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STEROLS AND POLYAMINES IN *IPT*-TRANSFORMED TOBACCO PLANTS

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Key Word Index—*Nicotiana tabacum*; Solanaceae; tobacco; polyamines; sterols; *ipt*; cytokinin.

Abstract—Free sterol and free polyamine contents were determined in the apex and the leaves of control and *Pssu-ipt* transformed tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1). The older leaves of *ipt*-transformed plants contained a much higher putrescine (put) content than those of control SR1 plants, whereas no significant differences for spermidine (spd) or spermine (spm) were found between control and *ipt* plants. Putrescine content corresponded well with endogenous cytokinin (free-bases) content and with ornithine- and ornithine-decarboxylose (ODC and ADC) activities. Plants transformed with *ipt* were characterized by a higher sterol content in the leaves and by a delay in the increase in the stigmasterol/sitosterol ratio that occurs from the upper to the lower leaves. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Cytokinins are a major class of phytohormones involved in a variety of plant growth and developmental processes. Several developmental stages of plants are correlated with changes in endogenous cytokinin levels. Cytokinins affect the release of axillary buds from dormancy [1], retard leaf senescence [2] and stimulate the development of chloroplasts [3]. Root initiation and growth is inhibited at a relatively high concentration of cytokinins [4].

In contrast with other phytohormones, no mutants of cytokinin biosynthesis are known at present. A promising approach to studying the role of cytokinins in whole plants during their development is the use of stable genetically transformed plants [5]. To this end the T-DNA isopentenyl transferase (*ipt*) gene of *Agrobacterium tumefaciens* has been inserted into the plant genome under the control of its own promotor or other plant promotors. This gene encodes an isopentenyl transferase that catalyses the conversion of 5′-AMP and isopentenyl pyrophosphate into isopentenyl-adenosine-5′-monophosphate, the first and rate-limiting step in the biosynthesis of cytokinins [6]. Constitutive overexpression of the *ipt* gene from its own promotor results in several abnormalities plants, e.g. a complete inhibition of root formation. Therefore, many investigators have tried to solve this problem using regulatable promoters to control the expression of the *ipt* gene, for example fused with a heat-regulated [7] or a light-induced [8] promoter.

A correlation between endogenous cytokinin levels and developmental phenomena in transgenic plants has been demonstrated by several investigators [7–11]. However, the mode of action of cytokinins remains obscure [12]. Both cytokinins and polyamines (pa) are known to delay senescence [13, 14]. In mung bean seedlings the increase in the stigmasterol/sitosterol ratio was related to physiological age of the hypocotyl tissue [15, 16]. Physiologically older hypocotyl segments were also characterized by an increased membrane permeability, probably due to the enhanced stigmasterol/sitosterol ratio of the membranes [17]. In excised mung bean hypocotyl segments, the stigmasterol/sitosterol ratio nearly doubled when incubated in water. Incubation in 5×10^{-5} M cytokinin reduced the senescence as measured by a reduced stigmasterol/sitosterol ratio [16]. Thus cytokinin, pa and sterols are all related to senescence. The availability of *ipt*-transformed plants gives the opportunity to study the possible mode of action of cytokinin in the delay of senescence. Therefore, we compared the sterol and pa content in leaves of different physiological ages of control (SR1) and *ipt*-transformed tobacco plants in order to try to unravel some aspects of the mode of

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Table 1. Cytokinin content (free bases and ribosides in pmol g^{-1} dry weight; conjugated in nmol g^{-1} dry weight) of different leaves of control (C) or *ipt*-transformed tobacco plants*

