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P. Bogani[a]; M. C. Intrieri[a]; M. Buiatti[a]
[a] Department of Evolutionary Biology "Leo Pardi", University of Florence, Via Romana, Florence, Italy

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Genetic and epigenetic factors in the control of dedifferentiation/ tumourisation in *Nicotiana* species and hybrids

P. BOGANI, M.C. INTRIERI, & M. BUIATTI

Department of Evolutionary Biology ''Leo Pardi'', University of Florence, Via Romana, 17 -50125 Florence, Italy

Abstract
In the present study, we analysed the effect of integration of single *Agrobacterium rhizogenes rol* genes or their combinations in the *rol*-containing *Nicotiana glauca*, in the non-*rol Nicotiana langsdorffii*, in their tumorous hybrid *N. glauca* × *N. langsdorffii* and in a non-tumorous mutant of it. We investigated whether the integration of transgenes could change the genotype's hormonal balance, thus inducing different *in vitro* morpho-physiological patterns. In the mutant, the presence and expression of endogenous *rol* genes and the effect of their induction were also studied. Transformation of *N. langsdorffii* with the whole *A. rhizogenes* complex and the integration of *rolB* and *rolC* genes in the mutant induced the production of autotrophic calluses exhibiting a tumorous morphology. Polymerase chain reaction (PCR) analysis of untransformed plants confirmed the presence of *rolB* and *rolC* in *N. glauca* and hybrids, whereas both genes were absent in *N. langsdorffii*. However, reverse-transcriptase PCR showed the loss of expression of both genes in the mutant. Treatment of the non-tumorous mutant plants with the demethylating agent 5-azacytidine revived *rolB* and *rolC* expression and induced the acquisition of hormone autotrophy.

Keywords: *DNA methylation, genetic tumours, Nicotiana, non-tumorous hybrid, rol gene expression*

Abbreviations: 5-AzaC, 5-azacytidine; BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; LS, Linsmaier and Skoog medium; NAA, naphthalene acetic acid; *rol*, root locus.

Introduction

Recent progress in the study of plant developmental processes is revealing the importance of epigenetic mechanisms such as DNA methylation, histone modifications, chromatin remodelling and RNA interference (Henderson & Jacobsen 2007) in the control of gene expression patterns. In particular, DNA methylation plays a major role in the control of transcriptional and post-transcriptional silencing of transgenes (Matzke et al. 1989; Matzke & Matzke 2004) and transposable elements (Lippman et al. 2004), and in the remodelling of genome structure and regulation patterns occurring in inter-specific hybrids and allopolyploids (Liu & Wendel 2003; Levy & Feldman 2004; Pires et al. 2004; Wang et al. 2004). In allopolyploids, DNA methylation and RNA interference permanently silence key genes for plant growth and development (Wang et al. 2006; Chen & Rajewsky 2007; Chen et al. 2008). Moreover, it was recently shown in *Arabidopsis* that DNA methylation of a putative negative *cis*-element (silencer) leads to de-repression of transcription of the downstream gene (Shibuya et al. 2009).

Since the early 1980s, habituation, i.e. self-sustained cell proliferation on media without growth regulators due to overproduction of phytohormones sharing morphological and biochemical similarity with genetic tumours, has been claimed to be an epigenetic phenomenon (Meins 1989a, 1989b). In the genus *Nicotiana*, the interaction between different genotypes (species) in interspecific hybrids is known to produce imbalances in the amounts and ratios of key phytohormones, which in turn induce spontaneous 100% tumour formation at late developmental stages (Kostoff 1930; Näf 1958; Smith 1965, 1972; Ahuja 1968; Meins 1989b). Tumour inception can be accelerated by stress (M. Buiatti

Correspondence: Dr. Patrizia Bogani, Department of Evolutionary Biology ''Leo Pardi'', Via Romana, 17/19, 50125 Firenze, Italy. Fax: +39-055-228850. Email: patrizia.bogani@unifi.it

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unpublished results) or chemical treatment notably affecting RNA synthesis (Buiatti 1968; Bayer 1982) and only occurs in crosses between species belonging to two different groups called ''plus'' and ''minus'' by Näf (1958). In two systems, namely *Nicotiana glauca* × *Nicotiana langsdorffii* (Ames & Smith 1969) and *Nicotiana longiflora* × *Nicotiana debneyi – tabacum* (Ahuja 1968), it has been proposed that the hormonal imbalance leading to tumour formation derives from the interaction between an oligogenic complex from one concurrent species, located on a single chromosome and a polygenic system from the other. In the former system (*N. glauca* × *N. langsdorffii*), a non-tumorous mutant of the tumorous hybrid was isolated from an irradiated population (Izard 1957) and was later shown to segregate as a single gene when crossed with the parental genotype (Durante et al. 1986). This mutant is co-dominant in the sense that heterozygous plants do not spontaneously form tumours but respond to wounding with active cell proliferation (Smith 1988). The homozygous non-tumorous plants show reduced auxin (Bayer 1982) and cytokinin (Nandi et al. 1990) synthesis and are less responsive to exogenous treatment with phytohormones (Collina Grenci et al. 1980) than their tumorous counterparts. The genetic nature of this mutant has been re-examined in the light of evidence suggesting that the non-tumorous condition may also involve mutations affecting tumour expression (Ahuja 1996).

