Spatial and Temporal Distribution of Polyamine Levels and Polyamine Anabolism in Different Organs/Tissues of the Tobacco Plant. Correlations with Age, Cell Division/Expansion, and Differentiation

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Polyamine (PA) titers and biosynthesis follow a basipetal decrease along the tobacco (Nicotiana tabacum) plant axis, and they also correlate negatively with cell size. On the contrary, the titers of arginine (Arg), ornithine (Orn), and arginase activity increase with age. The free (soluble)/total-PA ratios gradually increase basipetally, but the soluble conjugated decrease, with spermidine (Spd) mainly to determine these changes. The shoot apical meristems are the main site of Spd and spermine biosynthesis, and the hypogeous tissues synthesize mostly putrescine (Put). High and low Spd syntheses are correlated with cell division and expansion, respectively. Put biosynthetic pathways are differently regulated in hyper- and hypogeous tobacco tissues: Only Arg decarboxylase is responsible for Put synthesis in old hypergeous vascular tissues, whereas, in hypogeous tissues, arginase-catalyzed Orn produces Put via Orn decarboxylase. Furthermore, Orn decarboxylase expression coincides with early cell divisions in marginal sectors of the lamina, and Spd synthase strongly correlates with later cell divisions in the vascular regions. This detailed spatial and temporal profile of the free, soluble-conjugated, and insoluble-conjugated fractions of Put, Spd, and spermine in nearly all tobacco plant organs and the profile of enzymes of PA biosynthesis at the transcript, protein, and specific activity levels, along with the endogenous concentrations of the precursor amino acids Arg and Orn, offer new insight for further understanding the physiological role(s) of PAs. The results are discussed in the light of age dependence, cell division/expansion, differentiation, phytohormone gradients, senescence, and sink-source relationships.

Polyamines (PAs) are small polycations that represent important intrinsic developmental signals, linked to important developmental phenomena, including cell growth and division (Chattopadhyay et al., 2002; Theiss et al., 2002), morphogenesis (Paschalidis et al., 2001; Fos et al., 2003), stabilization of nucleic acids and membranes (Thomas and Thomas, 2001), protein synthesis and chromatin function (Mattheus, 1993), and biotic and abiotic stresses (Kurepa et al., 1998; Perez-Amador et al., 2002; Navakoudis et al., 2003; for review, see Bouchereau et al., 1999). Eukaryotic cells synthesize putrescine (Put) directly from Orn through the activity of Orn decarboxylase (ODC). Plants and some bacteria also synthesize Put indirectly from Arg, by the activity of Arg decarboxylase (ADC). An aminopropyl group derived from decarboxylated S-adenosylmethionine (dSAM) is transferred to Put by spermidine synthase (SPDS) to form spermidine (Spd), and another aminopropyl group is added to Spd by spermine synthase (SPMS) to form spermine (Spm). The dSAM is formed by the activity of S-adenosyl-L-Met decarboxylase (SAMDC; for review, see Wallace et al., 2003).

The positive charge of PAs enables them to interact electrostatically with polyanionic macromolecules within the cell. Spd and Spm can bridge the major and minor grooves of DNA, acting as a clamp holding together either two different molecules or two distant parts of the same molecule (Mattheus, 1993). Mutations in genes that control PA biosynthesis exert various effects on cell proliferation and viability, depending on the organisms (Wallace et al., 2003). For example, in Saccharomyces cerevisiae and Schizosaccharomyces pombe, null mutants of a gene encoding for SAMDC have an absolute requirement for Spd or Spm for growth (Chattopadhyay et al., 2002), whereas in Arabidopsis (Arabidopsis thaliana), SPDS genes are essential for survival, but not Spm (Imai et al., 2004). PAs in cells exist as free (soluble [S]), soluble-conjugated (soluble-hydrolyzed [SH]), or insoluble-conjugated (pellet-hydrolyzed [PH]) forms. The exact role of each of these fractions remains unknown.

