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Free and conjugated polyamines in wild and transgenic types of *Nicotiana tabacum* calli

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ABSTRACT

Homospermidine synthase (*HSS*) is a branch-point enzyme that links the secondary pathway (pyrrolizidine alkaloids) to the primary metabolism (polyamines). Since the diamine putrescine is a precursor of homospermidine and nicotine in tobacco, we performed expression of a *Senecio vernalis* homospermidine synthase gene (*hss*) in *Nicotiana tabacum* and determined the effect on free and conjugated polyamine levels in the callus. The *hss* gene from *Senecio vernalis* was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter in *Agrobacterium tumefaciens* Ti-plasmid and both constructs were transformed into tobacco plants (transformation was previously done, under publication). Seeds of the first generation were used in this study. Expression of the *hss* gene in callus was verified by western blot. The transgenic sense line was generated showing strong expression of *HSS*. These transgenic sense calli showed a significantly decreased level of free spermidine while the pool of total spermidine was not affected. Homospermidine was not detected in wild type tobacco under our conditions, while its level was highly increased in the transgenic line.

Key words: *Nicotiana tabacum*, homospermidine synthase, callus, polyamines, alkaloids.

INTRODUCTION

Manipulation of plant secondary metabolites biosynthesis through genetic engineering is an attractive but challenging goal. A possible but not always a successful strategy for manipulation of secondary metabolism is the expression of genes that regulate specific biosynthetic pathways. In the past, many examples have demonstrated that enhancing the rate-limiting enzyme activity through constitutive expression of a corresponding heterologous gene is promising for achieving distinct increases in target secondary products. The usefulness of this approach has been demonstrated for cadaverine and anabasine biosynthesis by expressing a bacterial lysine decarboxylase gene in *Nicotiana tabacum* (Fecker *et al.,* 1993). The overexpression of tryptophan decarboxylase gene of a foreign plant resulted in a 5- to 10-fold increase in serotonin levels in transgenic cell cultures of *Peganum harmala* (Berlin *et al.*, 1993). However, attempts to produce transgenic potato plants with a 35S *S*-adenosylmethioninedecarboxylase (SAMDC) sense construct failed since engineered SAMDC has proved to be lethal to the plant (Kumar *et al.*, 1996). We are interested in the regulatory function of the branch-point enzyme homospermidine synthase (EC.2.5.1.44) linking primary metabolism (putrescine) to secondary metabolism (pyrrolizidine alkaloids) (Fig. 1). The enzyme catalyzes the formation of the "uncommon" polyamine homospermidine which is a precursor in the formation of pyrrolizidine alkaloids (PAs) in various plant species. PAs represent a class of secondary compounds that play an important role in plant defence against herbivores (Hartmann and Witte, 1995). PAs are widely distributed in the plant