Sequences highly homologous to the genes *rolB*, *rolC* and open reading frames (ORFs) 13–14 of *Agrobacterium rhizogenes* (Furner et al. 1986; Ichikawa & Syono 1991; Aoki et al. 1994) have been found in a number of *Nicotiana* spp. (including *N. glauca* but not *N. langsdorffii*), and their non-random presence in the phylogenetic tree, derived from ancestral infections by *A. rhizogenes*, suggested their possible role in evolution (Bogani et al. 1997; Intrieri and Buiatti 2001). It has also been suggested that the expression of these genes could have had an adaptive role in the establishment of new species (Aoki & Syono 1999).

To further investigate this intriguing proposal, an expression study of these genes in different genetic and physiological contexts (different genotypes) may help unravel the mechanisms underlying their regulation pattern. In this study, we analysed the effect of integration of single *A. rhizogenes rol* genes or their combinations in the genotypes of *rol*-containing *N. glauca*, non-*rol N. langsdorffii*, their tumorous hybrid *N. glauca* × *N. langsdorffii* (*glauca–langsdorffii*) and a non-tumorous mutant of it (*glauca–langsdorffii* non-tumorous). In particular, we investigated whether the integration of transgenes modified the genotype's hormonal balance, thus inducing different *in vitro* morpho-physiological patterns. Finally, the presence and expression of endogenous *rol* genes in normal plants was also analysed along with the effects of their induction.

Materials and methods

Plant materials and tissue culture conditions

Seeds of *N. glauca* Graham, *N. langsdorffii* Weinman, their tumorous amphidiploid hybrid (*N. glauca* × *N. langsdorffii*) and a non-tumorous mutant of it (Izard 1957) were aseptically sown on Linsmaier and Skoog (LS) basal medium (Linsmaier & Skoog 1965), and seedlings were grown for 2 months at $24 \pm 1^{\circ}$C under continuous light. For infection experiments, leaf discs (5-mm diameter) were obtained from a pool of leaves from axenic plantlets using a paper punch and placed upside down on hormone-free LS medium for 48 h before infection.

Bacterial strains and cultures

The *Agrobacterium* strains were wild-type *A. rhizogenes* 1855 grown in yeast mannitol broth medium (YMB) medium (Hooykaas et al. 1977) and *Agrobacterium* strain LBA4404 (Ooms et al. 1982) harbouring Bin 19 (Bevan 1984) plasmids containing ORF 11 (*rolB*), ORF 12 (*rolC*), ORFs 10–11–12 (*rolABC*) or ORF 15 (*rolD*) from pRi 1855 (Cardarelli et al. 1987). *Agrobacterium* strain LBA4404 with the binary vector pBI121 (Jefferson et al. 1987) was used as a control. Media for strains harbouring Bin 19 and pBI121 were supplemented with 100 μg/ml kanyamycin and 40 μg/ml rifampicin.

Plant transformation

The *Nicotiana* species and hybrids were transformed following a modified protocol of Horsch et al. (1985). Forty-eight hours after cutting, leaf discs were incubated for 25 min in 10^8 cells/ml exponentially growing cultures of each *Agrobacterium* strain, blotted dry and transferred to Petri dishes containing LS medium supplemented or not with 1 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l naphthalene acetic acid (NAA). During *Agrobacterium* co-cultivation, the *Nicotiana* control discs were maintained in LS containing the corresponding volume of the bacterial growth medium sterilised by filtration. After 48 h, the discs were harvested and transferred to agar plates with fresh medium containing carbenicillin 500 mg/ml and kanamycin 60 μg/ml and analysed for callus growth and differentiation. Transgenic calluses and shoots were then exposed to further selection with kanamycin 100 μg/ml.

Finally, shoots were rooted on hormone-free LS medium under 300 μg/ml kanamycin selection. Transgenic and control plants were screened for their dedifferentiation/habituation-tumorisation capacity.

DNA and RNA isolation

DNA was extracted from leaf and callus tissue according to the method of Doyle and Doyle (1989). Total RNA and poly-A^+ mRNA were isolated from untransformed leaves using, respectively, the RNeasy^{TM} Plant Total RNA kit (QIAGEN, M-Medical Genenco, Florence, Italy) and the QuickPrep® Micro mRNA Purification kit from GE Healthcare (Bio-Sciences Ltd., Milan, Italy), according to the suppliers' instructions.

PCR analysis for the presence of transgenes and introgressed rol genes

Transformation of all calluses and regenerated plantlets after infection was tested through polymerase chain reaction (PCR) amplification of the *npt*II gene for kanamycin resistance. PCR was performed with the two specific primers 5′>GTC GCT TGG TCG GTC ATT TCG<3′ and 5′>GTG ATC TCA CCT TGC TCC TGC C<3′, according to Manders et al. (1994).