The complexity in control of cell division, expansion, growth, and senescence in the tobacco (Nicotiana tabacum) plant is well documented (Chen et al., 2001). Growth-promoting or -limiting elements exist, and it is reasonable to presume that PA gradients within meristems, leaves, petioles, internodes, and roots could be part of this network. Because these processes are associated with an enhanced carbon and nitrogen demand, a link to the regulation of sink-source relations is also suggested. During development, leaves undergo a transition from a sink (carbon-importing) to

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a source (carbon-exporting) organ (Oparka et al., 1999). On the other hand, nitrogen is supplied to sinks either via uptake from roots or retranslocation from old leaves (Hikosaka, 2005). PA contents, and mainly the S form, have been analyzed in various plant organs/tissues in in vitro systems (Tiburcio et al., 1997; Bouchereau et al., 1999). In addition, a number of enzymes of PA metabolism have been studied, mostly at the activity and protein level (Bouchereau et al., 1999). However, our in vivo detailed developmentally dictated gene expression analysis of the PA biosynthetic enzymes, in a truly tissue-specific manner, is essential to extend our knowledge about the molecular functions of PAs. In this work, we present the temporal and spatial distribution of the endogenous S-PA (Put, Spd, Spm), SH-PA (Put, Spd, Spm), and PH-PA (Put, Spd, Spm) contents throughout the entire tobacco plant, as well as the expression profiles of all biosynthetic genes at the transcriptional, translational, and posttranslational levels. The results suggest that PA synthesis and conjugation are likely to be an essential part of the homoeostatic mechanism that controls PA levels and their involvement in processes such as vascular differentiation. The genes of the PA biosynthesis network are down-regulated during development and are spatially regulated in a tissue/organ-specific manner. Put biosynthetic pathways work independently in tobacco, whereas Orn is mostly produced from Arg via arginase, as part of the urea cycle. Changes in PA metabolism during leaf development may be part of a mechanism controlling the transitions from cell division to expansion, depending on PA concentrations maintained within strict limits.

RESULTS

PA Levels Follow a Basipetal Decrease along Tobacco Plant Axis

An inverse correlation is generally observed between leaf ontogenic stage and the titers of all fractions of total PA (Put + Spd + Spm; Fig. 1) independently of growth conditions (data not shown). PA concentrations are extremely high in sink leaves of less than 10 mg in weight and decrease drastically when cell division ceases and cell elongation is the sole process of leaf growth. The trend is same regardless the mode of expression of PA levels (nmol g⁻¹ FW or nmol mg⁻¹ protein; data not shown). However, when PA levels are expressed per milligram of protein, differences were lower. This could be explained by the fact that the size of the vacuole and water content increase with cell age. The sums of S, SH, and PH fractions of total PA from the apical meristem to the 10th leaf from apex were 3,180 ± 142 and 684 ± 46 nmol g⁻¹ FW, respectively (4.6-fold decrease), or 157 ± 12 and 49 ± 4 nmol mg⁻¹ protein, respectively (2.2-fold decrease). The sum of S, SH, and PH fractions of total PA in leaves (lamina and petiole) also decreases from 2,577 ± 136 in the youngest (circle area of the first leaf) to 124 ± 3 nmol g⁻¹ FW in the oldest (circle area of the 25th) leaf (Fig. 1). The sums of total Put (S + SH + PH; total Spd, and total Spm are high in sink tissues and gradually decrease 34-fold, 22-fold, and 7-fold, respectively, from the first developing (first) to the senescing (25th) leaf (Fig. 2). In petioles and internodes, total-Put, total-Spd, and total-Spm titers remain relatively constant compared to the respective values in leaf margins and centers from the first to the fifth developmental stage (Fig. 2), suggesting that the young vascular tissues mediate basipetal transport of PAs. Furthermore, the internodes contain significantly higher levels of total Put, total Spd, and total Spm than the acropetal and basipetal parts of the petioles (Fig. 2), indicating that PAs are transported and accumulate in shoots. Endogenous PAs were also analyzed using in vitro-grown plants, and the results reinforce the correlation reported herein between PA titers and developmental stages (data not shown).
The Free/Total-PA Ratios Increase Basipetally from Apex to Base of the Plant, whereas the Conjugated Decrease

SH-Spd is the most abundant form in the hypergeous (aerial) plant organs (data not shown). The ratios of S-PAs/total PAs (white parts of circles/total circles, Fig. 1) increase 2.4-fold basipetally from shoot apex to the base of the plant, whereas the ratios of SH-PAs/total PAs decrease 1.5-fold (Fig. 1). The ratio S-Spd/total S-PAs increases 1.7-fold with increasing age in hypergeous tissues, whereas the ratios S-Put/total S-PAs and S-Spm/total S-PAs decrease 1.9- and 2.6-fold, respectively (Table I). Furthermore, the ratio S-Spd/total Spd also increases 4-fold, whereas S-Put/total Put increases only 2-fold and S-Spm/total Spm decreases 4-fold. Thus, taking into account also the respective conjugated ratios, it can be concluded that the basipetal increase in the S-PAs/total-PAs ratio is presumably due to decreased conjugation of Put and particularly Spd.