kingdom and are most common in *Senecio* and *Eupatorium* species (Asteraceae), *Symphytum* and *Heliotropinum* (Boraginaceae) and *Crotalaria* (Fabaceae). Alkaloids which are presumably derived from homospermidine namely solamine, solapalmitine and solacaproine have been isolated from different Solanaceous plants (Evans *et al*., 1977). Although to date there has been no proof for the occurrence of homospermidine in these plants. PAs contain the necine base retronecine which is esterified with a necine acid. In the pathway leading to retronecine either arginine or ornithine is decarboxylated to putrescine. Putrescine and spermidine are important precursors for the formation of the intermediate *N*-(4-aminobutyl)-1,4 diaminobutane (homospermidine). The incorporation of homospermidine into retronecine has been demonstrated (Khan *et al*., 1985). In the enzymatic studies performed with purified enzyme preparations from the bacteria *Rhodopseudomonas viridis* (Tait, 1979) and *Acinetobacter tartarogenes* (Yamamoto *et al*., 1993), and the *Chlorella* virus PBCV-1 (Kaiser *et al*., 1999), homospermidine was shown to be formed from 2 moles of putrescine in a NAD^+-dependent reaction catalyzed by *HSS*. However, it was shown recently that plant *HSS*, from the pyrrolizidine alkaloid containing *Senecio vulgaris* root culture, catalyzes the formation of homospermidine using spermidine and putrescine (Graser and Hartmann, 2000). In bacteria an aminobutyl moiety from putrescine is bound to the enzyme in the first step of the reaction, while in plants this moiety is derived from spermidine (Graser and Hartmann, 2000). In the second step, a butylamine moiety is transferred from the enzyme-imine intermediate to a second putrescine moiety. NAD^+ functions as a coenzyme, intramolecularly catalyzing the hydrogen transfer in a stoichiometric manner (Fig. 1). Meanwhile, homospermidine synthase genes from various sources have been isolated. The first *hss* gene was cloned, sequenced and characterized from the bacterium *Rhodopseudomonas viridis* (Tholl *et al*., 1996) and shares 34% amino acid identity with the *hss* gene from the virus PBCV-1 that infects an eukaryotic-like chlorella green alga (Kaiser *et al*., 1999). The prokaryotic and viral enzymes strictly catalyze the formation of homospermidine from two moles of putrescine. The first plant *hss* genes were cloned from *Senecio vulgaris* and *Senecio vernalis* (Kaiser,

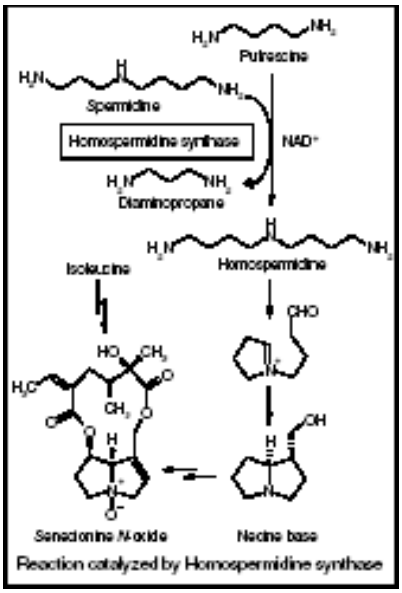


Fig. 1: Homospermidine synthase catalyzes the formation of homospermidine as the first specific step in PA biosynthesis (Ober *et al*., 2003).

1999; Ober and Hartmann, 1999b). The amino acid identity of both genes revealed significant homology to human deoxyhypusine synthase. A possible approach to study the regulatory function of homospermidine synthase in primary and secondary metabolism is the heterologous expression of the corresponding gene (*hss*) in transgenic plants. We had to use *Nicotiana tabacum* for transformation. In the present work we describe the transformation of tobacco plants with *hss* sense constructs from *Senecio vernalis.* To evaluate the effect of the constitutively expressed *HSS* on the polyamine pools in the callus, we determined the free and conjugated polyamine levels in the transgenic sense and the wild tobacco lines.

MATERIAL AND METHODS

Plant material

Sterile *Nicotiana tabacum* plants from *in vitro* culture were used to obtain the starting leaf material required for the tobacco transformation. Plants were grown on solid Murashige Skoog (MS) medium (Murashige and Skoog, 1962) containing 2% sucrose as carbon source without any phytohormones under controlled condition at 25°C

and 16 h light/8 h dark cycle. The medium was refreshed at 4-weeks intervals.