	Cytokinin	C4	C7	C15	*ipt*4	*ipt*7	*ipt*15
Free bases	z	50	20	<10	482	287	853
	dhz	40	26	<10	312	274	145
	ip	80	60	<14	<10	79	138
Total free bases		170	106	<34	804	640	1136
	Stimulation				4.7×	6.0×	33.4×
Ribosides	zr	74	18	60	100	633	2912
	dhzr	26	6	55	40	856	131
	ipr	46	20	<15	30	98	45
Total ribosides		146	44	130	170	1587	3088
	Stimulation				1.1×	36×	23.7×
Conjugated	zng	0.01	<0.02	<0.02	0.02	0.02	0.07
	dhzng	0.01	<0.02	<0.02	0.04	0.04	0.03
	z7g	<0.15	2.50	2.75	4.31	9.18	80.47
	dhz7g	0.62	0.71	0.15	0.81	3.63	41.50
	zrog	<0.07	<0.06	<0.07	<0.11	0.30	1.77
	dhzrog	<0.07	<0.06	0.06	<0.11	0.40	6.52
	zrp	0.06	0.17	0.06	0.43	0.82	0.03
	dhzrp	0.04	0.33	0.06	0.72	0.25	0.003
	ipng	<0.01	<0.01	<0.01	<0.01	0.008	<0.01
	zog	0.006	0.005	0.05	0.25	0.42	2.10
	dhzog	0.004	0.002	0.04	0.31	0.40	1.35
Total conjugated		1.06	3.89	3.28	7.12	14.73	127.98
	Stimulation				6.7×	3.8×	39×

z, zeatin; zr, zeatin riboside; dhz, dihydrozeatin; dhzr, dihydrozeatin riboside; dhzog, dihydrozeatin-*O*-glucoside; zog, zeatin-*O*-glucoside; dhzrog, dihydrozeatin roboside-*O*-glucoside; zrog, zeatin riboside-*O*-glucoside; zrp, zeatin-5′-monophosphate; zng, zeatin-3 or 9*N*-glucoside; dhzng, dihydrozeatin-*N*-glucoside; z7g, zeatin 7-glucoside; dhz7g, dihydrozeatin 7*N*-glucoside; ip, N^6-(Δ^2-isopentenyl)adenine; ipa, N^6-(Δ^2-isopentenyl)adenosine; 2ipmp, N^6-(Δ^2-isopentenyl)adenosine-5′-monophosphate; dhzrp, dihydrozeatine-5′-monophosphate; ipng, N^6-(Δ^2-isopentenyl)adenine-3 or 9 N-glucoside.

action of cytokinin. Our first approach was to localize the most pronounced effects of an enhanced cytokinin level on the free sterol and pa contents.

RESULTS AND DISCUSSION

The plants obtained from transgenic *Pssu-ipt* seedlings show the characteristic cytokinin phenotype [8]. The growth is strongly retarded due to a much reduced root system, and apical dominance is inhibited. The leaves of the *ipt* plants are wrinkled and the chlorophyll content in the transgenic plants is about 1400 ± 100 $\mu g\ g^{-1}$ fresh weight, which is slightly but significantly higher than in control SR1 plants (1120 ± 70 $\mu g\ g^{-1}$ fresh weight). Increased endogenous cytokinin level affects strongly the balancing of the steady-state photosynthesis and the sink–source relationship of photosynthetic products [10].

Cytokines (both free and conjugated) were determined in samples of leaves 4, 7 and 15 (Table 1). Leaf-numbering was from top to bottom (i.e. leaf 1 was the youngest). In control leaves the total content of the free bases decreased with increasing leaf number from 170 pmol g^{-1} dry weight in leaf 4 to less than 34 pmol g^{-1} dry weight in leaf 15. As expected, *ipt*-transformed plants had a much higher cytokinin content. Zeatin and dihydrozeatin were simulated at all leaf positions, whereas the amount of ip increased only in the lower leaves. Compared with leaf 15, total amounts of the free bases were significantly lower in leaves 4 and 7. Whereas the total free cytokinin base content in control leaves decreased from top to base, the inverse was found for *ipt* plants, with a somewhat lower amount in the middle leaves. The stimulation in *ipt* leaves was by a factor of 4.7, 6 and 33.4 for leaves, 4, 7 and 15, respectively. The accumulation of free cytokinins was highest in the lower leaves of *ipt* plants. In control tissue the total amount of ribosides was somewhat lower in the middle leaves. In the transgenic plants, the total amounts increased with leaf age, particularly due to the increase of zeatin riboside (zr). Very large amounts of z-7- and dz-7-glycosides were found in the leaves of control plants, as well as in those of *ipt* plants. In *ipt* plants the total amounts of these conjugates were increased by a factor 6.7, 3.8 and 39 in leaves 4, 7 and 15, respectively. In *ipt* plants the