Total-PA levels in primary and secondary root tissues do not differ significantly (Fig. 1), although total Spd is greater and total Put is lower in the secondary roots (Fig. 2), whereas all S fractions and SH-Spd are higher in the secondary roots (data not shown).

PAs Correlate with the Cell Division/Expansion Profile in the Youngest Leaves

Cell expansion in tobacco leaves increases, whereas nuclear DNA synthesis (K. Paschalidis and K. Roubelakis-Angelakis, unpublished data) and cell division (Chen et al., 2001) decrease, progressively with leaf age. All PA fractions as well as activities, transcripts, and proteins of the PA biosynthetic enzymes also decrease with age (Figs. 2 and 3). In the 1-cm-long tobacco leaf (first), cell division occurs throughout the leaf and petiole, but mostly at the peripheral regions (Chen et al., 2001). However, when the leaf reaches a length of more than 1.5 cm, cell divisions cease first at the apex and progressively further down the leaf, and gradually they are restricted to the basal part of the leaf blade, before ceasing completely (Poethig and Sussex, 1985; Chen et al., 2001). To investigate if the basipetal cessation of cell divisions and the developmental acquisition of cell size increases are mirrored by a similar reduction in PA content, all fractions of PAs were determined separately in the following sections of the leaves: the apical and marginal region, the central leaf region, the acropetal (apical) petiole, the basipetal (basal) petiole, and the internodes. Cells on the apical and marginal regions of the youngest tobacco leaf, where cells have the highest capacity for auxin-induced growth, maximal division rates, and minimal cell area (Jones et al., 1998), also have the highest titers of all PA fractions (Fig. 2), the highest biosynthetic activities (ADC, ODC, SAMDC, and SPDS; Fig. 3), and the highest odc transcript levels (Fig. 3), but lower Orn levels (Fig. 4), as compared to the acropetal petiole regions. Therefore, PA synthesis, abundance, and distribution seem to correlate positively with cell division, but negatively with the extent and distribution of cell expansion and cell size.

Table I. Free/total-PA ratios in the hypergeous organs of the tobacco plant

<table>
<thead>
<tr>
<th>S-Pu/total S-PAs</th>
<th>S-Spd/total S-PAs</th>
<th>S-Spm/total S-PAs</th>
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<tbody>
<tr>
<td>Shoot apex</td>
<td>0.29 ± 0.02</td>
<td>0.42 ± 0.03</td>
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<tr>
<td>First leaf</td>
<td>0.40 ± 0.02</td>
<td>0.45 ± 0.02</td>
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<tr>
<td>10th leaf</td>
<td>0.26 ± 0.01</td>
<td>0.61 ± 0.04</td>
</tr>
<tr>
<td>25th leaf</td>
<td>0.21 ± 0.01</td>
<td>0.72 ± 0.05</td>
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The transcripts of *adc* are consistent with the activity levels in the first leaf; they are high in leaf margin and decrease basipetally to the petiole (Fig. 3, C and D). However, the transcripts of *adc*, *samdc*, and *spds* and the protein level of ADC do not coincide with specific activities, being lower in leaf margin and gradually increasing in leaf center and petiolar parts (Fig. 3, C–E, and data not shown). The basipetally increasing gradients of transcripts continue also to the internode; the *spds* transcript in the first internode is almost 10-fold higher than in the marginal region of the first leaf (Fig. 3D). The opposite trend of *adc* transcript on one hand and ADC protein and activity levels on the other could suggest that regulation of ADC in tobacco plants is at the posttranslational level, as has been the case with ADC in oat (*Avena sativa*; Malmberg and Cellino, 1994), Arabidopsis (Watson and Malmberg, 1996), and grapevine (*Vitis vinifera*; Primikirios and Roubelakis-Angelakis, 2001). On the contrary, ODC activity is regulated at the transcriptional and the translational levels (Marton and Pegg, 1995). Lack of correlation between *samdc* and *spds* transcript levels and SAMDC and SPDS enzyme activities could also suggest a posttranscriptional, translational, and/or posttranslational regulation. SAMDC in mammals and plants is produced as a proenzyme maturing by autocatalytic proteolysis between two Ser residues (Heby and Persson, 1990; Schroder and Schroder, 1995), whereas SPDS is suggested to exist as a stable multienzyme complex (Panicot et al., 2002) or may be posttranslationally (Zhang et al., 2003) or posttranslationally (Yoon et al., 2000) modified. Because of the large volume of data, only those of the marginal leaf regions, the acropetal petiolar ones and the internodes, at each developmental stage and parameter, are shown.