Overexpression of HSS by transformation of tobacco plants with cDNA encoding HSS

Specific primers were designed for cloning of the cDNA encoding *HSS* of *Senecio vernalis* into the vector pGPTV-BARB (Becker *et al*., 1992). These primers introduced a *Sma*I and an *Xba*I restriction sites directly preceding the start codon and behind the stop codon of the amplified full-length cDNA, respectively. The construct named pBAR-35S-HSS was transformed into *E.coli* DH5á cells, then into *Agrobacterium* GV3101/MP90 strain by triple mating technique (Shaw, 1995), which was used for the introduction of T-DNA into injured cells of leaf discs (Gallios and Paulo, 1995). Tobacco leaf discs were immersed in the *Agrobacterium* culture for infection, then transferred onto callus induction medium (MS medium containing 0.2 mg/l NAA and 1 mg/l BAP) supplemented with ticarcillin/clavulanic acid 500 mg/l. Phosphinothricin 25 mg/l was added to the callus induction medium as selective material for induction of transgenic callus cells, which harboured the T-DNA of the pBAR-35S-HSS construct.

Western blot

Fresh callus (100-200 mg) were homogenized into a fine powder with liquid nitrogen using mortar and pestle and extracted with 1xPBS buffer containing 5% (w/v) Polyclar® AT and 2.5% (w/v) sodium ascorbate to extract the soluble proteins, whose concentration was determined using the Bradford photometric assay (Bradford, 1976). Protein storage took place at -20°C for further use. After separation of the proteins via SDS-PAGE, the proteins were transferred to a polyvenylidene fluoride (PVDF) membrane (Immobilon P, Millipore). The transfer of the protein from the gel to the PVDF membrane took place by an electric current (160 mA, 15 V for 1 hour) supplied with the electrophoresis apparatus (Pharmacia LKB-MultiDrive XL) connected to the western blot apparatus (Pharmacia LKB Multiphor II). Affinity-purified antibody (specific to the protein of interest) was used as a primary antibody and Goat anti-Rabbit anti-body as a secondary one. The development and detection of the membrane took place with "ECL western blotting detection reagent"- kit (Amerscham Biosciences). The membrane was exposed to radiographic film X-Omat (Kodak). The films were developed with RPX-DMAT Developer/Replenisher (Kodak) then fixed with RPX-DMATLO Fixer/Replenisher (Kodak).

Qualitative and quantitative determination of the polyamines by HPLC analysis

Polyamines occur in plants in three forms: free soluble polyamines, soluble conjugated polyamines and non-soluble conjugated polyamines. Conjugated polyamines were hydrolyzed according to Torrigiani *et al*., 1997 and Gemperlova *et al*., 2005. Free polyamines were benzoylated according to Remond and Tseng, 1979, in order to be able to detect polyamines under the UV detector at 230 nm. For HPLC analysis the samples were dissolved in 50-100 µl methanol. The HPLC analysis was accomplished by a Merck/Hitachi L-6200 intelligent pump and L-4200 UV/Visible detector. The sample was loaded to the HPLC column by a Rheodyne 7125 injection system (sample loop volume 20 µl). For separation an Rp 18, Nucleosil 20-Column (5 µm grain size, 250 mm of length, 4 mm id, Macherey&Nagel) was used. As isocratic solvent system a mobile phase mixture of acetonitrile/1.5% phosphoric acid (40:60 v/v) (Shih *et al*., 1982; Graser, 1997) was used with a flow rate of 1.0 ml/min. The detection of benzamides took place at 230 nm (Sander, 1991) with the Chromato-integrator L-4200 (Merck/Hitachi) with the software Winflow. Cadaverine 125 nmol was added for each sample directly before benzoylation as an internal standard, and quantification was achieved using a correction factor.

Toxicity studies

Animals

Male swiss albino mice (The Nile Company for Pharmaceutical and Chemical Industries, Cairo, Egypt) weighing 20-30g were housed in plastic cages with maximum 6 mice per cage. Animals were kept at a temperature of 23 ± 2°C under a 12 hr light/dark cycle. Standard rodent food pellet and tap water were available *ad libitum*. The mice were acclimatized to these conditions for a minimum of 1 week before initiating experiments. All experiments were conducted between 8 a.m. and 3.00 p.m.