O-glycosides of the free bases and the ribosides accumulated mainly in the older leaves. Practically no accumulation was observed for 3*N*- or 9*N*-glycosides. In the control plants (SR1) a decrease in the concentrations of putrescine (put), spermidine (spd) and spermine (spm) occurred from the young leaves (1) to the older leaves (15) (Fig. 1(A)). The apex with its very small leaves had pa levels comparable to those in the youngest leaves. In *ipt*-transformed plants the spd and spm concentrations showed a similar decreasing trend as seen in the control plants (Fig. 1(B)) and their concentrations were not significantly different from those in SR1 plants. The large differences between SR1 and *ipt* plants were observed in the put levels. In leaves 1 to 4, the concentrations of put were comparable in SR1 and *ipt* plants (Fig. 1(A) and (B)). In leaves 5 to 8 the put concentration was 40–50% lower in *ipt* plants. However, from leaf 9 onwards, *ipt* plants showed a very pronounced increase in put concentration, whereas the put concentration in SR1 plants decreased further. This led to very large differences in the oldest leaves, about 2.5 times more put occurring in the old leaves of the *ipt* plants (Fig. 1(C)). Altogether, the results of cytokinin analysis correlate well with the put analysis.

Beside put, spd and spm, some unknown peaks occur mainly in the HPLC chromatograms of the *ipt* plants. These components occurred at a R_t of 1.52 and 3.15 relative to that of the internal standard (diaminoheptane (2Dns)). Their level was estimated at about 10% of the total pa content. Due to the low concentrations of pa that are usually detected by a fluorescence detector, complete chemical identification of these substances will be very difficult.

The ornithine decarboxylase (ODC) activity was very high in young leaves (3+4) and decreased with leaf age from top to bottom in both control leaves and in leaves from *ipt*-transformed plants (Table 2). ODC activity in young *ipt* leaves did not differ significantly from that in control leaves, but in leaf 7 the activity was only about half that of corresponding control leaves. On the contrary, in leaves 10 and 14 of *ipt* plants ODC activity was enhanced by a factor of 2.7 and 4.5, respectively, above that in control leaves. In young leaves arginine decarboxylase (ADC) activity was much lower than that of ODC, and the activity decreased with leaf age. In leaves 3+4 and 7 no significant difference in ADC activity was observed between control and *ipt* leaves. From leaf position 10 on, ADC activity in *ipt* leaves surpassed the activity in control leaves by a factor of 1.47 and 10.3 in leaves 10 and 14, respectively. The tissue concentrations of pa (especially put) are quite well related to ODC and ADC activities, which were also high in young leaves and decreased with leaf age. However, in leaves 5 to 8 of *ipt* plants the put levels were 40–50% lower than in control plants, as were ODC and ADC activities. The lower concentration of free cytokinin bases in leaf 7 corresponded with the low put content in this leaf as well.

