Phyton (Horn, Austria)	**Vol. 37**	Fasc. 2	229–239	2. 3. 1998

Nicotine Content in Transformed Haploid and Dihaploid Tissues of Tobacco (*Nicotiana tabacum* L.)

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With 1 Figure

Received June 9, 1997

Key words: Dihaploids, haploids, *Nicotiana tabacum*, nicotine content, transformation.

Summary

Ćurković Perica M., Gillet F., Jacquin-Dubreuil A., Krsnik-Rasol M. & Jelaska S. 1998. Nicotine content in transformed haploid and dihaploid tissues of tobacco (*Nicotiana tabacum* L.). – Phyton (Horn, Austria) 37 (2): 229–239, 1 figure. – English with German summary.

Three anther-derived haploid and three haploid-derived dihaploid tissue lines of tobacco were transformed with three strains of *Agrobacterium tumefaciens*: B6S3, pGV2215 and pGV2255. Unorganised, shooty and rooty tumours were induced, respectively. Tumour morphology and the growth rate of transformed tissues depended mainly on the bacterial strain. The presence of T-DNA genes in transformed tissues was confirmed by polymerase chain reaction (PCR). By means of high performance liquid chromatography (HPLC) nicotine was found only in the control plants and in the roots transformed with *Agrobacterium tumefaciens* pGV2255. Its content did not depend on the ploidy level of the tissue. Transformed roots could not be subcultured

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because of callogenesis. The callus which overgrew transformed roots did not produce nicotine.

Zusammenfassung

ĆURKOVIĆ PERICA M., GILLET F., JACQUIN-DUBREUIL A., KRSNIK-RASOL M. & JELASKA S. 1998. Nikotingehalt in transformierten haploiden und dihaploiden Geweben von Tabak (*Nicotiana tabacum* L.). – Phyton (Horn, Austria) 37 (2): 229–239, 1 Abbildung. – Englisch mit deutscher Zusammenfassung.

Drei von Antheren stammende haploide Gewebelinien und drei aus solchen haploiden Linien gewonnene dihaploide Gewebelinien von Tabak wurden mit drei Stämmen von *Agrobacterium tumefaciens:* B6S3, pGV2215 und pGV2255 transformiert. Unorganisierte, sproß- sowie wurzelähnliche Tumore wurden induziert. Die Morphologie der Tumore und die Wachstumsrate der transformierten Zellen hängt hauptsächlich vom Bakterienstamm ab. Das Vorhandensein von T-DNA-Genen in transformierten Geweben wurde mittels PCR bestätigt. Mittels HPLC konnte Nikotin nur in den Kontrollpflanzen und in den Wurzeln, welche mit *Agrobacterium tumefaciens* pGV2255 transformiert wurden, gefunden werden. Der Gehalt hängt nicht vom Ploidiegrad des Gewebes ab. Transformierte Wurzeln konnten wegen der Kallusbildung nicht subkultiviert werden. Der Kallus, welcher die transformierten Wurzeln überwuchs, produzierte kein Nikotin.

Introduction

Nicotine is the major alkaloid in commercial tobaccos, and its level is affected by many factors (TSO 1990). The relationship between alkaloid content and ploidy of plant material had been studied, but no clear correlation has been demonstrated (JACQUIN-DUBREUIL & al. 1991). BURK & MATZINGER 1976, SCHILTZ & al. 1979–1980 showed higher alkaloid content in haploid and dihaploid plants than in diploid source. BURK & CHAPLIN 1976 reported that in tobacco an increase in alkaloid level can result from changes of the genome size from diploid to polyploid status, but the correlation between nicotine content in haploids and derived dihaploids was not discussed.

Transformation by means of *Agrobacterium tumefaciens* alters hormone level in plant tissue (GAUDIN & al. 1994). Expression of T-DNA oncogenes causes a disturbance of the natural growth regulator balance and leads to proliferation of tumorous cells (REAM 1989). A tumour morphology is mainly determined by the *aux* and *cyt* loci. Inactivated auxin genes *1* and/or *2* or cytokinin gene *4* induce shooty or rooty tumours, respectively (BARRY & al. 1984, THOMASHOW & al. 1984). In some plant species genes *5* and *6b* were found to be responsible for tumour morphology (TINLAND & al. 1989, KÖRBER & al. 1991). Gene *3* codes the synthesis of octopine synthase (HOOYKAAS & SCHILPEROORT 1992).

