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ACCUMULATION OF NICOTINE AND DITERPENOIDS IN CYBRIDS OF *NICOTIANA* WITH *PETUNIA* CHLOROPLASTS

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Key Word Index—*Nicotiana tabacum*; *Petunia hybrida*; Solanaceae cybrids; nicotine; diterpenoids; cembranoids.

Abstract—Cybrids with nuclei of *Nicotiana tabacum* and chloroplasts from *Petunia hybrida* in general accumulate higher amounts of nicotine and (1*S*,2*E*,4*R*,6*R*,7*E*,11*E*)- and (1*S*,2*E*,4*S*,6*R*,7*E*,11*E*)-2,7,11-cembratriene-4,6-diol than do ordinary tobacco plants. The composition of the cembranic diterpenoids present in the cuticular wax of the cybrid leaves, was, with one exception, found to be similar to that encountered in the leaves of *N. tabacum*.

INTRODUCTION

Cybrids are products obtained via protoplast fusion where the organelles of one species are combined with the nucleus of another. The protoplast of a cultivar (Samsun) of *Nicotiana tabacum* having a maternally inherited chlorophyll deficiency was fused with γ-irradiated protoplasts of *Petunia hybrida* cv Comanche. Photoautotrophic fertile plants were obtained which were able to synthesize chlorophyll due to the establishment of *Petunia* chloroplasts in the resulting cybrids [1]. In the living plant, nicotine (**1**) is stored within plastid vacuoles and between the outer and inner membranes of the plastid envelope [2]. Diterpenoids of the cembrane type are synthesized and stored in the heads of the hairs on the leaf surface of *Nicotiana* species [3]. In tissue cultures, light and the presence of chloroplasts is normally a prerequisite for terpene biosynthesis, although HMG-CoA-reductase is also found within the E.R. and cytoplasm [4, 5]. The level and composition of these secondary metabolites can thus be used as valuable markers for the function of the chloroplasts within the cybrids.

RESULTS

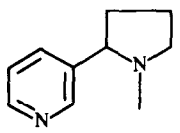
Nicotine content

The nicotine content of the plants examined, expressed as a percentage of the nicotine content of the parent plants, is given in Table 1. The nicotine content of the parent *N. tabacum* species varied (a: 0.18%, b: 0.48%, c: 0.20% of dry weight), while no nicotine was detected in *P. hybrida*. It follows from Table 1 that the nicotine content of the cybrids, relative to that of *N. tabacum*, varied between 63 and 238%, except in the case of the cybrid A6 (a) which is the sole cybrid having a much lower nicotine level, i.e. 6% of that of the reference. Because the result could not be obtained in subsequent experiments, it is considered erroneous and not included in Table 1.

Diterpenoid content

In order to confirm the chemical structures of the major secondary metabolites, these were isolated, using HPLC, and their identities were established by spectroscopic methods (^{1}H NMR and MS). The relative content of (4*R*)- and (4*S*)-2,7,11-cembratriene-4,6-diol (**2**+**3**) in the different plants is summarized in Table 1. The ratios between the two isomeric cembratriene-4,6-diols (**2**, **3**), which were measured with the aid of HPLC, were in all cases found to be *ca* 1:3.

All cybrids, except A11, produced higher amounts of **2**+**3** than did the parent plant. In a detailed study, including HPTLC (normal and reversed phase) and HRGC/MS, it was revealed that A11 contained cembratriene-monools (**4**, **5**), compounds which could not be detected in the other plants. This study also indicated that



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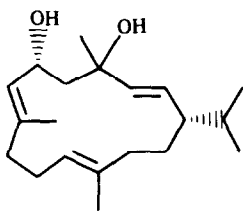
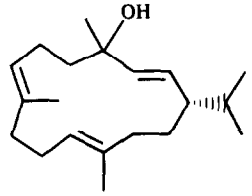


2 4*R*
3 4*S*



4 4*R*
5 4*S*

Table 1. Concentrations of nicotine and cembratrienediols in cybrids of *N. tabacum* and *P. hybrida* expressed as a percentage of the amounts of respective compound in the parent *N. tabacum* plant

Plant	% Nicotine	% Cembratriene-diols
P. hybrida	not detected	not detected
N. tabacum	100	100
A6[b]	127	*
A11[a]	128	74
A18[b]	63	*
A28[c]	238	704
A30[c]	137	412
A38[c]	149	464
C1[b]	103	*
C9[b]	88	*
C13[b]	133	*
C22[c]	95	621
D13[b]	142	*
Mean value	128	550[c]

*Not analysed

the level of oxygenated 2,7,11-cembratriene-4,6-diol derivatives [6] was lower in A11 than in the other plants.

DISCUSSION

The variation of the nicotine content in the *Nicotiana* cybrids is of the same order as that normally observed for other secondary metabolites in a group of field grown individuals of *N. tabacum*. It follows that the present cybrids probably possess an intact chloroplast capable of storing the nicotine produced.

It is noteworthy that, in contrast, cybrids between *Nicotiana* and *Atropa* have been reported to produce only trace amounts of alkaloids [7]. Part of the variation in the cembrane levels can be ascribed to a normal variation between biological individuals. The high mean value—5.5 times that of the parent plant—must, however, be attributed to some other effect.