The primers used for PCR amplification of *A. rhizogenes* or *N. glauca rolB* and *rolC* genes were designed on T-DNA sequences published by Furner et al. (1986) and corresponded to *A. rhizogenes*-specific *rolB* primers, top 5′>CAC TTG CCT TTT TCG TAA CTA<3′ and bottom 5′>TTA GGC TTC TTT CTT CAG GTT<3′; *A. rhizogenes*-specific *rolC* primers, top 5′>CTT TTT ATT ATT TGC TCC A<3′ and bottom 5′>TAA AGG GAA ACA AGT GAC<3′; *N. glauca rolB* primers, top 5′>ATG GCT TCC CAA TTC<3′ and bottom 5′>AAC TAT GTA GTG CCG AAA GCT<3′ and *N. glauca rolC* primers, top 5′<ATG GCT GAA GTT GAC CTG TGT<3′ and bottom 5′>CCA TCA GTT CAT TCC AAA TTT<3′. PCR reactions were performed in 50 μl of 10 mM Tris-HCl pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 200 μM each of deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP) (Pharmacia, Milan, Italy), 200 nM of each primer (Genenco, M-Medical) and 100 ng of template DNA. Amplification was carried out in a PTC-100^{TM} Thermal Cycler (MJ Research), programmed for one denaturation cycle of 5 min at 94°C and a further 25 cycles of 1 min at 94°C, 1 min at 49°C and 1 min at 72°C. Taq polymerase (Roche Diagnostics, Milan, Italy) was added to the reaction tubes after a hot start.

Introgressed rol gene expression analysis

To examine the possible role of DNA methylation in the regulation of introgressed rol gene expression in untransformed, non-tumorous mutants, we treated 2-month-old plantlets with 30 μM 5-azacytidine (5-AzaC; Sigma-Aldrich, Milan, Italy) for 48 h. After treatment, leaf explants from different plantlets were obtained and maintained on hormone-free LS medium to test their capacity for dedifferentiation and cell growth without phytoregulators. For this purpose, 30 explants from treated and untreated plants were incubated on LS without hormones or supplemented with 0.4 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), and the presence of callus was scored at the end of the first 30 days of culture. Callus growth was measured as the increase in fresh weight [(fw − iw)/iw; fw=final weight; iw=initial weight] for 60 days (two further transfers) on the same media. The same plants were used for DNA and RNA isolation.

Reverse-transcriptase (RT)-PCR was performed as in Van Der Straeten et al. (1992) with minor modifications. One microgram of total RNA or 10 μl of an mRNA purification was mixed with 100 ng of the 3′-oligonucleotide in 50 μl of 1 × buffer [67.2 mM Tris–HCl pH 8.8, 16.6 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 1.68 mg/ml bovine serum albumin (nuclease free), 0.72% 2-mercaptoethanol and 33 units RNasin (Promega, Milan, Italy)], and incubated for 5 min at 85°C and then for 5 min at 45°C. The tubes were put on ice and 50 μl of a reaction mixture containing 100 ng of the 5′-oligonucleotide, 1 × buffer, 2 U of Taq polymerase and 8 U of AMV Reverse Transcriptase (Promega), 66 U RNasin and 0.2 mM of each dNTP were added. Reverse transcription was performed at 40°C for 45 min. The PCR reaction for the amplification of *N. glauca rol* genes followed immediately, as described above.

After PCR, 10–20 μl of products were loaded and electrophoresed on a 1% agarose gel in Tris-acetate-EDTA (TAE) buffer (Maniatis et al. 1982) containing 0.5 μg/ml ethidium bromide and photographed under UV light. The Roche Marker VI (pBR328 BglI/HinfI) was used as a size marker in all gels.

Restriction endonuclease digestion of DNA and methylation analysis

The genomic DNA was extracted from leaves of non-tumorous *glauca–langsdorffii* plantlets, untreated or treated with 30 μM 5-AzaC and 5 μg were digested to completion at 37°C with methylation-sensitive restriction enzymes MspI and HpaII

(Roche), according to the supplier's instructions. Digested DNAs were separated by electrophoresis on a 1.2% agarose gel in TAE buffer at 30 V overnight. The relative amount of methylation was determined after densitometric scanning (Pharmacia LKB, Ultra Scan XL) of the negative gel images and transferring of data files to GEL SCAN XL software.

Southern blot analysis

Amplified DNA or c-DNA samples were transferred to positively charged nylon membranes (Roche) by alkali blotting and fixed to the membranes with a UV cross-linker (XL 1000, Spectronics Corporation). Filters were then hybridised overnight at 42°C with dig-UTP labelled DNA fragments (non-radioactive DNA labelling and detection kit, Roche) corresponding to *rolB* and *rolC Agrobacterium* genes purified by elution from agarose gel (Geneclean kit, BIO 101 Inc., La Jolla, CA). Post-hybridisation washes were carried out twice at room temperature with 2× saline-sodium citrate (SSC), 0.1% SDS for 10 min and twice at 65°C with 0.1× SSC, 0.1% sodium dodecyl sulphate (SDS) for 30 min. Filters, incubated in disodium 3-(4-methoxyspiro {1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1]decan}-4-yl) phenyl phosphate (CSPD) (Roche) as chemiluminescent substrate, were then exposed to Kodak X-OMAT AR films with intensifying screens at room temperature for 2 h to overnight.