Since the alkaloid content depends on hormonal balance in plant tissue, an altered nicotine content could be expected in transgenic tissues. SAITO & al. 1991 transformed tobacco by different strains of *A. tumefaciens*

and *A. rhizogenes*. Nicotine was produced by rooty tumours and by hairy roots, but not by shooty tumours. The production of alkaloids was high in hairy roots and in rooty tumours, but it was low in unorganised tissue of *Nicotiana* (ROBINS & al. 1987).

Alkaloid content was tested in a variety of transformed plant tissue, but the comparison of nicotine content in transformed haploid and dihaploid plant tissues of the same genotype has not been reported.

Our aim was to find out if dihaploids produced more nicotine than haploids, which they were derived from, and how the transformation of haploids and dihaploids affects the nicotine production.

Material and Methods

Plant tissue culture methods

Anther culture of tobacco (F1 generation of a cross between variety Virginia D × GV3NN11 line) was established following the procedure of NITSCH & NITSCH 1969. Buds were collected from plants grown in the field, and treated at 4 °C for 24 hours. Anthers were sterilised in 70% ethanol (10 s) and in 1.5% Izosan-G (a chlorine product of "Pliva", Zagreb) (5 min), and placed on the medium recommended by BAJAJ 1983. Haploid plantlets that emerged from anthers were transferred to the medium for further growth (KASPERBAUER & WILSON 1979). Diploidization was achieved according to the procedure of KASPERBAUER & COLLINS 1972. Shoots were regenerated from leaf explant of haploids on the same medium supplemented with 2 mgl^{-1} kinetin. Separated shoots were transferred to the MS medium (MURASHIGE & SKOOG 1962) supplemented with organic constituents according to KASPERBAUER & WILSON 1979. All media were adjusted to pH 5.7 before autoclaving. Haploids and regenerants in the culture were incubated at 24–27 °C under 16–h photoperiod.

Cytological analyses

The ploidy level of plants generated by anther culture and of those regenerated after diploidization was determined, according to SHARMA & SHARMA 1972. Root meristems were treated with hydroxyquinoline (4 hours), fixed in a mixture of methanol and acetic acid (3 : 1 v/v) for 10 min, treated in 1N HCl at 60 °C (12 min) and stained with 0.2 % orcein (30 min). Specimens for light microscopy were prepared performing a squash technique.

Transformation

Tumours were induced on leaf disks (HORSCH & al. 1985) of three haploid (15 H, 44 H, 46 H) and three from them derived dihaploid (15 DH, 44 DH, 46 DH) tobacco plants grown in vitro. Three *A. tumefaciens* strains were used for transformation: B6S3 (wild type of plasmid), pGV2215 (shooty mutant lacking gene *2*) and pGV2255 (rooty mutant lacking genes *4*, *6a* and *6b*).

We indicated the transformed lines induced by B6S3 strain with a suffix "B". Suffixes "S" and "R" were added to the line codes transformed with pGV2215 and pGV2255 plasmid mutants, respectively.

DNA isolation

Plant DNA was isolated according to the modified procedure of DELLAPORTA & al. 1983. Plant tissue (approximately 3 g) was frozen in liquid nitrogen and ground with mortar and pestle. DNA extraction buffer (100 mM Tris/HCl, 50 mM EDTA, 500 mM NaCl; 2 mM β-mercapto ethanol; pH 8.0) in an aliquot of 15 ml and 1 ml of 20% SDS were added and the mixture was incubated for 10 min at 65 °C. Five ml of 5 M KCl were added; the samples were placed on ice for 20 min and centrifuged at 15000 × g for 20 min. The supernatant was filtered through Miracloth (Behring Diagnostic) into 10 ml of ice-cold isopropanol. Precipitated DNA was centrifuged at 25000 × g for 20 min and the precipitate was dried and resuspended in 50 mM Tris and 10 mM EDTA, pH 8.0. Samples were centrifuged at 15000 × g and the supernatant was collected and mixed with 75 µl of 3 M Na-acetate and 500 µl of cold isopropanol. The DNA precipitate was spooled, washed in 70% ethanol and resuspended in buffer (10 mM Tris and 0.1 mM EDTA, pH 8.0). Precipitation was repeated by adding 30 µl of 3 M Na-acetate and 600 µl of 96% ethanol. The DNA was dried and resuspended in water. The concentration was measured spectrophotometrically.