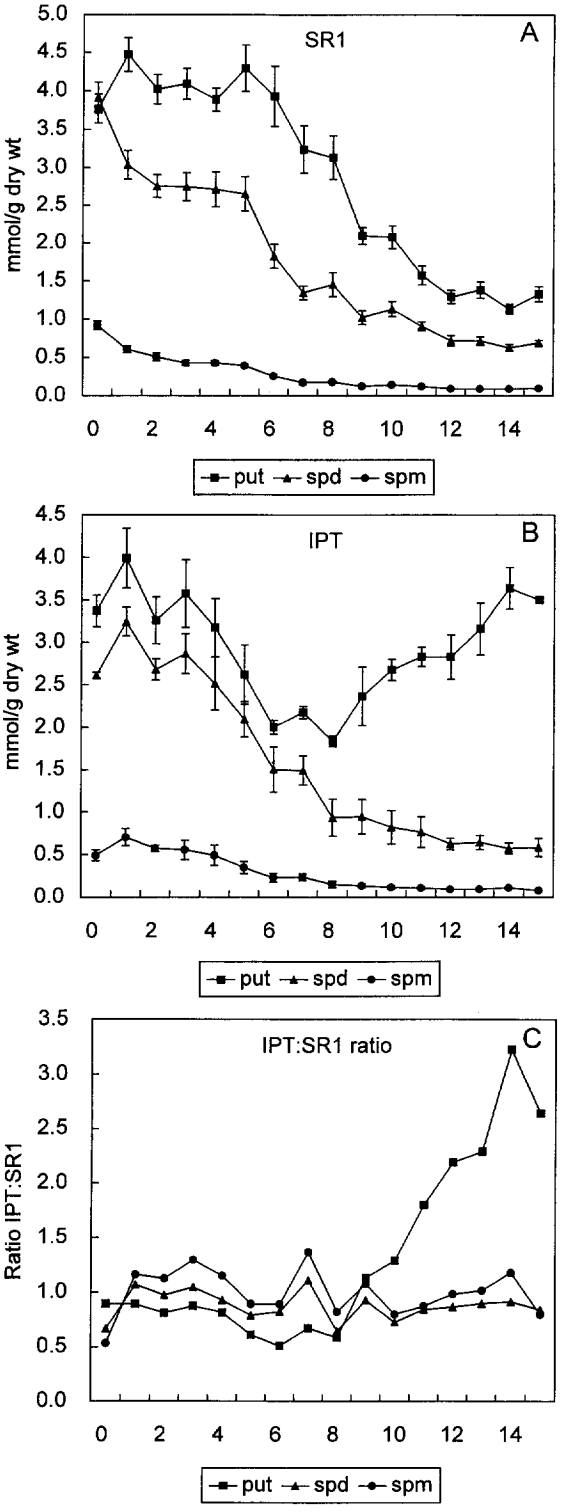

Fig. 1. Polyamines in the apex (0) and leaves 1 (upper) to 15 (lower). (A) SR1 control; (B) *ipt* plants; (C) ratio of the pa found in *ipt* plants to that in control SR1 plants. Mean of three experiments.

In the control plants, pa levels were high in the young leaves and decreased in the older leaves. Similar differences in pa titre as a function of tissue age have

Table 2. ODC and ADC activity (pkat mg^{-1} protein) in leaves 3+4, 7, 10 and 14 of control and of *ipt*-transformed plants ($n = 3$)

	ODC		ADC	
Leaf	Control	*ipt*	Control	*ipt*
3+4	8.4±0.27	8.2±0.24	0.18±0.02	0.18±0.007
7	0.7±0.08	0.4±0.02	0.09±0.01	0.1±0.01
10	0.4±0.05	1.1±0.09	0.06±0.08	0.09±0.01
14	0.2±0.007	0.8±0.06	0.02±0.004	0.22±0.03

been observed in mung bean [17] and tomato [18]. The influence of enhanced cytokinin levels in *ipt* plants on put levels was observed in the older leaves only, there being no influence on the upper part of the plants. The endogenous cytokinin content in young control leaves was possibly sufficient to maintain pa biosynthesis. In lower control leaves, the decrease in pa level could be related to the extremely low cytokinin free base content, whereas in old leaves of *ipt*-transformed plants the high cytokinin content maintained put biosynthesis. Although the put content increased in old *ipt* leaves, no increase in spd or spm content was found. Further studies are necessary to ascertain whether this is due to a block in the pathway between put and spd provoked by high cytokinin content, or to a strong homeostatic control of spd level with, for example, an enhanced conjugate formation of spd. This enhanced pa synthesis might reduce ethylene synthesis, thus delaying senescence [19, 20]. Conversely, cytokinins have been reported to stimulate ethylene biosynthesis in *Arabidopsis thaliana* [21].

A lower pa content in root-inducing left hand transfer (RiT) DNA transformed tobacco has been reported [22, 23]. A phenotype with wrinkled leaves, shorter internodes, increased branching, delayed flowering, partial sterility and altered geotropic behaviour in roots was observed. It was suggested that leaf wrinkling resulted from the lower pa titre. Control plants treated with difluoromethylornithine (DFMO), a known inhibitor of put biosynthesis, showed leaf wrinkling that was almost completely reversed by the addition of put. In our plants leaf wrinkling occurred, but this was accompanied by an enhanced put content in the older leaves. Therefore, it seems doubtful that leaf wrinkling is induced by low put levels and that the observed changes in phenotype are due to interference with pa metabolism as suggested by Burtin *et al.* [23]. Possible functions of the enhanced put levels are an inhibition of ethylene production [19, 20] or a stabilization of membranes, thus influencing membrane permeability [25–29], or inhibition of pigment breakdown [26, 30].