Low SPDS Coincides with Cell Expansion in Tobacco

In expanding leaves, the activities of ADC and ODC (Fig. 3A) follow the basipetally decreasing gradient, from leaf margin to petiole, which is also found at the first developmental stage. On the contrary, in leaves of the fifth and 10th developmental stages, SPDS- and SPMS-specific activities increase from leaf margins to petioles following a reverse, to the first developmental stage, gradient (Fig. 3A). Both enzyme and transcript levels of SPDS are almost 2-fold higher in petioles than in leaves at the 10th developmental stage (Fig. 3, A, C, and D). In mammals (Marton and Pegg, 1995) and

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**Figure 3.** Expression patterns of ADC, ODC, SAMDC, SPDS, and SPMS in the soluble fractions from tobacco plant tissues. A, Shoot apex; L, apical and marginal leaf lamina (○); P, acropetal petiole (△); I, internode (dotted line); R, primary root; r, secondary root. A, Specific activities in L, P, and I at different developmental stages. B, Specific activities of ADC, ODC, and SAMDC (nmol CO₂ h⁻¹ mg⁻¹ protein), SPDS (nmol Spd h⁻¹ mg⁻¹ protein), and SPMS (nmol Spm h⁻¹ mg⁻¹ protein) in shoot apex, primary, and secondary roots. C, RNA gel-blot analysis of the expression of *adc*, *samdc*, and *spds* genes. D, Quantification of mRNA levels (mRNAs/actin mRNAs). E, Western-blot analysis of ADC protein. Numbering started from the apical organ being designated as first. Error bars represent ±s.
Chlamydomonas reinhardtii (Theiss et al., 2002), cell divisions are abolished by the addition of 4-methyl-cyclohexylamine, an inhibitor of SPDS, whereas addition of the ODC inhibitor DL-α-difluoromethylornithine does not abolish C. reinhardtii cell divisions but causes an early accumulation of dividing cells (Theiss et al., 2002). Furthermore, significantly higher ODC activities are measured after addition of the SPDS inhibitor 4-methyl-cyclohexylamine (Theiss et al., 2002), whereas inhibition of ODC by DFMO causes an increase in SAMDC activity, resulting in increased synthesis of Spd (Marton and Pegg, 1995).

As ODC activity has been correlated with cell divisions in many mammalian and higher plant systems (Marton and Pegg, 1995; Bouchereau et al., 1999), our results, together with the above, could suggest a tissue- or age-dependent expression of either ODC or SPDS in dividing tobacco cells, competing with each other for the progression of cell divisions. In the youngest tobacco leaf margin, ODC activity and transcript levels are almost 3- and 1.5-fold higher than in the youngest petiole, respectively (Fig. 3, A and C).

Put Is Synthesized via ODC in Root Tissues and via ADC in Old Hypergeous Organs; Biosynthetic Enzymes of Spd and Spm Are Absent in the Particulate Fractions

The specific activities of all biosynthetic enzymes in the soluble fraction generally decrease with age in tobacco, with the exception of SPMS (Fig. 3, A and B), and are in agreement with the endogenous PA levels. ODC soluble-specific activity is significantly higher in the hypogeous than in the hypergeous organs, including shoot apical meristems, which also exhibit high ODC activity (Fig. 3, A and B). SAMDC activity is also high in roots, whereas ADC is lower in roots than in old and fully developed tissues (Fig. 3, A and B).

ADC and ODC activities in the particulate fractions of aerial tobacco tissues (leaves, petioles, and internodes) follow the same trend as the soluble ones (Table II). ODC particulate activity is approximately half of the soluble one, while in young roots it is extremely low as compared to the activity in the soluble fraction (Table II; Fig. 3), which is almost 30-fold higher than the respective ADC (Fig. 3, A and B). In contrary, higher ADC than ODC specific activities are present in the old hypergeous organs. Root apical tissues contain higher soluble but lower particulate ADC and ODC activities than the older root tissues. Furthermore, SAMDC, SPDS, and SPMS activities are absent from the particulate fractions.
Arginase Activities and Arg and Orn Levels Increase with Age; Put Synthesis in Hypogeous Tissues Is Due to Successive Actions of Arginase and ODC

In tobacco plant, arginase-specific activities, as well as Arg and Orn levels, increase with increasing organ age (Fig. 4). Differences are more pronounced in internodes, where arginase, Arg, and Orn increase from the youngest to the oldest one, 3.9-, 1.3-, and 1.8-fold, respectively. The above three parameters generally increase from marginal leaf regions to petioles and internodes. The newly formed roots exhibit higher arginase and Orn but lower Arg levels than the primary roots (Fig. 4B). Furthermore, Put biosynthesis in roots (Fig. 2) uses Orn as a substrate and is due to successive actions of arginase (Fig. 4B) and ODC (Fig. 3B).

