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BRIEF COMMUNICATION

Resistance to ACNU induced toxicity in transgenic tobacco suspension cultures with *ada* gene transferred from *Escherichia coli*

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Abstract

The protein coding region of the *E. coli* DNA repair gene *ada* combined with the CaMV 35S promoter has been transferred to tobacco by means of *Agrobacterium tumefaciens* Ti plasmid. In transgenic plants having the *ada* gene in a sense orientation, detectable amounts of O^6-alkylguanine-DNA-alkyltransferase has been found whereas in non-transformed plants this activity is absent. Cell suspension cultures derived from the former plants showed lower sensitivity to the toxic (growth inhibiting) effects of the bifunctional alkylating agent 1-(2-chloroethyl)-1-nitroso-3-(aminomethyl-1,3-diazinylo)-methylurea compared with cell cultures derived from a control non-transformed plant or from transgenic plants harbouring the *ada* gene in an opposite, non-sense orientation.

The *E. coli* DNA repair gene *ada* encodes enzyme O^6-alkyl-guanine- and alkylphosphotriester-DNA-alkyltransferase (ATase) which removes simple alkyl groups from O^6-alkylguanine, O^4-alkyl-thymine and from an SP stereoisomer of alkylphospho-triesters (Lindahl *et al.* 1988). The transfer of this gene into the genome of tobacco enables us to explain the role of resulting DNA lesions and their

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Abbreviations: ACNU - 1-(2-chloroethyl)-1-nitroso-3-(aminomethyl-1,3-diazinylo)-methylurea; ATase - O^6-alkylguanine- and alkyl-phospho-triester-DNA-alkyltransferase; CaMV - cauliflower mosaic virus
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repair in the toxic and mutagenic effects of alkylating agents in plants.

Our previous papers (Bříza *et al.* 1989, Babůrek *et al.* 1991, Angelis *et al.* 1992) reported results on the toxicity and mutagenicity of alkylating agents in transgenic clones of *Nicotiana tabacum* var. *xanthi* n.c. obtained by an *Agrobacterium tumefaciens* mediated transformation with plasmid pAD2034 harboring the *ada* gene under the control of dual 1'-2' promoter from Tr-DNA (Velten *et al.* 1984). However, there was no or negligible reduction of the somatic mutation frequency after the action of *N*-methyl-*N*-nitrosourea in transgenic plants compared with non-transformed ones (Babůrek *et al.* 1991). This may be caused by the low activity of the dual 1'-2' promoter in the leaf meristems (Landridge *et al.* 1989). Therefore, we decided on a formation of another series of transgenic tobacco plants with the *ada* gene under the control of the stronger 35S from CaMV promoter.

ACNU (*Sankyo*), one of the bifunctional derivatives of chloroethyl-nitrosourea, was used as a mutagen. It forms interstrand DNA crosslinks via alkylation of guanine at the O^6 position (Tong *et al.* 1982).

Dealkylation of O^6-chloroethylguanine by means of ATase prevents therefore the production of these cross-links which block DNA replication and are most probably responsible for the toxic effect in cells (Margison and Brennand 1987).

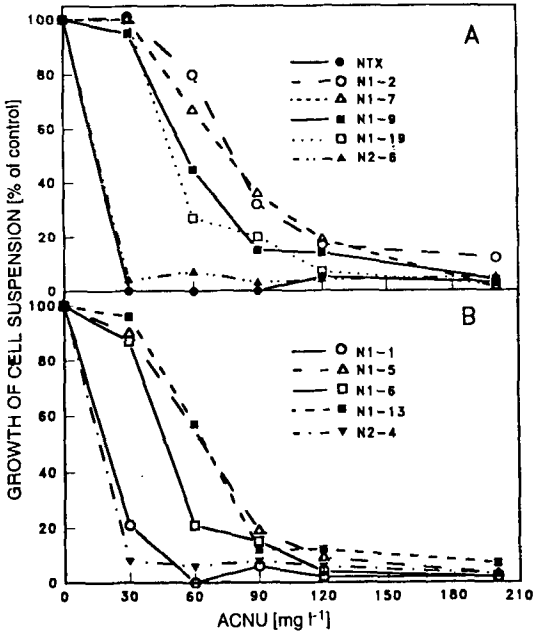

Fig. 1 A,B. Growth inhibition of ACNU treated cell suspensions derived from non-transformed tobacco plant (NTX) and from plants transformed with plasmid pKA1 (N1-1, N1-2, N1-5, N1-6, N1-7, N1-9, N1-13, N1-19) or plasmid pKA2 (N2-4, N2-6) after 18-d cultivation in growth medium. Data represent mean of three values.

Coding sequence of the *ada* gene (1.1 kb), obtained from G.P. Margison from the Paterson Institute of Cancer Research, CRC, Manchester, England, was cloned both in serial (pKA1) and in opposite orientation (pKA2) in the Ti plasmid pGA643 carrying NPT II chimeric gene for kanamycin resistance and the 35S promoter from CaMV.