The put content in SR1 leaves decreased with age, as did the total zeatin content. In *ipt* plants the put levels as well as the ODC and ADC activities followed a similar pattern as the total cytokinin free base content (decrease to the middle leaves and then increasing in the lower leaves). These results suggest a direct involvement of cytokinin free bases in the regulation of put concentration, although the causal relationship between these two parameters requires further study. Furthermore, it is not known which of the cytokinins is responsible for the observed changes.

Sterol analyses revealed that in leaves of control plants the total sterol content decreased with leaf age from about 1500 $\mu g\ g^{-1}$ dry weight in the top leaves to about 850 $\mu g\ g^{-1}$ dry weight in basal leaves (Fig. 2(A)). In all leaves the content of stigmasterol was greater than that of sitosterol. The absolute stigmasterol content fluctuated around a constant value in all leaves, whereas sitosterol decreased as function of leaf age (Fig. 2(A)). Thus, as percentage composition, stigmasterol increases and (sitosterol decreases with increasing leaf age (Fig. 2(C)). The stigmasterol/sitosterol ratio increased four-fold from the young leaves to the older ones (Fig. 2(C)). Cholesterol made up about 10.5% of the total sterol mixture in the upper leaves and about 6.6% in the four lower leaves. The campesterol concentration varied around 17.5% in the upper leaves and around 25% of total sterols in the lower leaves. A known precursor of sitosterol, 28-isofucosterol, was present in low amounts in the apex as well as in the first six leaves (Fig. 2(A)).

The total sterol content was about two times higher in *ipt* plants than in control plants. The total amount of sterol decreased with increasing leaf age. Contrary to the control plants, in *ipt*-transformed plants the absolute stigmasterol level was lower than that of sitosterol in the four youngest leaves (Fig. 2(B)). The stigmasterol level remained almost unchanged as a function of leaf age, whereas sitosterol decreased from the fifth or sixth leaf on. The stigmasterol/sitosterol ratio was very low in the five youngest *ipt* leaves and then started to increase as in the control plants (Fig. 2 (C)). Thus, in *ipt* plants the 'ageing' or membrane maturation, as measured by the increase in the stigmasterol/sitosterol ratio, starts at the fifth or sixth leaf with a similar rate as that occurring in control plants from the first leaf on (Fig. 2(C)). Cholesterol and campesterol levels did not change significantly between different leaves. In the apex and the upper leaves 28-isofucosterol is also found. Enhanced cytokinin levels had a double effect on sterols in *ipt* plants: they increased total sterol levels and delayed the increase in the stigmasterol/sitosterol ratio. In