Results

Transformation of leaf discs of Nicotiana species and hybrids with Agrobacterium rol genes

The effect of infection on differentiation and dedifferentiation capacity of infected leaf discs on hormone-free medium. One of the known causes of changes in gene expression in transgenic plants is the interaction between the transgene and partially or totally homologous endogenous plant genes (see Matzke & Matzke 2004). With the following experiments, we wanted to test the effect of *rol* gene transformation on differentiation and dedifferentiation of *Nicotiana rol* and non *rol*-containing genotypes. Two independent experiments were carried out leading to the transformation of a total of 60 leaf discs (30 each experiment) per genotype for each *Agrobacterium* strain. Moreover, the binary vector pBI121 was used as an empty vector control. Figure 1 shows the effect of infection with the wild-type 1855 *Agrobacterium* strain and other strains bearing plasmids with different single *rol* genes or *rol* gene combinations on cell proliferation and differentiation in the first 60

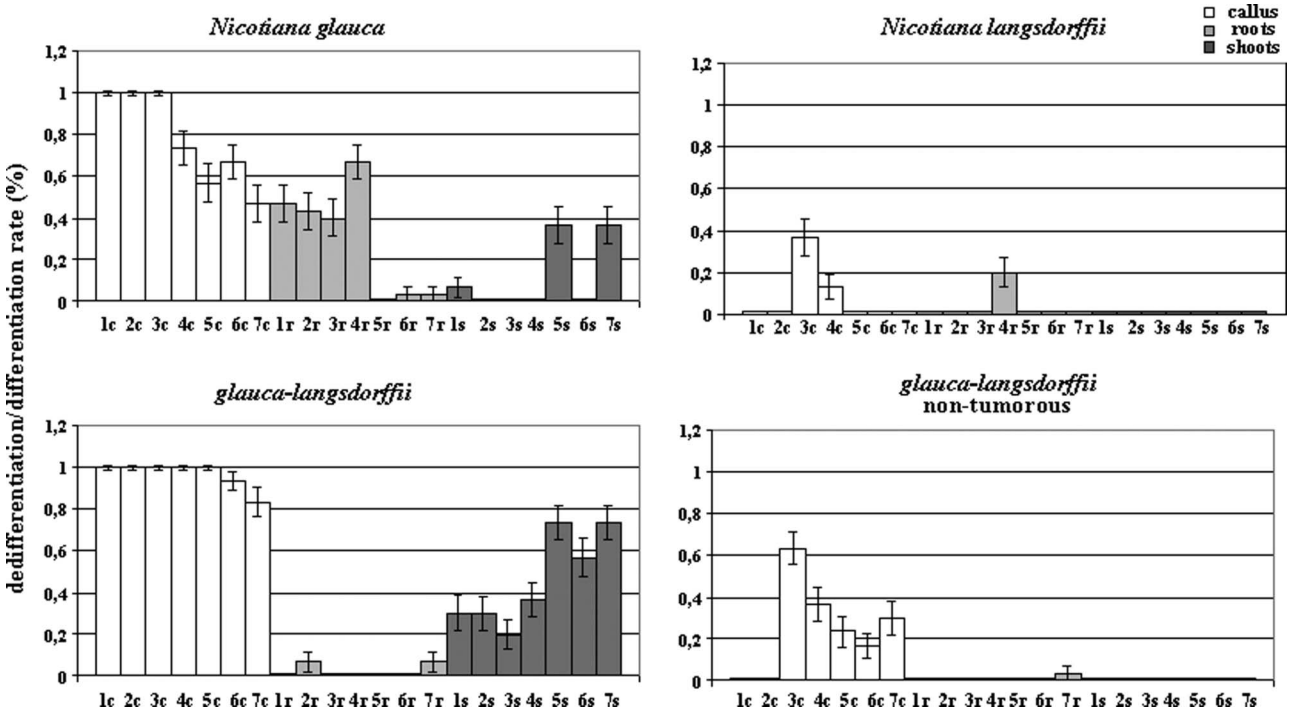

Figure 1. Effect of transformation of *Nicotiana* genotypes with *A. rizhogenes* strains bearing different *rol* genes on tumour induction (c), root (r) and shoot (s) regeneration. 1: Untransformed control explants; 2: pBI121 transformed explants; 3: 1855 transformed explants; 4: *rolB* transformed explants; 5: *rolC* transformed explants; 6: *rolD* transformed explants; 7: *rolABC* transformed explants. Percentage values are the average between two independent experiments.

days after infection. To obtain information on their endogenous hormonal pattern, infected explants were maintained on media without exogenous auxins and cytokinins and analysed for morphogenetic behaviour. As shown in Figure 1, only control tissues of *N. glauca* and the tumorous genotype *glauca–langsdorffii*, both containing the whole set of *rol* genes, showed active cell proliferation (callus formation) after cutting. As expected in the presence of a negative interaction between the integrated *rol* genes and endogenous homologous sequences, in *N. glauca*, the percentages of proliferating explants were reduced by infection in all cases in comparison with wild-type infected explants. Moreover, while only infection with *rolABC* reduced the spontaneous autotrophic cell proliferation in genetically tumorous cells, callus formation was induced in *N. langsdorffi*, a non *rol*-containing species, by infection with *rolB* and 1855. The same effect was observed in the non-tumorous mutant after infection with all bacterial strains, particularly 1855. However, while 1855- and *rolB*-transformed cells continued to divide indefinitely, maintaining a tumorous healthy phenotype on hormone-free medium, calluses transformed with the other *A. rhizogenes* complements displayed a ''flat'' phenotype in culture in which cells periodically died and, in all cases, showed very slow growth. Root formation was significantly increased by infection with *rolB* in *N. glauca* and *N. langsdorffii*, while shoot formation only occurred in a few cases in *N. glauca* and in the tumorous genotype, in which infection with *rolC* or *rolABC* had an enhancing effect. No significant differences were observed between non-transformed genotypes and pBI121 transgenic ones. The tumorous phenotypes and all regenerated putatively transgenic plantlets were analysed by PCR for the presence of the *nptII* gene coding for neomycin phosphotransferase II, which confers resistance to the antibiotic kanamycin, and for the presence of each single *rol Agrobacterium* gene. In both cases, the results demonstrated the integration of structurally intact copies of transferred genes (data not shown).