Plant material, transformation procedure as well as culture conditions for both plants and cell suspensions were the same as previously described (Angelis *et al.* 1991).

An assay for ATase activity was performed *in vitro* in leaf extracts (with Tris-HCl buffer, pH=8.3) of both the transgenic and control plants. The extract of leaves (from 0 to 80 µl) was mixed with 20 µl [^{3}H]-methylated substrate DNA (0.5 mg l^{-1}, total activity about 20 000 d.p.m.) and with assay buffer (from 80 to 0 µl). The mixture was incubated at 37 °C for 50 min and then cooled to 0 °C. After addition 0.75 mg bovine serum albumin hydrolysis was performed in 1M HCl at 70 °C for 30 min. The mixture was cooled, proteins were precipitated by 5 % TCA and their radioactivity measured in the scintillation cocktail (*KP/b, Beckman*). Radioactivity of sediment is proportional to the activity of ATase (Margison *et al.* 1985).

The toxicity assay (growth inhibition) of ACNU was performed with cell suspension cultures derived from individual transgenic or non-transformed plants according to Angelis *at al.* (1991). Briefly, ACNU treatment was carried out in the stationary phase of suspension growth for 24 h at 25 °C and pH 5 in the dark. After washing with a 3 % (m/v) saccharose solution and filtering through nylon mesh approx. 1 g of the cells was inoculated into 30 cm^3 of liquid growth medium and the cell volume of 5 cm^3 sample (3 samples per dose) was measured each 5th or 7th day.

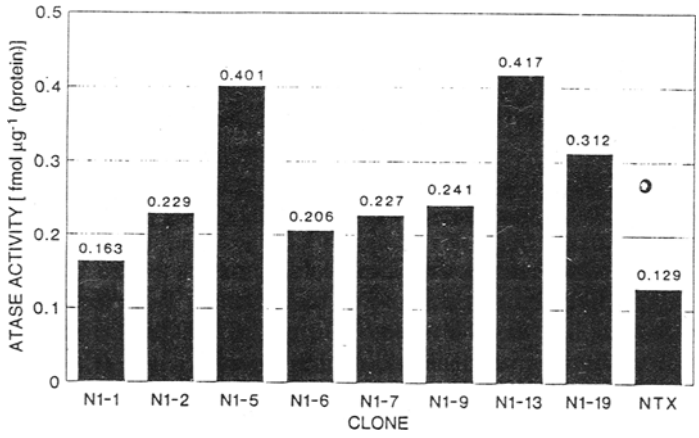

Fig. 2. ATase activities in leaf extracts from non-transformed tobacco plants (NTX) and from plants transformed with plasmid pKA1 (N1-1, N1-2, N1-5, N1-6, N1-7, N1-9, N1-13, N1-19). Each value represents mean of 2 or 3 experiments.

The growth of suspension culture derived from non-transformed plant (NTX) was inhibited even with the dose of 30 mg l^{-1} ACNU (Fig. 1A). After the same doses of

ACNU, growth inhibition of suspension cultures derived from transgenic plants containing the *ada* gene in an opposite orientation (N2-4, N2-6) was almost same as in NTX (Fig. 1A and 1B). On the contrary, 7 of 8 tested suspension cultures derived from a plant harbouring the *ada* gene in normal orientation (N1-2, N1-5, N1-6, N1-7, N1-9, N1-13, N1-19) demonstrated a highly enhanced tolerance against the toxic effects of ACNU (Figs. 1A, 1B). Growth values reached 50 % of the control (*i.e.* suspension culture without ACNU treatment) at a concentration range of 50-80 mg l^{-1} ACNU, whereas the growth values of NTX, N2-4 and N2-6 reached 0-8 % of the control even at a concentration of 30 mg l^{-1}.

Considerably increased ATase activity in comparison with NTX was found in clones N1-5, N1-13 and N1-19 (240-320 % of NTX, Fig. 2). Enhancement of ATase activity in clones N1-2, N1-6, N1-7 and N1-9 was slightly lower (160-190 % of NTX).

The clone N1-1 showed only 26 % enhancement of ATase activity compared with NTX (Fig. 2). This is in agreement with the low level of tolerance against toxic effects of ACNU observed in the clone N1-1 in the toxicity assay (Fig. 1B).

The presented data suggest that the introduction of the *ada* gene into the tobacco genome is resulted in the reduction of sensitivity to the toxic effects of ACNU in cell cultures transformed with plasmid pKA1. This supports an assumption that O^6-chloroethylguanine has been repaired by the product of the *ada* gene before the DNA-DNA crosslinks were formed. These lesions seem to be, therefore, responsible for a great deal of the growth inhibiting effect of ACNU in tobacco suspensions. The evaluation of the response of alkylating agents in mutation induction in transgenic tobacco plants is now under way.

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