Mouse acute toxicity determinations

Wild and transgenic callus extracts were dissolved in a 0.9% NaCl saline solution and injected

i.p. at doses ranging from 0.1 to 2 g/kg. Concentrations were adjusted so that total volume injected was 10 µl/gm body weight.

Fifty four mice were classified into 9 groups, 6 mice each and were treated as follows:

Group 1 : 0.9% NaCl saline solution i.p.
Group 2 : wild extract 0.1g/kg i.p.
Group 3 : wild extract 0.5g/ kg i.p.
Group 4 : wild extract 1g/ kg i.p.
Group 5 : wild extract 2g/ kg i.p.
Group 6 : transgenic extract 0.1g/kg i.p.
Group 7 : transgenic extract 0.5g/ kg i.p.
Group 8 : transgenic extract 1g/ kg i.p.
Group 9 : transgenic extract 2g/ kg i.p.

Immediately after injection, mice were placed in a plastic cage with bedding. Behavioural responses including Straub tail, tremors and tachypnea were recorded for 15 min.

An arbitrary scale was used to assess sensitivity to extracts as follows: 0, no obvious effects; 1, locomotor effects including sedation and increased exploratory activity; 2, tremors, tachypnea and back arching; 3, rapid movements of the legs; 4, complete loss of righting reflex and seizures; 5, death (Franceschini *et al*., 2002).

StatPlus 2005 Professional Build 3.5.3.0 based on Finney method, 1971 was used in the calculation of LD_{50} from log dose-probit response curve. Comparisons between wild extract and transgenic extract treated groups were performed by Student's *t*- test using Instat 2 software.

RESULTS

Molecular characterization of transgenic *hss* sense and wild type tobacco lines

The *hss* sense plants were analysed by western blot to confirm that the transgenic line contained the *hss* gene. The integration of the *hss* gene was determined in the transgenic regenerated callus. In parallel, randomly chosen wild type callus were checked by western analysis to confirm that they do not express the *hss* construct (Fig. 2).

Polyamine measurements

The content of free polyamines in the callus tissues of the transgenic and wild tobacco was determined by HPLC analysis (Fig. 3). The results presented in Fig. 4 and table 1 demonstrate that the tobacco plant transformed with the recombinant plant vector contains homospermidine (959 nmol/g fresh weight) which represents the major polyamine in the transgenic type. However, spermidine (1186.92 nmol/g fresh weight) is the major polyamine in the wild type. Compared to the wild callus, the sense callus showed significantly reduced levels of spermidine and putrescine. The spermidine content of the transgenic callus decreased 86% as compared to the wild tobacco callus. Putrescine levels were slightly reduced in the transgenic line (25%). However, a 100-fold increase in homospermidine level occurred in the transgenic sense callus compared to the wild type.

Determination of total free polyamines showed that their content in the transgenic and wild type callus did not differ significantly. The transgenic sense plant showed a remarkable decrease in the spermidine level. The level of homospermidine in the transgenic line had extremely evolved which may compensate for the decrease in the level of

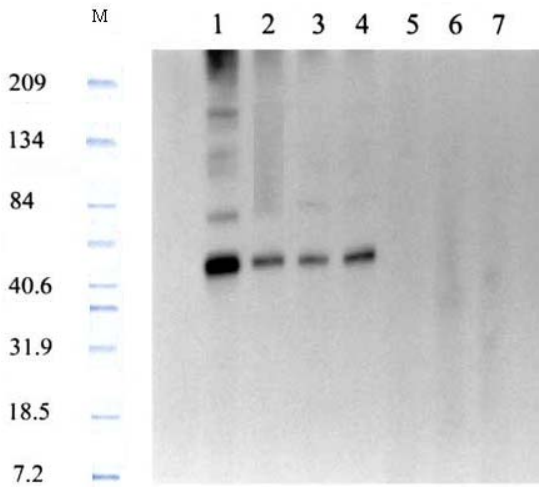


Fig. 2: M, is the marker in KD; 1, is protein extract of *S. vernalis* as a positive control; 2, 3 and 4 represent the protein extract of the callus of the first generation of the transgenic tobacco plant; 5, 6 and 7 represent the protein extract of the callus of the first generation of the wild type tobacco plant as a negative control.

spermidine. These results confirm that there is no effect on the free polyamine pools in the sense construct.

