), suggesting that *Ngrol* genes might be involved in the formation of genetic tumors. Tumor formation usually is caused by

activation of oncogenes or inactivation of tumor suppressor genes that both may be a consequence of genetic rearrangements. It seems possible that for instance a gene expressing a growth factor is regulated differently in the hybrid compared to the maternal organisms.

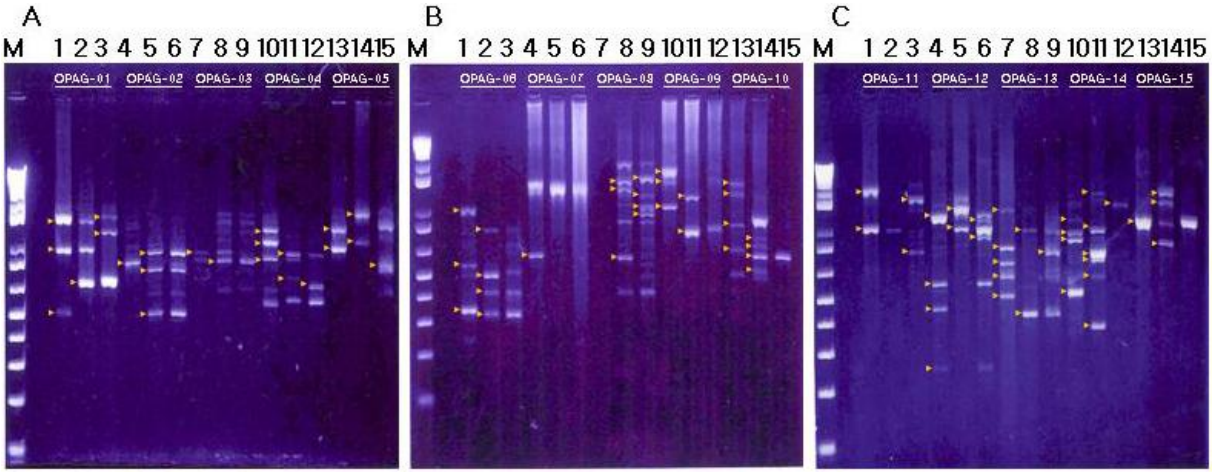



**Figure 5:** Randomly amplified polymorphic DNA patterns in hybrid plants and parents. The amplification products were run side by side on the gel, and bands were compared on the basis of molecular size. Ten µl of each polymerase chain reaction product was loaded on a 1% agarose gel. M refers to the 1 kb ladder marker loaded in the first lane of each gel. Lanes 1, 4, 7, 10, 13 are fragments from *Nicotiana. langsdorffii*; 2, 5, 8, 11, 14 are from *Nicotiana. glauca*; 3, 6, 9, 12, 15 are from hybrid plants. Gel A has been obtained with primers OPAG 1-5, gel B with primers OPAG 6-10, and gel C with primers OPAG 11-15.

**Table 1:** DNA sequences of primers used for RAPD analysis and the amplification products obtained with the *Nicotiana hybrid* and parents.

| Primer | Sequence (5' to 3') | The number of amplification products in the *Nicotiana* hybrid and parents | | |
|---|---|---|---|---|
| | | hybrid | *N. glauca* | *N. langsdorffi* |
| OPAG-01 | CTACGGCTTC | 3 | 3 | 3 |
| OPAG-02 | CTGAGGTCCT | 3 | 3 | 1 |
| OPAG-03 | TGCGGGAGTG | 4 | 5 | 2 |
| OPAG-04 | GGAGCGTACT | 3 | 2 | 4 |
| OPAG-05 | CCCACTAGAC | 3 | 2 | 2 |
| OPAG-06 | GGTGGCCAAG | 3 | 4 | 4 |
| OPAG-07 | CACAGACCTG | 1 | 1 | 2 |
| OPAG-08 | AAGAGCCCTC | 7 | 6 | 0 |
| OPAG-09 | CCGAGCGGTT | 1 | 2 | 3 |
| OPAG-10 | ACTGCCCGAC | 1 | 2 | 5 |
| OPAG-11 | TTACGGTGGG | 3 | 1 | 2 |
| OPAG-12 | CTCCCAGGGT | 4 | 2 | 3 |
| OPAG-13 | GGCTTGGCGA | 4 | 2 | 5 |
| OPAG-14 | CTCTCGGCGA | 1 | 5 | 3 |
| OPAG-15 | CCCACACGCA | 1 | 4 | 1 |

In conclusion, we found a correlation between the morphology of a tumor and its hormonal status (content or sensitivity). The calluses formed by the parental plants grew slowly in comparison to the hybrid plants with genetic tumors. Our observations underline the complexity of phytohormone interactions and provide information on the effect of hormones on the hybrid plants. The differences in RAPD products revealed in the present work may result from either DNA rearrangements or genetic modification during hybridization. In either event they show that the analysis of genetic polymorphism is a powerful technique for differentiating between parent species and hybrid.

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