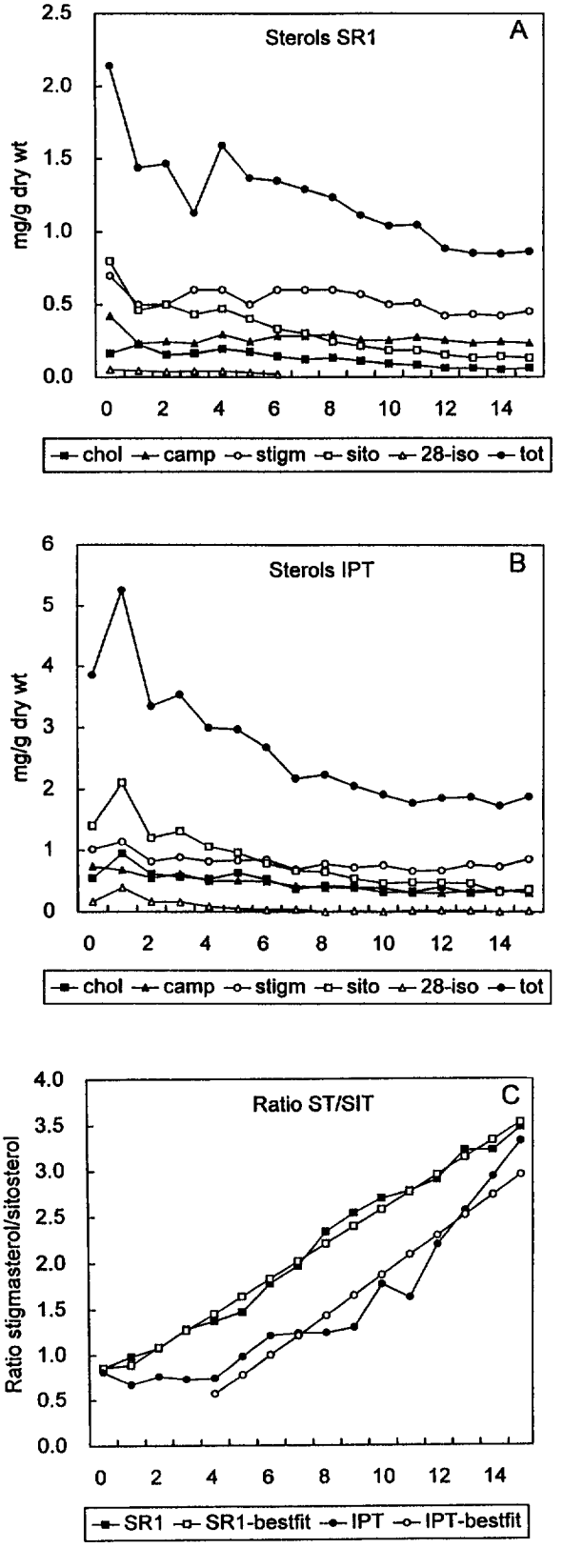

Fig. 2. Sterols in the apex (0) and leaves 1 (upper) to 15 (lower). (A) Control SR1 plants; (B) *ipt* plants; (C) stigmasterol/sitosterol ratio and the best-fit curves. Mean of three experiments, S.E. between 5 and 12% (not shown) for clarity.

mung bean seedlings the increase in the stigmasterol/sitosterol ratio was seen as an indication of tissue ageing or maturation, and this increase was found along the hypocotyl axis from top to bottom [15]. If growing mung bean hypocotyl segments were cut and floated on water, the ageing of the segments as well as the increase in the stigmasterol/sitosterol ratio occurred much faster than in marked segments on the intact plants. However, when excised segments were floated on 5×10^{-5} M cytokinin, ageing and the increase in the stigmasterol/sitosterol ratio were delayed [16]. It was also shown that sterol biosynthesis was localized in the young shoot tissues, which also contained Δ^7-avenasterol and 28-isofucosterol, both precursors of sitosterol [31, 32]. The change in the stigmasterol/sitosterol ratio as a function of tissue age has already been observed in tobacco [33, 34]. Moreover, mung bean seedlings under heavy metal stress (Cd, Cu, Zn, As) showed biochemical reactions similar to those occurring in ageing and they also responded with an increased stigmasterol/sitosterol ratio (Geuns *et al.*, unpublished).

It can be deduced from Fig. 2(B) that sterol synthesis is enhanced and probably maintained for a longer time in plants expressing the *ipt* gene. Accordingly, the amount of sitosterol was higher than that of stigmasterol in the upper four leaves. Enhanced cytokinin levels in the leaves delayed the increase in the stigmasterol/sitosterol ratio. Whether this is due to the disappearance of sitosterol or a conversion of sitosterol to stigmasterol, which is then metabolized further, cannot be deduced from our results. Also, in mung bean segments and barley leaves floating on a 5×10^{-5} M cytokinin solution, cytokinin delayed ageing and lowered the stigmasterol/sitosterol ratio. The changes in sterol composition were observed in all the cell membranes and organelles analysed [16]. α-Naphthalene acetic acid increased sterol biosynthesis, also resulting in a decreased stigmasterol/sitosterol ratio [35].

Free sterols are membrane components and the sterol composition is very important for the maintenance of membrane structure, function and integrity. An enhanced stigmasterol/sitosterol ratio enhances membrane leakage and reflects membrane senescence. Changes in the sterol and fatty acid compositions of membranes do influence membrane permeability and enzyme activity (e.g. ATPase, the activity of which is influenced by lipid environment [35–38]). In our *ipt*-transformed plants the observed effects of enhanced cytokinin levels on the sterol level and composition is one of the possible modes of action of cytokinin in the delay of senescence. By keeping the sterol level high and the stigmasterol/sitosterol ratio low in the different membranes, cytokinin might be able to maintain membrane integrity.