Results in table 1 showed that polyamines are mainly present in their conjugated forms. The distribution pattern compared to that of free polyamines differs considerably. Spermidine represents the major polyamine in the analysed fractions of conjugated polyamines in the wild type callus. While homospermidine represents the major polamine in the transgenic callii of tobacco that harbour and express the cDNA of *hss*. Spermidine is mainly present in its conjugated form, however, a significant decrease of free spermidine was observed in case of the transgenic line when compared to the wild type.

Table 1: Concentrations of polyamines in wild and transgenic tobacco

	Wild tobacco				Transgenic tobacco			
Concentration in callus (nmol/g fresh weight)	**put**	**spd**	**spm**	**hspd**	**put**	**spd**	**spm**	**hspd**
Free polyamines	224.7	506.62	12.2	0	180	76.8	0	389
Conjugated polyamines	433	680.3	25.4	0	313	90.1	0	570

Table 2: Onset time (sec) of tremors and tachypnea in animals treated with wild and transgenic tobacco extracts. Values represented are means ± SD; *n=6*

	Wild extract		Transgenic extract	
	1g/kg	**2g/kg**	**1g/kg**	**2g/kg**
Tremors	102± 21.6	40±12.2	177± 63.7*	103.3±40.8**
Tachypnea	138±34.2	42±10.9	178±68.6	101.6±37.1**

* $P<0.05$ compared to wild extract

** $P<0.01$ compared to wild extract

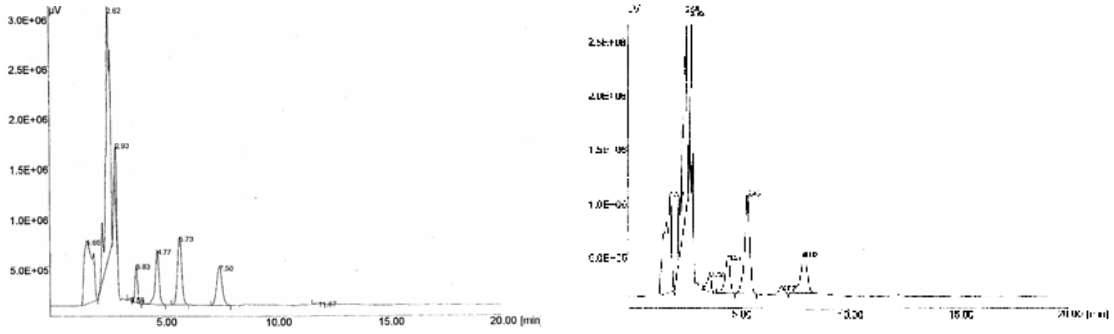


Fig. 3: HPLC analysis of benzoylated free polyamines in 0.1N HCl extract of the callus of wild and transgenic tobacco. 4.77, 5.73, 7.50 and 11.87 are the Rt of putrescine, cadaverine, spermidine and spermine in the wild type, respectively. 4.57, 5.45, 7.02 and 8.02 are the Rt of putrescine, cadaverine, spermidine and homospermidine in transgenic tobacco, respectively. Cadaverine is an internal standard.

Toxicity studies

Mice injected with wild and transgenic extracts showed impaired locomotor activity after a first exploratory phase. Few seconds later, they developed tachypnea and tremors. This was followed by a transient sedation and immobility.

None of the treated mice was given a score exceeding 2 during 15 min of observation following injection. However, in group 5, one mouse died 20 hours later, two died 48 hours later and one died 1 week later. No death occurred in other groups. LD_{50} was 1.87± 0.33 g/kg.