The Shoot Apical Meristems Are the Highest Biosynthetic Sites of Spd and Spm

It is evident that all the studied organs of the tobacco plant are capable of synthesizing PAs (Fig. 5). The highest relative Put biosynthetic capacity is localized in the hypogeous apical meristems followed by the shoot apex and young developing leaves. The highest relative biosynthetic capacity of the higher PAs (Spd and Spm) is localized in the shoot apex and in the youngest aerial tissues, but roots also synthesize Spd and Spm at high rates (Fig. 5). These trends apply to plants of different ages and are independent of photoperiod (data not shown). Spd and Spm synthesis is more uniformly distributed (with lower temporal differences) than Put synthesis throughout tobacco development. Lower but still significant rates of PA biosynthesis are exhibited in expanding leaves and in old vascular tissues.

DISCUSSION

Tobacco has been used as a model system to study the various events related to primary assimilation or remobilization of carbon and nitrogen during transition of sink to source leaves and senescence (Oparka et al., 1999; Masclaux et al., 2000). In addition, PAs are proposed to participate in the plasticity of carbon and nitrogen metabolism in higher plants (Bouchereau et al., 1999). Turano and Kramer (1993) determined the interaction of carbon and nitrogen availability on Table II. ADC and ODC (nmol CO₂ h⁻¹ mg⁻¹ protein) specific activities of the particulate fractions in protein extracts of tobacco

Activities were measured in the first, fifth, 10th, 15th, 20th, and 25th developmental stages (numbered from shoot apex) and in the shoot apex, primary root, and secondary root. Data are the means ± se. ND, Not detected.

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<tr>
<th></th>
<th>First</th>
<th>Fifth</th>
<th>10th</th>
<th>15th</th>
<th>20th</th>
<th>25th</th>
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<tbody>
<tr>
<td>ADC</td>
<td>1.2 ± 0.11</td>
<td>0.6 ± 0.04</td>
<td>0.4 ± 0.02</td>
<td>0.2 ± 0.00</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>P</td>
<td>0.6 ± 0.06</td>
<td>0.3 ± 0.02</td>
<td>0.2 ± 0.02</td>
<td>0.1 ± 0.00</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>I</td>
<td>0.5 ± 0.04</td>
<td>0.7 ± 0.05</td>
<td>0.4 ± 0.01</td>
<td>0.2 ± 0.01</td>
<td>0.2 ± 0.00</td>
<td>0.2 ± 0.0</td>
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<tr>
<td>ODC</td>
<td>L</td>
<td>2.6 ± 0.24</td>
<td>2.2 ± 0.12</td>
<td>0.8 ± 0.09</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.9 ± 0.09</td>
<td>0.7 ± 0.09</td>
<td>0.4 ± 0.03</td>
<td>0.2 ± 0.00</td>
<td>ND</td>
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<tr>
<td></td>
<td>I</td>
<td>0.5 ± 0.04</td>
<td>0.4 ± 0.03</td>
<td>0.5 ± 0.03</td>
<td>0.4 ± 0.01</td>
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<tr>
<th></th>
<th>Shoot Apex</th>
<th>Primary Root</th>
<th>Secondary Root</th>
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<tbody>
<tr>
<td>ADC</td>
<td>0.9 ± 0.07</td>
<td>0.2 ± 0.01</td>
<td>0.1 ± 0.00</td>
</tr>
<tr>
<td>ODC</td>
<td>1.3 ± 0.09</td>
<td>0.7 ± 0.06</td>
<td>0.3 ± 0.01</td>
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Figure 5. PAs as signal-integrating developmental processes throughout the tobacco plant. PA synthesis is estimated from the enzyme-specific activities in the soluble fractions. Activity levels are represented by magnitude levels depicted by the sizes of letters P for Put synthesis and S for synthesis of higher PAs (Spd and Spm). A, Sites of Put synthesis. Synthesis of Put is depicted as a sum of ADC and ODC activities. B, Sites of synthesis of higher PAs. Synthesis of higher PAs is depicted as a sum of SPDS and SPMS activities. Numbers 10.2 and 79 show (with arrows) the activity levels (in nmol [of the product measured] h⁻¹ mg⁻¹ protein) depicted by the sizes of the biggest P and S, respectively. All the other sizes of P and S are depicted proportionally to the respective biggest ones.
PA accumulation in soybean (*Glycine max*) by using a detached leaf system. Aziz (2003) suggests that Spd could regulate fruitlet abscission in grapevine by modulating the levels of sugars and amino acids in inflorescences, whereas, in tobacco, PAs and aromatic amines could be a limiting factor in sexual development (Martin-Tanguy, 1997) or play a role in apical dominance (Geuns et al., 2001). There is a lack of information on the relationship between the PA biosynthetic pathways and the metabolism of carbon and nitrogen or partitioning between source and sink organs in the vegetative plant. In tobacco, PA distribution follows a reduction from sink to source leaves, and the strongest reduction coincides with senescence.