Regeneration of transgenic rol plants from infected leaf discs grown in the presence of exogenous cytokinins and auxins: habituation/tumorisation capacity of transgenic regenerated plants. In this series of experiments, infected explants were grown on regeneration medium supplemented with high levels of BAP and low levels of auxin and with 300 μg/ml kanamycin as a selectable marker. Under these conditions, *N. glauca* infected with *rolB* and *rolD* formed tumours on the regeneration medium, but produced shoots in the presence of *rolC*, *rolABC* or the wild type 1855. *N. langsdorffii* and hybrids produced shoots after infection with all *rol* gene combinations, with all transgenic shoots of the tumorous hybrid and non-tumorous-1855 developing teratomas throughout

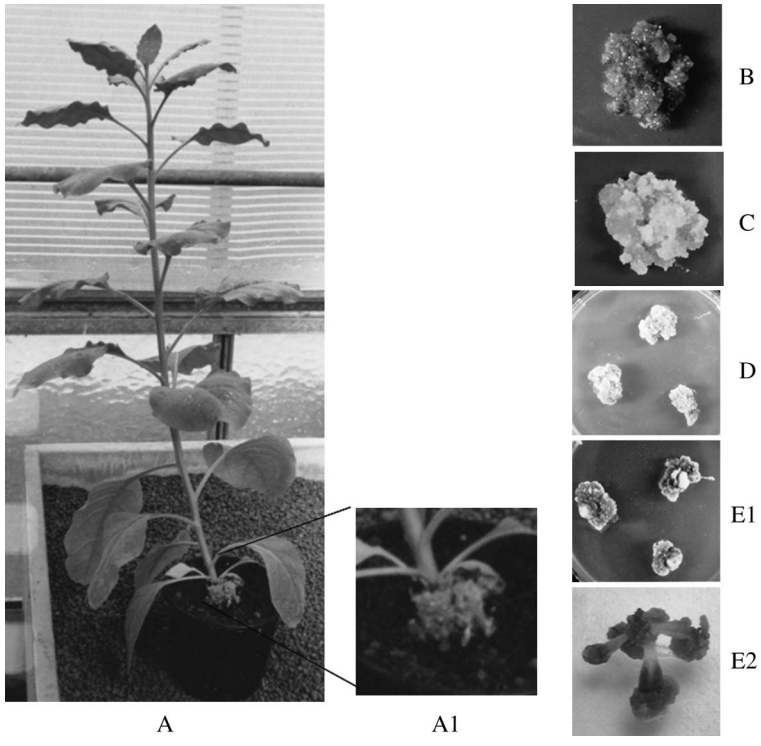

Figure 2. Autonomous proliferation capacity of leaf discs from *N. langsdorffii*-1855 (B), *N. langsdorffii-rolB* (C), and non-tumorous *glauca–langsdorffii-rolB* (D) and *rolC* (E1, E2) plants on hormone-free medium. (A) The *glauca–langsdorffii* wild type with a spontaneous genetic tumour (A1).

subsequent subcultures. Several transformed clones were isolated for each shoot-forming *Nicotiana* genotype. DNA and RNA from young leaves of 5- to 6-week-old plants were assayed for the presence and expression of transgenes through PCR and RT-PCR amplification (data not shown). Since wounds are known to induce cell proliferation in the tumorous amphidiploids, we tested the ability of *A. rhizogenes* genes to induce dedifferentiation competence in *N. langsdorffii* and to restore it in the non-tumorous hybrid by excising leaf discs from both genotypes transgenic for 1855, *rolB*, *rolC*, *rolABC* or the pBI121 control, and transferring them to hormone-free medium, supplemented with kanamycin 100 μg/ml. Only discs from *N. langsdorffii*-1855 or -*rolB* and non-tumorous hybrid-*rolB* and -*rolC* plants proliferated without the addition of exogenous hormones, with undifferentiated cells showing a fully autonomous tumorous phenotype (Figure 2). Moreover, non-tumorous-*rolC* plants spontaneously formed tumours when stressed by contact with agar (Figure 2-E2) or after wounding in transgenic plantlets (Figure 3).

Figure 3. Integration of *rolC* induces cell proliferation at a wounding site in transgenic, regenerated, non-tumorous *glauca–langsdorffii* plants.

RT-PCR analysis of introgressed rolC and rolB gene expression in untransformed rol-containing Nicotiana plants

PCR analysis of *rolC* and *rolB*, using the flanking sequences of the *N. glauca* corresponding genes as primers, confirmed the presence of these genes in the tumorous hybrid *glauca–langsdorffii* but also in its non-tumorous mutant. The PCR data were confirmed by Southern hybridisation with *A. rhizogenes rolB* and *rolC* genes used as probes. Moreover, a *rolB-C* encompassing fragment cloned from the non-tumorous hybrid did not show any sequence difference when compared with *N. glauca* or *glauca–langsdorffii* tumorous hybrid genes and promoters (data not shown).