CONCLUSIONS

Our results imply that the enhanced cytokinin levels in older *ipt* leaves delay or prevent their senescence:

the put content and ODC and ADC activities are enhanced, the stigmasterol/sitosterol ratio is lower, sterol biosynthesis is enhanced and the leaves contain more chlorophyll. The mode of action of cytokinin might be through an increase of put or of sterol biosynthesis and metabolism. Our results also emphasize the importance of discriminating between the different plant zones that are influenced by higher cytokinin levels (e.g. in studies on membrane isolation, or in experiments on the biosynthesis of pa or sterols under the influence of enhanced cytokinin levels). Furthermore, we demonstrate that pa and sterol metabolism were affected in a different way in different regions of *ipt*-transformed tobacco plants: in the upper leaves no influence of enhanced cytokinin levels on free pa metabolism was found, whereas in these leaves a very pronounced effect on sterol metabolism was found, probably associated with a delay of membrane maturation (measured as the stigmasterol/sitosterol ratio). It is not known whether the enhanced cytokinin levels also influence compartmentalization and membrane permeability or enzyme activity (e.g. ATPase) because of an altered pa and sterol metabolism.

EXPERIMENTAL

Plant material. Seeds of *Pssu-ipt*-transformed tobacco (*Nicotiana tabacum* L. cv. Petit Havana SR1) were of the same progeny as those described in ref. [8]. The chimeric *Pssu-ipt* gene consisted of gene 4 (*ipt*) from *Agrobacterium tumefaciens* coupled to the light-inducible *ssu*-promotor (*Pssu*) from the gene encoding for the small subunit of Rubisco of *Pisum sativum* L. Transgenic *Pssu-ipt* shoots were regenerated and grafted onto wild-type root stocks. After selfing, the F1 generation was germinated *in vitro*. After 4–6 weeks the *ipt*-transgenic (kanamycin-resistant) seedlings were transferred to soil and cultivated for 48 days under greenhouse conditions. Plants with a shoot length of 25 cm were used in the study. These plants are further named *ipt* plants.

Non-transformed tobacco seedlings from *in vitro* pre-culture were grown for 17 days in soil in the greenhouse. These control plants, denoted by 'SR1', also had a shoot length of 25 cm. The apex consisted of the apical meristem and the smallest leaves. The uppermost leaf of about 23 mm length was named the first leaf.

Polyamine analysis. A modified procedure was used for small amounts of tissue [39]. Ten mg of dry powdered tissue was homogenized in 1 ml 4% $HClO_4$ containing 1,7-diaminoheptane/2HCl (2 mg l^{-1}) as internal standard. After 1 hr extraction at 4° the homogenate was centrifuged. To 50 μl of the supernatant were added 100 μl 0.4 M borate/NaOH buffer (pH 11) and 100 μl of dansyl chloride soln (7 mg ml^{-1} acetone). After vortexing, the mixture was heated at 60° (dark) for 15 min. The dansylated pa were extracted in 0.6 ml toluol. After extraction, 0.6 ml hexane was added to the toluol to lower the polarity in order to obtain a quantitative adsorption on the silica gel purification columns. These were made of 250 mg sillicagel 60 (0.063–0.2 mm for column chromatography) loaded in 1 ml pipette tips containing a plug of glass wool. The toluol/hexane fraction was applied to the columns, which were then rinsed with 250 μl toluol and 250 μl toluol/ Et_3N (10:0.3 v/v). The columns were then sucked dry and the dansylated polyamines eluted with 2 × 0.3 ml EtOAC, which was evaporated in a vacuum centrifuge. The residue was dissolved in 250 μl MeOH, of which 10 μl was injected into a fully automated HPLC system. Column: 10 cm × 3 mm i.d., 5 μm Spherisorb S5 ODS 2 (Bio-Rad); solvent and column at 50°; solvent: 1.5 ml min^{-1}. Gradient 0–2 min, 58% MeCN in H_2O; 2–7.5 min, 58–91%; from 7.6 min, rinsing with 58% MeCN. Analysis time; 10 min. Detection: fluorescence detector (exc., 340 nm; em, 510 nm).