In this context, PAs could act either as carbon/nitrogen reserves or as signal molecule(s) regulating senescence and probably sink-source relationships. Also, PAs are present in thylakoid membranes, PSI, and the light-harvesting complex (Kotzabasis et al., 1993b; Šťich et al., 2004), suggesting a functional role in the photosynthetic activity during senescence or in the regulation of photoassimilate synthesis and/or partitioning between organs. PAs also inhibit ethylene biosynthesis and other degradative processes associated with senescence, including chlorophyll loss, increase in membrane leakage, and rise in RNAse and protease activities (Pandey et al., 2000). PA involvement in delay of senescence was confirmed by elegant transgenic approaches (Mehta et al., 2002; Tucker and Seymour, 2002). However, the underlying molecular basis for this PA implication is still not understood. Other phytohormones may also affect PA metabolism. A transient increase in ACD and ODC activities as well as in the amount of free PAs and a decrease of conjugated PAs during early parthenocarpic fruit development induced by auxins and gibberellins (Alabadi et al., 1996) has been observed; expression of ODC and SPD genes is also up-regulated (Alabadi and Carbonell, 1998, 1999). Fos et al. (2003) have suggested that parthenocarpy induced by the recessive mutation *pat-2* in tomato (*Lycopersicon esculentum*) activates PA biosynthesis through the ODC pathway leading to elevated free-Spm content in unpollinated ovaries, probably as a result of a higher concentration of active GAs in *pat-2* ovaries. Furthermore, jasmonate-induced up-regulation of PA biosynthetic genes (Biondi et al., 2001; Goossens et al., 2003) is differently modified by the presence/absence of exogenously supplied auxins and cytokinins (Biondi et al., 2003).

Although all organs of a tobacco plant are competent to synthesize PAs (Fig. 5), the shoot apical tissues exhibit the highest Spd and Spm synthesis and high Put synthesis. Shoot apical meristems and actively dividing young tissues contain also the highest contents of PAs, which decrease drastically following the completion of their active development and probably the sink-source transition (Fig. 1). Spd titers are generally higher throughout development and Spm exhibits low variation (Fig. 2), suggesting a tighter regulation of cellular Spm metabolism, compared to Put or Spd, in agreement with results from camellia (*Camellia sinensis*) and grapevine cells (Pedroso et al., 1997; Primikirios and Roubelakis-Angelakis, 1999; Paschalidis et al., 2001). The limited size of the Spd pool may not be large enough to allow the conversion of excess Spd to Spm. Bassie et al. (2000) proposed a threshold model in terms of the size of the Put pool to explain why rice (*Oryza sativa*) tissues expressing the oat *adc* cDNA driven by a very strong constitutive promoter are able to accumulate higher levels of Spd and Spm compared to plants engineered with the same transgene driven by a weaker promoter (Capell et al., 1998) that do not show any changes in the levels of higher PAs.

At relatively low level of auxin, it is suggested that the auxin-binding protein 1 acts to mediate cell expansion, whereas high auxin levels stimulate cell division via an unidentified receptor (Chen et al., 2001). Cells at the tip of a young leaf have the capacity to respond to auxin, and this capacity moves basipetally as the leaf expands (Chen et al., 2001). In tobacco, there is a negative correlation between the abundance of PAs, the epidermal and mesophyll cell size, and leaf area. Before the leaf is fully expanded, it undergoes the sink-source transition progressing basipetally from the apical lamina to the base (Oparka et al., 1999). In this leaf, relatively low synthesis of higher PAs (Spd and Spm) occurs at the apical regions where cell expansion rate is maximum and cell division rate is minimum. On the other hand, at locations and times when cell division is maximum, SPDS expression is high. These correlations prompted us to propose that there are two separable Spd (and Spm) synthesis responses: a low Spd synthesis response leading to cell expansion and a high Spd synthesis response leading to cell division. Furthermore, ODC expression correlates with cell divisions in the youngest peripheral lamina regions, whereas SPDS correlates with cell division in the transport system of expanding tobacco leaves (Fig. 3, A and C). In eukaryotic cells, Spd is required for the activation of the initiation factor eIF-5A (Jakuš et al., 1993) and, therefore, necessary for protein biosynthesis and cell growth, whereas in dividing and non-dividing tobacco protoplasts (Siminis et al. 1994), PAs improve totipotency by preventing reactive oxygen species generation and induction of the programmed cell death syndrome (Papadakis and Roubelakis-Angelakis, 2005).