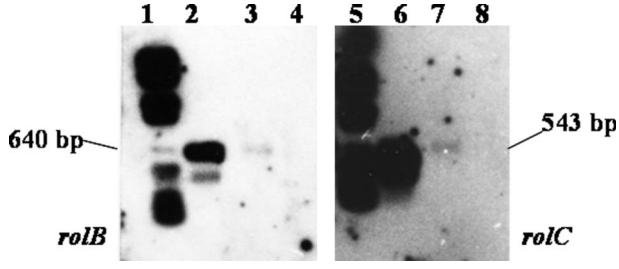

Figure 5. RT-PCR analysis of *rolB* and *rolC* gene expression on mRNAs from non-tumorous *glauca–langsdorffii*, treated with 30 μM 5-azacytidine (lanes 3 and 7) or untreated (lanes 4 and 8), after hybridisation with *A. rhizogenes rol* genes. Lanes 2 and 6 correspond to *rolB* and *rolC* genes amplified from genomic total plant DNA. Lanes 1 and 5: molecular weight marker pBR328, BgII, HinfI (Roche).

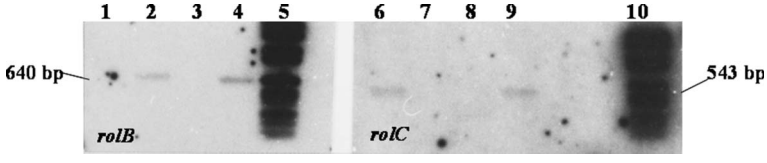

Figure 4. RT-PCR analysis of *rolB* and *rolC* transcription carried out on total RNA from *N. langsdorffii* (lanes 1 and 7), *N. glauca* (lanes 2 and 6), non-tumorous *glauca–langsdorffii* (lanes 3 and 8) and tumorous *glauca–langsdorffii* (lanes 4 and 9). Twenty microlitres of RT-PCR products were loaded on a 1% agarose gel and blotted on a Nylon filter (Roche). The filter was hybridised with the homologous sequences of *A. rhizogenes*. Lanes 1 and 10: molecular weight marker pBR328, BgII, HinfI (Roche).

To investigate whether the untransformed non-tumorous hybrid phenotype is related to the loss of expression of introgressed genes, we used RT-PCR transcription analysis rather than RNA Northern blotting to detect the very low levels of *rol* mRNAs. Figure 4 shows Southern blot analysis of RT-PCR reactions performed on different total RNA samples from the four *Nicotiana* genotypes using *N. glauca rol* primers. Interestingly, a positive signal was clearly visible in the tumorous hybrid and in *N. glauca* but was absent in the non-tumorous mutant.

Induction of rol expression in the non-tumorous hybrid by 5-AzaC

RT-PCR of mRNAs extracted from axenic, 2-month-old, non-tumorous *glauca–langsdorffii* plantlets that had been exposed to 30 μM 5-AzaC for 48 h showed de-repression of *rolB* and *rolC* transcription (Figure 5, lanes 3 and 7). In addition, 5-AzaC restored hormone autotrophy in non-tumorous cells as shown by the ability of treated leaf explants to dedifferentiate and grow on hormone-free medium. Table I shows the effect of treatment with the demethylating agent on the ability of leaf explants from normal non-tumorous mutant and *N. langsdorffii* plants to form callus after 30 days of culture on hormone-free medium. Calluses were then excised from explants and grown in the same hormone-free medium or in a medium supplemented with 0.4 mg/l of the auxin 2,4-D, in which *in vitro* cultured cells of both genotypes normally divide and grow. Figure 6 shows that treatment with 5-AzaC led to induction of a habituated phenotype in the non-tumorous *glauca–langsdorffii* mutant. These data agree with previous results on the behaviour of non-tumorous cell suspension cultures treated with 5-AzaC during the first phase of the culture cycle (Durante et al. 1989). As a control, untreated leaf explants were also put on hormone-free medium, with negative results in terms of dedifferentiation capacity. *N. langsdorffii* plants, lacking introgressed *rol* genes, were also treated with 5-AzaC and explants were induced to dedifferentiate on hormone-free medium. However, only a few explants produced calluses, with slow growth limited to the first 60 days of culture (Table I, Figure 6).

The demethylation of DNA in non-tumorous *glauca* × *langsdorffii* plantlets induced by 5-AzaC treatment was also directly shown by restriction analysis with MspI and HpaII methyl-sensitive isoschizomers and confirmed by densitometric analysis of the restriction patterns (Figure 7). When DNA extracted from untreated tissue was digested with MspI and HpaII (respectively lines d and b in Figure 7), the degree of digestion was lower than that of DNA from treated tissues (lines c and a). Curves e and f represent differences between restriction patterns, with, respectively, the HpaII and MspI isoschizomers of DNA from 5-AzaC-treated and -untreated plants.

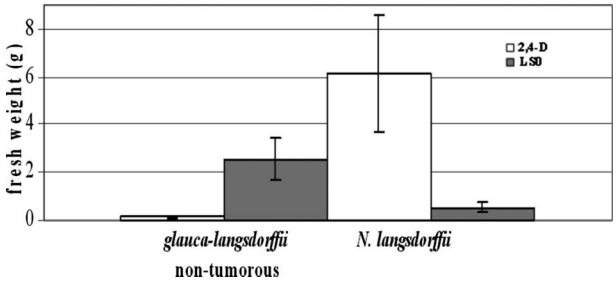

Figure 6. Increase in fresh weight of non-tumorous *glauca–langsdorffii* and *N. langsdorffii* calluses from leaf explants after treatment of normal plants with 30 μM 5-azacytidine. Calluses were cultured for 60 days on a medium without hormones (LS0) or supplemented with 0.4 mg/l 2,4-D.