PCR conditions

To prove that T_L-DNA was incorporated in haploid and dihaploid tobacco lines, the polymerase chain reaction (PCR) was performed in a PCR Thermal Reactor (Hybaid). Reaction mixtures of 25 µl containing 0.25 mM of each dNTP, Taq pol buffer (Promega), 1.5 mM $MgCl_2$, 5 pM of each primer for amplification of genes *6a* and *virB*, or 50 pM for the amplification of gene *5*, 12.5 nkat of Taq Polymerase and 800 ng of DNA were used. For amplification of the gene *6a*, (GIELEN & al. 1984) primers 5'-TGCTTCAGATGGATTGCTTGCC-3' and 5'-GATAGCACCATCTAACTCCACG-3' (Eurogentech) were used. The expected length of the amplified fragment was 336 base pairs. PCR was conducted under the following conditions: 30 cycles, 94°C, 60°C, 72°C, one min each. Gene *6a* was amplified in tobacco lines transformed with *A. tumefaciens* strains B6S3 and pGV2215. PCR for gene *5* was performed using the primers 5'-AAGTGGTCAAGTGCTCTCGC-3' and 5'-TCGGAAGAAGAGCAATGTAAGC-3' under the conditions: 30 cycles, 94 °C, 55 °C, 72 °C, one min each, and the expected length of the amplified fragment was 290 bp (base pairs). Gene *5* was amplified in DNA isolates from lines transformed with pGV2255.

PCR for the *virB* gene (WARD & al. 1990) was performed using the primers 5'-AGAGGCGGTGTTAGTTGC-3' and 5'-AACATCCAGCGAATGCCG-3' under the conditions: 30 cycles, 94 °C, 55 °C, 72 °C, one min each. The expected length of the amplified fragment was 317 bp.

All amplified fragments were resolved in a 1.5 % agarose gel (70 V, 2 h), using a Tris-acetate buffer and photographed after staining with ethidium bromide. For electrophoresis, 10 µl of each PCR sample were mixed with 3 µl of xylene cyanol loading buffer.

High performance liquid chromatography

According to MANCEAU & al. 1992 plant tissue was freeze-dried and 200 mg of dry material was extracted with $CHCl_3$: 27 % NH_4OH (49 : 1 v/v), twice under reflux, for 2 h. After filtration each crude extract was evaporated to dryness under reduced

pressure. Crude alkaloid extracts were purified by treating with 10 ml HPLC mobile phase at 40 °C for 15 min and filtered prior to chromatographic analysis.

A Novapack C_{18} 4 mm column (15 cm × 4 mm), a Waters pump and a 481 Lambda-Max spectrophotometer were employed. Chromatography was performed at room temperature using a mobile phase of 0.4% aqueous acetonitrile containing 0.1% (v/v) phosphoric acid buffered to pH 3.5 with triethylamine. The flow-rate was 0.8 ml/min. Alkaloids were detected at 260 nm and quantified using a Shimadzu ICR 1B automatic integrator that had been calibrated using standards purchased from Extrasynthese (Genay, France).

Results

Culture and transformation of haploid and dihaploid tobacco lines

The first plantlets developed from the anthers after 4 weeks of cultivation. Cytological analyses showed that they were all haploids. Ten percent of 389 regenerants obtained from haploid leaf segments after a diploidisation procedure on the medium with kinetin, were dihaploids. Three haploids: 15 H, 44 H, 46 H and three from them derived dihaploids: 15 DH, 44 DH, 46 DH were transformed with *A. tumefaciens* strains B6S3, pGV2215 and pGV2255.