Our understanding of the specific PA pathways is still not precise enough to allow a safe correlation of total biosynthetic and catabolic fluxes in all pathways simultaneously. To our knowledge, this is the first exhaustive report on detailed temporal and spatial distribution of the PA anabolic (this work) and catabolic (K. Paschalidis and K. Roubelakis-Angelakis, unpublished data) pathways in plants. The tobacco secondary roots contain significantly higher free Put than other plant tissues (Fig. 6), except the shoot apical tissues, which contain similarly high free Put (data not shown). The root tissues use mainly as the substrate...
Orn and the ODC pathway for Put production (Fig. 6C). Therefore, the amount of Orn in the tissues could become limiting. The addition of either Orn or Arg to the medium results in increased accumulation of Put in poplar (*Populus* spp.) cells (Bhatnagar et al., 2001), showing that these precursors may be limiting also in tobacco root cells. ODC in mammals is known to be regulated by feedback mechanisms that operate at transcriptional and translational levels (Nilsson et al., 1997; Cohen, 1998), and the turnover is promoted by excess PAs via the induction of an antizyme protein (Hayashi and Murakami, 1995), whereas in grapevine we observed a feedback regulation of ADC protein by exogenous Put (Primikirios and Roubelakis-Angelakis, 1999). In tobacco root apical tissues, high free-Put contents reflect extremely high ODC, but although Arg levels are higher in roots than in the aerial tissues, no relevant increase in ADC activity, protein, and transcript levels are found (Figs. 3 and 4). In the old hypergeous tissues, ADC activities are higher than those of ODC, supporting that Put synthesis is accomplished by ADC in these tissues and by ODC in roots. Thus, Put biosynthetic pathways are regulated in an organ-specific manner in tobacco. A steady-state equilibrium may also be established between the rates of Put biosynthesis and its degradation (K. Paschalidis and K. Roubelakis-Angelakis, unpublished data), as both of them are responsible for the regulation of endogenous concentrations of Put in cells. Our previous results suggest that under conditions of abiotic stress a similar equilibrium would continuously recycle NH₃, therefore minimizing its toxicity (Loulakakis et al., 2002).

A constitutive expression of the ODC enzyme in tobacco root cells could lead to a depletion of Orn pools. Since Orn is also the precursor of Arg, its depletion could reduce the availability of Arg and in turn limit the amount of Put synthesized via ADC (Fig. 6). Both low levels of Arg and ADC observed in newly formed roots, as compared to the primary root, together with high Orn, ODC, and arginase levels (Figs. 3 and 4) could support these hypotheses. Thus, most of the Orn in these cells is directly produced from Arg by arginase as part of the urea cycle and not from Glu. This production, however, is insufficient to saturate the available ODC enzyme in these cells. Although little is known about homeostatic regulation of Orn pools in plants (Bhatnagar et al., 2001), a homeostatic mechanism must exist to increase Orn production concomitant with its increased use.

The presence of alternative pathways for biosynthesis of PAs in plants (Bouchereau et al., 1999) has led to the hypothesis for differential regulation of the expression of each gene and compartmentalization of the respective proteins. SAMDC, SPDS, and SPMS are not present in the particulate fractions from tobacco organs at different developmental stages, whereas ADC and ODC exist in both the soluble and particulate fractions (Fig. 3; Table II). These results indicate that subcellular localization could be an additional means of regulation of PAs in individual cellular compartments during tobacco development.