Table I. Effect of treatment with 30 μM of 5-AzaC on the ability of leaf explants from normal, non-tumorous mutant *glauca-langsdorffii* and *N. langsdorffii* plants to form callus (habituation induction) after 30 days of culture in hormone-free medium (LS0).

Genotypes	% Leaf explants forming callus on LS0
glauca–langsdorffii non-tumorous	0
glauca–langsdorffii non-tumorous/5-AzaC	70.0 $\pm$ 0.09
N. langsdorffii	0
N. langsdorffii/5-AzaC	33.3 $\pm$ 0.08

Discussion

The aim of the present study was to investigate the possible connection between the formation of genetic tumours in *Nicotiana* hybrids and the presence and expression of *A. rhizogenes rol* sequences, as suggested by several authors (Furner et al. 1986;

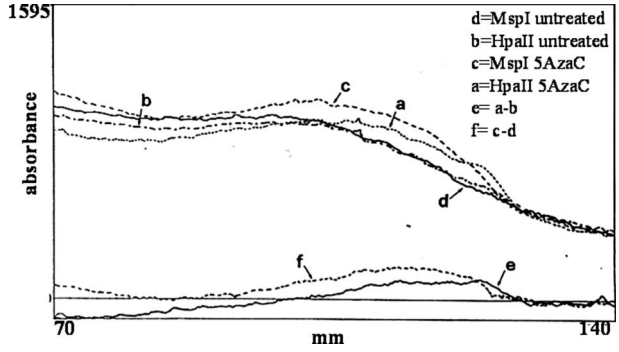

Figure 7. Densitograms of MspI and HpaII digestions of DNA from non-tumorous *glauca–langsdorffii*, untreated or treated with 30 μM 5-azacytidine. Digested DNA was electrophoresed on 1.2% agarose gel stained with ethidium bromide. Negative image of the gel was then scanned by videodensitometry (Pharmacia LKB, Ultra Scan XL). The X-axis represents the number of points that were scanned in each lane.

Ichikawa et al. 1990, Ichikawa & Syono 1991; Udagawa et al. 2004). For this purpose, we studied the effect of transformation with single *rol* genes and different combinations of them on the establishment of a tumorous or non-tumorous phenotype in *rol*-positive and *rol*-negative *Nicotiana* genotypes belonging to the amphidiploid hybrid *glauca–langsdorffii* and the *N. langsdorffii* system, including a non-tumorous "mutant" described for the first time by Izard (1957). Genetic tumours are neoplastic overgrowths that spontaneously develop in about 10% of *Nicotiana* inter-specific hybrids (Smith 1972). These overgrowths are characterised by cells proliferating *in vitro* without the aid of phytohormones, a feature also common to a naturally occurring phenomenon termed "habituation" in which undifferentiated callus cultures permanently lose hormone requirements (especially for cytokinins) in *in vitro* subcultures. It has been suggested that epigenetic processes such as DNA methylation contribute to the acquisition of phytohormone habituation (Durante et al. 1989; Meins 1989a, 1989b; Pischke et al. 2006).

As mentioned earlier, copies of *rolB*, *rolC*, ORFs 13 and 14 from *A. rhizogenes* are present in one of the parental species of our system, *N. glauca* (Furner et al. 1986; Aoki et al. 1994), but not in *N. langsdorffii*. In a study by Ichikawa et al. (1990), it was noticed that the *N. glauca* (Ng) *rolB-C* complex was expressed in genetic tumours of the hybrid *glauca–langsdorffii.* Transcription of NgORF13 and NgORF14 was also demonstrated in this hybrid (Aoki et al. 1994), with a high level of NgORF13 expression in tumours but not in leaf tissues (Udagawa et al. 2004). On the other hand, Smith (1988) showed, with a series of backcross experiments, that *N. glauca's* contribution to the tumour-prone, amphidiploid *glauca–langsdorffii* was limited to genes located on a single chromosome responsible for tumour inception, while tumour growth seemed to be regulated by a polygenic *N. langsdorffii* complex. These data agree with the hypothesis that genes homologous to the *A. rhizogenes* complex present in *N. glauca* contribute to tumour formation in hybrids with *N. langsdorffii* through complementation of a polygenic complex, leading to the high cytokinin level of this species (Nacmias et al. 1987).

These studies prompted our experiments aimed at the restoration of a tumour-prone phenotype in *N. langsdorffii* and in the non-tumorous mutant of the hybrid through transformation with *A. rhizogenes* genes. As summarised in Table II, transformation with the whole *A. rhizogenes* complex of non-tumorous *glauca–langsdorffii* and *N. langsdorffii* induced the production of autotrophic callus. Effects similar but not identical to those produced by transformation with *A. rhizogenes* 1855 were also caused by *rolB* or *rolC* alone depending on the physiological state of the tested plants or tissues. Leaf discs from *N. langsdorffii*-1855 transgenic plants spontaneously produced tumorous autotrophic calluses with a morphology similar to those of the tumorous hybrid *glauca–langsdorffii* when grown on hormone-free media. Excised explants from *rolC* transgenic *N. langsdorffii* and non-tumorous plants also produced autotrophic growth. Moreover, during the first phase of *in vitro* culture, transgenic non-tumorous plants spontaneously formed tumours when stressed by contact with agar or after wounding. These results fully confirm the role of *rol* genes in the induction of genetic tumours and, in view of their known effects on hormone balances (Meyer et al. 2000), suggest that the switch to autotrophic growth *in vitro* (a part of the tumorous phenotype) is induced by changes in the endogenous levels of free auxin and, in particular, cytokinin.