Transformation of tobacco lines

We could not transform the 46 H line with the B6S3 strain, and the 44 H line with the pGV2215 strain. Other lines were successfully transformed. Tumours were formed 5 weeks after infection of tobacco leaf explants with pGV2215, and 6 weeks after the infection with B6S3 and pGV2255. After a second subculture on the MS medium without carbenicilline (used to prevent a bacterial growth), the growth of rooty tumours (pGV2255) slowed down, and a cotton-like callus started to grow on their surface. During the third subculture, only unorganized tissue proliferated, and rhizogenesis was completely suppressed. All lines transformed with B6S3 grew as unorganized tumourous tissue. Shooty teratomas (pGV2215) never developed roots on the MS medium and never reached more than three cm of height. The growth rate of transformed tissues (estimated by fresh mass increase) depended mainly on the bacterial strain used for transformation (Table 1). Lines transformed with the pGV2255 grew faster than those transformed with the pGV2215 and the B6S3. Neither growth nor tumour morphology were influenced by the ploidy level of the tissues. All tumour lines transformed by the same bacterial strain had similar morphology.

Table 1

Fresh tissue mass yield in established transformed tobacco lines depending on the strain of *A. tumefaciens*. Fresh mass was measured 4 weeks after inoculation of 0.2 g of tissue on the MS medium.

Bacterial strain	Callus line	Mean fm of 4 inoculi (g±S.E.)
pGV2255	15 DHR	6.7±0.3
	15 HR	6.5±0.4
	46 DHR	5.4±0.5
	46 HR	5.2±0.2
pGV2215	15 DHS	2.1±0.1
	15 HS	1.9±0.2
	46 DHS	1.8±0.2
	46 HS	2.2±0.2
B6S3	15 DHB	3.6±0.3
	15 HB	3.7±0.2
	44 DHB	3.5±0.2
	15 DHR	2.8±0.1

Each pair of haploid and derived dihaploid line is marked with the same number. H – haploid line, DH – dihaploid line, B – tumor induced by B6S3 strain, S – shooty tumour induced by pGV2215, R – rooty tumour induced by pGV 2215.

PCR analysis of transformed tissues

DNA isolation from the three tumour lines and untransformed control plants yielded in each case about 30 µg DNA /g of fresh tissue.

In order to confirm the transformation, DNAs isolated from unorganised and shooty tumours were subjected to PCR amplification of a fragment (336 bp) of the gene *6a* from the T_L-DNA region, while DNA from rooty tumours was subjected to PCR amplification of a fragment (290 bp) from gene *5*. DNAs from untransformed tobacco leaves were used as a negative control and bacteria, as a positive control. PCR yielded the expected 336 bp fragment showing that gene *6a* was incorporated in the lines transformed with the B6S3 (Fig. 1) or with pGV2215. The fragment was not present in the control tissue. PCR analyses of lines transformed with pGV2255 yielded the expected 290 bp fragment. The *VirB* gene was found only in the bacterial positive controls, but was missing from transformed lines and control plants, showing that the PCR results were not the consequence of bacterial contamination.

Nicotine content in transformed haploid and dihaploid tobacco tissue

By means of HPLC nicotine was detected neither in tumours induced by B6S3 nor in shooty tumours, or calli developed on transformed roots.

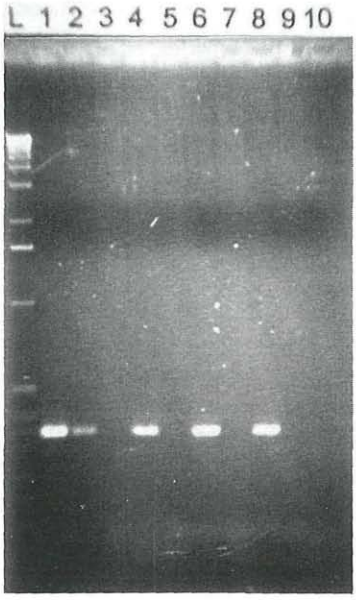

Fig. 1. Agarose gel electrophoresis of *6a*-gene fragment amplified by PCR in DNAs isolated from lines transformed with B6S3.
Lines: L – ladder (1 kb Ladder, Inc), 1 – bacterial strain B6S3 (positive control), 2 – 15 HB, 3 – 15 H, 4 – 44 DHB, 5 – 44 DH, 6 – 15 DHB, 7 – 15 DH, 8 – 46 DHB, 9 – 46 DH, 10 – untransformed plant (negative control). H – haploid, DH – dihaploid.