Straub tail (a nearly vertical tail) was induced by 1 and 2g/kg of wild extract as well as 2g/kg of transgenic extract. Percentage Straub mice produced by 1 and 2g/kg of wild extract was 20 and 66.6% respectively while only 33.3% of mice injected with 2g/kg transgenic extract displayed Straub tail Fig. 5.

Onset time of tachypnea and tremors is represented in table 2. Animals treated with transgenic extract showed delayed onset of recorded behaviour compared with animals receiving the same doses of wild extract. Control animals (group 1) didn't show response during 15 min.

DISCUSSION

The role of polyamines in plant growth and development remains to be fully clarified. In this study the expression of the homospermidine synthase gene, from *Senecio vernalis,* in transgenic tobacco was examined. *HSS* is a branch-point

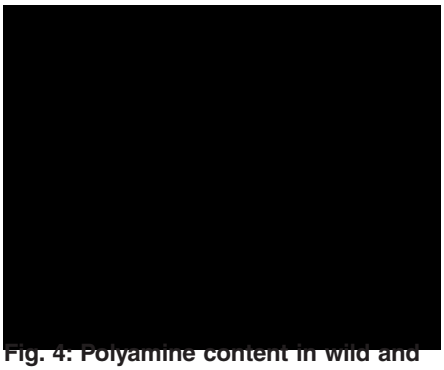
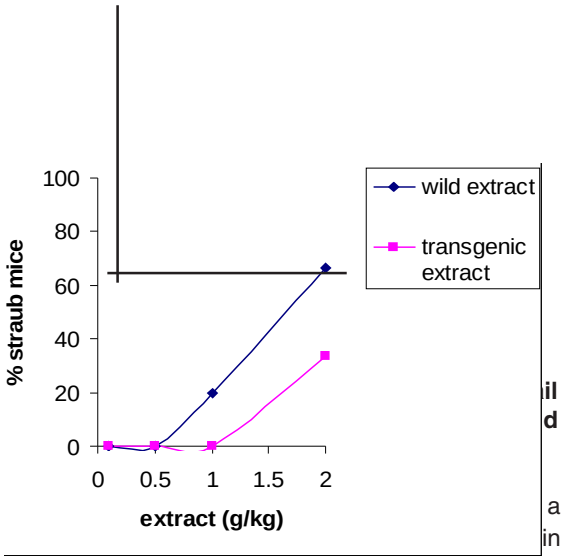


Fig. 4: Polyamine content in wild and transgenic tobacco. Free and conjugated polyamines were determined (nmol/g fresh weight). WT: wild type tobacco; Trans: transgenic tobacco



enzyme linking primary metabolism (polyamines) to secondary metabolism (PAs) in plants and so changes in its level might influence polyamine levels and formation of secondary metabolites. Although *Nicotiana tabacum* does not produce PAs, we used tobacco as a model system because a technique to regenerate transgenic *Senecio* species was not available. Also to examine the effect of any interruption in the synthesis of polyamines on the biogenesis of alkaloids in tobacco plant, we determined both the free and conjugated polyamine content in the wild and transgenic callii. The content of the polyamine putrescine was specifically watched as it is an important precursor in the formation of nicotine which is likely to affect the formation of this pyridine alkaloid.