This hypothesis seems to fit well with the general idea that genetic tumours will develop in the presence of both an optimal equilibrium between high auxin and cytokinin synthesis and the genes needed for their utilisation. The disruption of this equilibrium, by either a decrease or increase in auxins or cytokinins, may lead to inhibition of tumorogenesis, as shown in this study for *N. glauca* and partially for the tumorous genotype. However, as mentioned before, the presence of additional copies of single sequences may lead to co-suppression processes and thus may be the cause of the observed reduction in autonomous cell proliferation in habituation and tumour-prone genotypes.

The hormonal hypothesis fits well with a series of other data. In the first place, there seem to be high auxin levels in *N. glauca* and high cytokinin levels in *N. langsdorffii*, which would combine in the hybrid leading to the hormonal balance presumably needed for tumour formation. This is consistent with previous experiments carried out in our laboratory (Nacmias et al. 1987) in which the transformation of *N. glauca* and *N. langsdorffii* with *iaa*M and *iaa*H genes (both needed to synthesise auxins) and with *ipt*

Table II. Effect of the infection of *Nicotiana* species and hybrids with *A. rhizogenes rol* genes on callus formation on hormone-free medium.

Genotypes/ treatments	*N. glauca*	*N. langsdorffii*	Tumorous hybrid	Non-tumorous hybrid
Untrasformed control	++	−	++	−
1855	++	+	++	++
*rol*B	+	+	++	++
*rol*C	+	−	++	+
*rol*D	+	−	+	+
*rol*ABC	+	−	+	+

gene (encoding a key enzyme in cytokinin synthesis) from *A. tumefaciens* differentially influenced the genotypes' hormone balances. Furthermore, very recently, we demonstrated that the insertion of the rat glucocorticoid receptor gene drastically inhibited root regeneration in *N. glauca* reducing its endogenous auxin content, whereas a slight capacity for rooting and a slight increase in indole-3-acetic acid (IAA) content was noted in *N. langsdorffii* transgenic plants compared with the corresponding wild-type plants (Giannarelli et al. 2010). All these findings are in accordance with previous observations reported in the literature concerning differences in hormone content in *N. glauca* and *N. langsdorffii* genotypes (Bayer 1982; Bogani et al. 1985; Aoki et al. 1994; Intrieri & Buiatti 2001). Moreover, Feng et al. (1990) also showed restoration of a tumorous phenotype *in vitro* (plants were not regenerated) in the non-tumorous genotype by integration of the *ipt* gene, and they confirmed a lower cytokinin content in the mutant.

Thus, the results of previous experiments and the data presented here seem to support the initial hypothesis that the tumorous phenotypes could partly derive from complementation between a species with high auxin synthesis and the required genes from *A. rhizogenes* (*N. glauca*) and another species (*N. langsdorffii*) with high cytokinin levels and other polygenic enhancing factors. The critical question at this point concerns the nature of the modification of the non-tumorous hybrid *glauca–langsdorffii* that led to the suppression of the tumorous phenotype. The reasons for such behaviour could be the deletion of *rol* genes in the mutant or their repression, possibly through methylation. The PCR analyses reported in this study showed the presence of conserved *rol*-like genes in the mutant but the lack of their expression. However, treatment of normal plants with the demethylating agent 5-AzaC both restored *rol* gene expression and made treated leaf explants competent for autonomous cell division and growth, as shown by their dedifferentiation capacity on hormone-free medium. This feature, termed chemical tumorogenesis or habituation, was inhibited in normal non-tumorous plants and in *N. langsdorffii* (the *rol*-negative species), in the latter also following 5-AzaC treatment. These results suggest that DNA methylation in the non-tumorous hybrid inhibits *rol* gene expression and autotrophic cell proliferation on hormone-free medium.

Plant cell development is controlled by hormonal regulation of DNA methylation (Finnegan et al. 1998), and it is known that hormonal control of epigenetic processes such as modifications of the expression profiles of genes involved in DNA methylation plays a role in habituation (Meins 1989a, 1989b; Pischke et al. 2006). Therefore, our results suggest that the non-tumorous genotype of the *glauca–langsdorffii* amphidiploid behaves like an ''epi-mutant'', the restoration of its *rol*-like activities fitting well with the initial assumption that these sequences contribute to the tumorous phenotype of the hybrid. It should be remembered that the non-tumorous mutant derives from a single non-tumorous plant found by Izard (1957) in a field of plants that had been irradiated and thus exposed to stress, a possible cause of epigenetic changes. The *in vitro* behaviour of this strain was shown to mimic that of *N. glauca* also at the molecular level, as there was cell proliferation of both of them but not of the tumorous genotype. On the other hand, tissues of the F1 between the two strains were found to behave like the tumorous genotype, confirming the ''recessive'' nature of the ''mutant'' (Smith 1988). If confirmed, the results of our study would place Izard's mutant in the fast-growing list of cases of epigenetic inheritance in plants.

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