Table 2

Nicotine content in haploid and dihaploid tobacco control plantlets and in roots transformed with pGV2255. Each value represents the average of triplicate measurements.

Control tobacco plantelets grown in vitro Lines	Nicotine content (% dry mass ± S.E.) Leaves	Roots	Transformed roots obtained with pGB2255 Lines	Nicotine content in transformed roots (% dry mass ± S.E.)
15 H	0.56 ± 0.03	0.89 ± 0.06	15 HR	0.76 ± 0.03
15 DH	0.83 ± 0.04	0.71 ± 0.05	15 DHR	0.82 ± 0.04
44 H	0.57 ± 0.03	0.69 ± 0.05	44 HR	0.63 ± 0.03
44 DH	0.38 ± 0.03	0.58 ± 0.05	44 DHR	0.40 ± 0.02
46 H	0.44 ± 0.03	0.53 ± 0.04	46 HR	0.42 ± 0.02
46 DH	0.58 ± 0.04	0.60 ± 0.03	46DHR	0.60 ± 0.04

Low nicotine content was determined in the control plants and transformed roots (Table 2). Nicotine content did not depend on the ploidy level of the tissue.

Discussion

The morphology of crown gall tumours depends on the integration and expression of *onc* genes in transformed tissues, on the plant species, genotype and physiological condition of the host (ISHIKAWA 1988). In our experiments morphology and growth of transformed tobacco lines were mainly dependent on the bacterial strain used for transformation. Ploidy level of the transformed tissues had no effect, neither on the morphology nor on the tumour growth. Lines transformed with pGV2255 had not a characteristic rooty phenotype, and changed to unorganized growth on MS medium without hormone supplements. A similar result was reported by DEBLAERE 1986, who is an author of this strain. The growth rate of unorganized tissue transformed with pGV2255 was twice that of tumours transformed with the wild strain B6S3. GARFINKEL & al. 1981 reported that tumours induced by a plasmid lacking gene *6b* grew faster than the ones induced with a wild type of plasmids. It could explain the higher growth rate of the pGV2255 transformed tissue.

SIMPSON & al. 1986 and SPIELMANN & SIMPSON 1986 reported that 35 % of tobacco crown-gall lines had irregular T-DNA structure. In most of the cases the irregularity was not severe, affecting only the T-DNA termini, it remained without any phenotypic implication. Although gene *5* is a terminal gene of T_L-DNA, it was detected in all lines transformed by pGV2255, while gene *6a* was found in the pGV2215 and B6S3 transformed lines. Detecting the gene *5* or *6a* by PCR we confirmed a transformed character of the tissues. The absence of *vir B*-gene excluded the bacterial contamination of tissues.

Since young plantlets from in vitro cultures were used for transformation and measurements of nicotine, low nicotine content was expected. The nicotine content of dihaploids was not determined by nicotine content of haploids, from which dihaploids were derived. Its production in dihaploids could not be predicted by its measuring in haploids. Measurements of nicotine amount in the plants having the same genotype, but grown in the field (data not shown) support this result. Nicotine is mainly synthesized in the roots of *Nicotiana tabacum* and transported to the aerial parts of the plant (TSO 1990). Unorganized callus tissue of *Nicotiana* are generally poor in nicotine and related alkaloids, though under optimal culture conditions the amount comparable to parental plants have been achieved (ROBINS & al. 1987). We did not detect nicotine in unorganized tissues or shoots of any transformed line. These results coincide with those of SAITO & al. 1991. Transformed roots produced nicotine but they could not be

subcultured because they were overgrown by unorganised callus which did not produce nicotine. The genes for nicotine production seem to be inactivated by callogenesis. No correlation between nicotine content and ploidy level of transformed lines was found.

We can conclude that ploidy level does not affect nicotine content in control nor in transformed tissue.