the callus under the control of the (CaMV) 35S promoter. The wild type callus was used as a control. The most significant changes in the polyamine pools after expression of the transgenic gene were observed in the levels of free and conjugated polyamines. The content of homospermidine was not detected in the case of wild type tobacco under our conditions, while it was detected by huge amounts (959 mol/g fresh weight) in case of the sense transgenic callus. The triamine spermidine is one of the substrates of deoxyhypusine synthase (DHS) (Liao *et al*., 1988) catalyzing the formation of deoxyhypusine residue on the precursor of the eukaryotic initiation factor eIF-5A. Native

deoxyhypusine synthase from tobacco was recently cloned and characterized (Ober and Hartmann, 1999a). It was observed that the spermidine pool decreased by 7.11-fold in case of the transgenic callus when compared to the wild type line. It was also observed that the putrescine pool decreased in the case of transgenic tobacco by 1.33-fold when compared to wild type tobacco. As mentioned before, the putrescine represents the main precursor of spermidine and nicotine. Spermidine can serve as an acceptor for the aminobutyl moiety of putrescine which is transferred by *HSS*. In this reaction homospermidine is formed. Hence, the decrease in the level of free spermidine and putrescine in the transgenic line is due to the formation of homospermidine, which subsequently affects the amount of conjugated polyamines, as they are hydrolysed to compensate the decrease of free polyamines. Spermidine-hydroxycinnamoyl-conjugates are known to be involved in callus formation (Cohen, 1998). Obviously the occurrence of homospermidine in native tobacco plants can be attributed to deoxyhypusine synthase activity since homospermidine can be formed in two different ways, i.e. by *HSS* and by deoxyhypusine synthase (Kaiser *et al*., 1999; Ober and Hartmann, 1999a). However, we were unable to detect *HSS* activity in native tobacco callus under our conditions.

A comparison between free and conjugated polyamines revealed significant differences. The determination of the polyamines showed that they are mainly present in their conjugated form in both the wild and transgenic calli (Table 1). Conjugated derivatives of putrescine are mainly formed under environmental stress conditions. High levels of endogenous putrescine are toxic for the vegetative growth of the plant. These effects have been intensively studied by inducible overexpression of oat arginine decarboxylase in transgenic tobacco (Masgrau *et al*., 1997).

We hypothysed the decrease in the content of putrescine and spermidine in the transgenic calli to their role in the formation of homospermidine. The functional role of homospermidine remains unknown. Homospermidine in wild type tobacco presumably is the precursor of simple alkaloids which have been isolated from different Solanaceae species like *Cyphamandra betacea* and *Solanum tripartitum* (Hartmann and Witte,1995). These alkaloids share common features such as possessing two dimethyl amino groups and a central substituted nitrogen. However, the biosynthesis of these alkaloids needs to be elucidated. Many studies in the past have shown that enhanced nicotine accumulation can only be achieved by increasing the metabolic flux through putrescine. In this context overexpression of yeast ornithine decarboxylase in transgenic roots of *Nicotiana rustica* lead to a two-fold increase of nicotine (Herminghaus *et al*., 1996).

Decreased putrescine as well as increased homospermidine level in the transgenic line calli compared to wild type line may cause the difference in toxicity studies observed between their extracts. This may be due to increased nicotine level in the wild type line compared to the transgenic line. Nicotine elicits a wide range of behavioural effects when administered to rodents. Increasing doses of nicotine in mice can cause sedation, decrease nociception, alter memory and learning, decrease body temperature and at high concentrations induce a series of behavioural effects that culminate with clonic–tonic seizures and death (Brioni *et al*., 1997; Fonck *et al*., 2003). Straub tail was described by Spande *et al*., 1992 as a response to nicotinic agonists. In this study, we showed that no death occurred in the mice groups injected with transgenic callus extract up to 2g/kg while LD_{50} was 1.87± 0.33 g/kg for wild type callus extract. The number of animals injected with 2g/kg of wild type extract showing Straub tail was double the number of animals injected with the same dose of transgenic extract. Animals treated with transgenic extract showed delayed onset of recorded behaviour compared with animals receiving the same doses of wild extract. This may be attributed to the changes in the polyamine content and consequently the alkaloid content.

It would be an important issue for the future to analyse polyamine pools in the regenerated transgenic plants of different generations and to determine their alkaloid content. From there it might be possible to engineer tobacco plants with an altered alkaloid content as will be investigated in future studies.

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