Light stimulation of terpene accumulation in cells and tissues containing chloroplasts is a well known phenomena even if chloroplast development and terpene biosynthesis are not directly coupled [8]. In the present case, the higher accumulation of cembranes in the cybrids could be seen as a restoration of the chloroplast-defective *Nicotiana* genome involving also restoration of the cembrane accumulation capability, cf. ref. [9].

Our results clearly show that a co-operation between the nucleus of *N. tabacum* and the chloroplasts of *P. hybrida* is obtained. As secondary metabolism plays a role in the plant defence system, including resistance towards pathogens, this observation may increase the importance of cybridization in plant breeding. It is interesting to note that the cybrid A11[a] produced lower levels of oxygenated cembratrienediols than did the other plants and that it was the only plant that accumulated cembratriene-monools (**4**, **5**). A plausible explanation for this is that this cybrid has a defective oxygenation system, which makes it attractive for biosynthetic studies of cembrane biosynthesis using radioactive material.

EXPERIMENTAL

Plant material. Plants of *N. tabacum* c.v. Samsun and *P. hybrida* c.v. Comanche and cybrids of *Nicotiana* with chloroplasts from *Petunia* were grown in a greenhouse under normal conditions. The cybrids were produced as reported previously [1]. Plants were cultured and analysed at three different occasions, referred to as a, b and c. Leaves from these plants, were harvested and analysed, when the plants had reached a size allowing two pairs of leaves to be fully developed.

Analytical procedures. Instruments specified in ref. [10] were used. TLC of the cuticular waxes was performed on Merck HPTLC-RP18 plates. Nicotine (**1**) was determined according to a standard method [11].

Extraction of the cuticular components was performed according to a previously published method [9]. The mass spectral determinations of the 2,7,11-cembratriene-4,6-diols (**2**+**3**) were carried out in a full scan mode under ammonia chemical ionization conditions. The temp. of the ion source in the mass spectrometer was 150° and the ammonia reagent gas pressure, measured in the source housing, was 5×10^{-5} torr. The internal standard, 2-bromobenzoic acid, was added to the extract and 2 μl of the resulting soln was deposited on a direct-inlet probe. The sample was then allowed to evap. into the ion source by heating the probe to 200°. Full CI-mass spectra were acquired repetitively using a scan cycle time of 2 sec during the evapn of the sample and for a total runtime of *ca* 3 min. Using the DS90 software, ion-chromatograms were then calculated for the ions of *m/z* 289 $(M-17)^+$ and *m/z* 271 $[M-35]^+$ derived from the compounds **2**+**3** and the ions of *m/z* 220 and *m/z* 218, corresponding to the two $[M+NH_4]^+$ ions of the internal standard.

Typically, Gaussian-shaped ion profiles were obtained employing an evaporation time of 0.5 min for 2-bromobenzoic acid and 1 min for the compounds **2**+**3**. The amount of the last two compounds in the samples were then calculated by the software through comparison of the areas under the ion profiles of *m/z* 289 and *m/z* 271 with those of the int. standard and correcting for differences in response factors. The determination of the content of the compounds **2**+**3** in an extract was carried out in triplicate.

HPLC of the cuticular waxes of the different cybrids using a column packed with Spherisorb 5 ODS1 (MeOH–H_2O, 4:1) gave two compounds which were identical (^{1}H NMR and MS) to authentic specimens [12] of compounds **2** and **3**. Flash chromatography of the cuticular waxes of A11 on silica gel using a gradient elution (EtOAc–hexane) gave a compound which on HRGC/MS had a R_t and a MS identical to that of an authentic sample of **4** and **5**.

REFERENCES

1. Glimelius, K. and Bonnet, H. T. (1986) *Theor. Appl. Genet.* **72**, 794
2. Neumann, D. (1985) in *Biochemistry of Alkaloids* (Mothes, K., Schülle, H. R. and Luckner, M., eds), p. 49. VEB, Berlin.
3. Keene, C. K. and Wagner, G. J. (1985) *Plant Physiol.* **79**, 1026.
4. Björk, L. (1986) *Found. Biotechn Ind. Ferment. Res.* **4**, 177.
5. Garg, V. K. and Douglas, T. J. (1983) *3-Hydroxy-methylglutaryl-coenzyme A Reductase* (Sabine, J. R., ed.), p. 29. CRC Press, Boca Raton, Florida.
6. Wahlberg, I. and Enzell, C. R. (1987) *Nat. Prod. Rep.* **4**, 237.
7. Gleba, Y. Y. (1987) *Molec. General Genetics* **209**, 159.
8. Scheibner, H., Björk, L., Schultz, U., Diettrich, B. and Luckner, M. (1987) *J. Plant Physiol.* **130**, 211.
9. Severson, R. F., Arrendale, R. F., Chortyk, O.T., Johnson, A.

W., Jackson, D. M., Gwynn, G. R., Chaplin, J. F. and Stephenson, M. G. (1984) *J. Agric. Food Chem.* **32**, 566.
10. Wahlberg, I., Arndt, R., Nishida, T. and Enzell, C. R. (1986) *Acta Chem. Scand* **B40**, 123.
11. Coresta Standard Method No 20 (1968) *Coresta Inform. Bull* 1969-2, 25.
12. Roberts, D. L. and Rowland, D. L. (1962) *J. Org. Chem.* **27**, 3989.