Acknowledgements

We thank Dr. D. TEPFER (INRA, Versailles, France) for his help with performing PCR analyses and for useful suggestions regarding the manuscript. We also thank Dr. J. SCHELL (Max-Planck Institute, Köln, Germany) for providing the bacterial strains we used in this work and Dr. B. ŠMALCELJ (Tobacco Institute, Zagreb, Croatia) for plant material which was used to generate haploid and dihaploid tobacco plants.

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Phyton (Horn, Austria) 37 (2): 239–240 (1998)

Recensio

The Garden. Journal of the Royal Horticultural Society. Vol. 122 (1): 1–70, (8): 537–610 and (11): 763–836, 1997. – Lex. 8°, zahlr. Abb., geheftet. – The Royal Horticultural Society, 80 Vincent Square, London SW 1P 2PE. – £ 2.75 pro Heft. – ISSN 0308-5457.

Die Zeitschrift der Royal Horticultural Society [zuletzt besprochen in Phyton 36 (2): 171–172] enthält neben rein gärtnerischen Artikeln nahezu immer auch solche, die aus der Sicht der Systematischen Botanik interessant sind, insbesondere farbig bebilderte Berichte aus pflanzengeographisch interessanten Gebieten und Zusammenstellungen gängiger Kultursorten. Aus dem Band 122 seien Beiträge aus drei Heften als Beispiele genannt. Der durch sehr stark vorstehende Knoten auffällige Bambus *Quiongzhuea tumidinoda* wird von lokaler Industrie in Szetschuan zu Spazierstöcken verarbeitet (R. LANCASTER, p. 46–47, 3 Abb.). Expeditionsbericht aus Chile betr. *Araucaria araucana*-Bestände (Abb. v. *Desfontainia spinosa, Loganiaceae* oder *Desfontainiaceae*; vgl. auch 122 (5): 324–325), *Fitzroya cupressoides* auf Chiloé (Abb. v. *Asteranthera ovata, Acanthaceae* und *Sarmienta repens, Gesneriaceae*) und *Berberidopsis corallina* (*Flacourtiaceae*, Nutzung für Flechtwerk, durch Waldzerstörung in der Existenz bedroht, vgl. auch Plant Talk 6: 28–29, 1996) (P. BROWNLESS & al., p. 50–53, 10 Abb.). Bericht über *Anthemis tinctoria*-Sorten; von 14 alten Cultivaren wird vermutet, daß sie verlorengegangen sind (A. LESLIE, p. 552–555, 8 Abb.). Strauchige *Hypericum*-Sorten (R. LANCASTER & al., p. 566–571, 25 Abb.). Kultivierte *Impatiens*-Arten (C. GREY-WILSON, p. 583–587, 20 Abb.). Über Schadwirkung und Unterscheidungsmöglichkeiten von sechs *Armillaria*-Arten (D. WHITEHEAD & al., p. 790–792, 6 Abb.). Kommentar aus Anlaß des 400. Jahrestages des Erscheinens von John GERARDS The Herball or Generall Historie of Plantes (PRITZEL Nr. 3282) (B. ELLIOTT, p. 793–795, 5 Abb.). Expeditionsbericht aus NO-Sikkim „Auf den Spuren Sir Joseph HOOKERS", u. a. mit Abb. von *Cardiocrinum giganteum* (*Liliaceae*) und den Wollkerzen *Eriophyton wallichii* (*Lamiaceae*), *Rheum nobile* und *Saussurea gossypiphora* (H. J. NOLTIE, p. 806–809, 11 Abb.). Passionsblumen mit eßbaren Früchten in Blüte und Frucht (15 Arten, mit Kulturanleitungen; 50% der *Passiflora*-Arten selbstinkompatibel; J. VAN DER PLANK, p. 816–821, 11 Abb.).

Auf p. 19 findet sich wieder [vgl. Phyton 22 (2): 339–340 bzw. Garden 107 (1): 27,29] ein kurzer Bericht über das den irreführenden Vulgärnamen Garden Huckleberry tragende *Solanum scabrum* MILLER (C. SIMMS, 1 Abb.). Es wird zwar die Ähnlichkeit mit *S. nigrum* angesprochen, es sind aber keine Unterscheidungsmerkmale