Sterol analysis. Dry powdered tissue (50 mg) was extracted with 4 × 3 ml acetone after the addition of 20 μg 5α-cholestanol as internal standard. The vials were continuously sonicated for 30 min, at a maximum temp. of 40°. After extraction the acetone was evaporated under N_2. The residue was dissolved in Et_2O and spotted on TLC plates of silica gel. Spots of 5α-cholestanol and lanosterol at 1.5 cm from the bands of the extracts were used to locate the free sterol bands (resp. sterols and 4,4-dimethylsterols). After development of the plates in petrol/Et_2O/HOAc (40:10:1), the sterol bands were visualized under UV (366 nm) after spraying the plates with berberine · HCl in EtOH. The bands scraped off from the TLC plates were extracted with 10 ml Et_2O, which was sucked over *ca* 0.5 g Al_2O_3 (Brockman grade III) on a glass filter and evaporated under N_2 into very small sample vials made of the tips of Pasteur pipettes. The free sterol samples were dissolved in 50 μl $(ClCH_2)_2$, of which 0.2–0.4 μl was injected directly into a capillary column by using a homemade injector, in which the column is pulled through the hot injection port above the column oven. As the injection of the sample solution occurs at room temp., problems associated with on-column injection, discrimination of high boiling compounds and peak broadening are avoided. The solvent evaporates at room temp. in the column end above the GC oven and the sterol sample is deposited as a narrow band ($\pm$2 mm) on the cold column end. After the elution of the solvent peak, the injector is pushed down, thus bringing the column end with the sterol sample condensed on it into the hot injection port and automatically starting the printer–plotter. Problems associated with cooling down and warming up of the column oven are also avoided [40]. GC conditions: column, WCOT fused silica, 25 m × 0.32 mm, CP-Sil-8 CB (Chrompack), df = 0.12 μm, oven at 250°; carrier, H_2 at 0.7 bar; FID at 290°; injection port, 285°; injector, homemade [40].

Extraction, purification and analysis of cytokinins. To determine the endogenous cytokinin contents,

dried and powdered tissue was extracted overnight at −20° in $CHCl_3$/MeOH/H_2O/HCOOH (5:12:2:1) [41]. Deuterated cytokinin standards of [$^2H_5$]z, [$^2H_5$]zr, [$^2H_5$]z9g, [$^2H_5$]zog, [$^2H_5$]zrog, [$^2H_5$]2ip, [$^2H_5$]2ipa, [$^2H_5$]2ip9g, [$^2H_3$]dhzmp (Apex, Honiton, U.K.) were added for recovery purposes. The supernatant was evaporated until the water phase, and the pH was adjusted to 7. This extract was then purified on DEAE–sephadex (HCO_3^- form and RP-C_{18} columns), followed by immunoaffinity chromatography [42]. The cytokinin fractions were analysed further by HPLC linked to a Quatro II mass spectrometer equipped with an electrospray interphase ((+)ES Lc-MS/MS) [43]. Samples (10 μl) were injected and eluted with MeOH/NH_4OH 0.01 M (70:30) at 800 μl min^{-1}. Using a post-column split of 1/20, the effluent was introduced into the electrospray source (source temp. 80°C, capillary voltage +3.5 kV, cone voltage 20 V). Quantitation was obtained by multiple ion monitoring (MRM) of (MH^+) and the appropriate produce ion. Endogenous concns were calculated, taking into account the abundance ratio of the diagnostic ions corresponding to the unlabelled and labelled (deuterated) compounds. Results are given of a typical experiment. Cytokinin dosages were also performed by immunoenzyme assays on plants of the same progeny, and similar results were obtained [44].

ODC and ADC measurements. Ornithine decarboxylase (EC 4.1.1.17) and arginine decarboxylase (EC 4.1.1.19) activities were measured on crude enzyme preparations of leaves 3+4 (combined), 7, 10 and 14, as described in ref. [20]. Addition of 2 mM EDTA and 8 mM cysteine did not reduce CO_2 release, indicating that the assays for ODC and ADC are not influenced by oxidative decarboxylation as reported in ref. [45].

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