In conclusion, our data give a clearer picture of the homeostatic characteristics of PA biosynthesis and conjugation in tobacco plant organs during development. Biosynthetic capacity and PA pool size decrease basipetally in the aerial parts during growth and senescence, potentially implicating the sink-source transition. The ratios of soluble and conjugated PAs also change with development (Fig. 1). As the size of the total-PA pool and the relative ratio of SH-PAs/total PAs decrease, the relative ratio of S-PAs/total PAs increases, suggesting that, in addition to biosynthesis, conjugation exerts a significant role in the homeostatic mechanism of cellular PAs. The pool size of PAs in the hypogeous organs is comparative to their size in the mature and old hypergeous tissues (Fig. 1), even though the biosynthetic capacity of roots is severalfold...
higher (Fig. 5), suggesting different turnover rates between the organs. Moreover, the contribution of the ODC and ADC pathways to Put biosynthesis and the genes of Spd and Spm synthesis are developmentally regulated and exhibit different subcellular localization. Arginase-derived Orn is the substrate for Put production in roots via ODC, whereas Put in old hypergeous tissues is synthesized via the ADC pathway. Furthermore, ODC and SPDS depend on the spatial and temporal status of the plant for a role in the progression of cell division/expansion, whereas a high Spd synthesis response leading to cell division and a low response leading to cell expansion are proposed.

MATERIALS AND METHODS

Plant Material

Explants were removed from glasshouse-grown (25°C ± 2°C, 16/8 h photoperiod, and total energy of 55 μE m⁻² s⁻¹) 6- to 8-week-old tobacco (Nicotiana tabacum L. cv Xanthi) plants and numbered from shoot apex. They were divided in tip and leaf margin, leaf center, acropetal or apical petiole, and basipetal or basal petiole. Furthermore, shoot apex, primary or main root, and secondary or newly formed root explants were also removed.

PA, Arg, Orn, and Protein Determination

Free, soluble-conjugated, and insoluble-conjugated PAs were determined according to Kotzabasis et al. (1993a), with a HP 1100 HPLC (Hewlett-Packard, Waldbronn, Germany).

Arg and Orn were colorimetrically determined according to Cericotti and Spandrio (1957) and to Roubelakis and Kliever (1978), respectively.

Total proteins were extracted according to Papadakis et al. (2001) with 100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM leupeptin, 10% (v/v) glycerol, and 0.2% Triton X-100. The homogenates were centrifuged and the supernatants (soluble fractions) were desalted in Bio-gel P-6 (Bio-Rad, Hercules, CA). The pellets (cell walls, nucleus, plastids, and mitochondria; de Marco and Roubelakis-Angelakis, 1996) were dissolved in half-strength buffer, supplemented with 1 mM NaCl, centrifuged, and gave the particulate fractions. Protein concentration was determined according to Lowry et al. (1951) and by gel visualization.

Enzyme Assays

An isothiocyanate method (Primikirios and Roubelakis-Angelakis, 1999) was used for the determination of ADC, ODC, and SAMDC activities by measuring the release of 14CO2. L-[1-14C]Arg, L-[1-14C]Orn, and adenosyl-[3H]-Met 5-[carboxyl]-14C, respectively (ARC, St. Louis), were used as radioactive substrates. Usually, 100 μL of the protein extract were incubated with 25 μL (0.078 μCi)/1-14C-Arg (0.626 mCi/ mmol), 3.13 μCi/ mL, 1 mL final concentration of Arg, 25 μL (0.078 μCi)/1-14C-Orn (0.626 mCi/ mmol, 3.33 μCi/ mL, 1 mL final Orn concentration), and 25 μL (0.0156 μCi)/1-14C-adenosyl-[3H]-Met (0.1252 mCi/ mmol, 0.626 μCi/ mL, 1 mL final adenosyl-[3H]-Met concentration) in polypropylene tubes. For the ADC assay, 200 μM N5-hydroxy-L-Arg (a competitive inhibitor of arginase; Iniesta et al., 2001) was also added in the reaction mixture. Labeled CO2 was counted in an LS 6000SE (Beckman, Fullerton, CA) scintillation counter.

Arginase activity was colorimetrically measured as the rate of Orn formation, as described by Roubelakis and Kliever (1978), in a reaction mixture containing 100 mM Tris-HCl, pH 9.7, 25 mM L-Arg (adjusted to pH 9.7), 0.1 mL of total protein extract, and 1.5 mL MrCl (10 min of preincubation at room temperature) in a total volume of 1 mL. The reaction was terminated after 1 h by addition of 0.5 mL of 15% perchloric acid. The arginase activity was a linear function of incubation time, at least for 1 h, of substrate concentrations up to 20 μM and of the amount of protein extract under these conditions. Boiled enzyme preparations and reactions terminated with perchloric acid before incubation were used as controls, whereas 200 μM N5-hydroxy-L-Arg inhibited arginase activity in tobacco tissues by 96%.

SPDs and SPMS were assayed by measuring the formation of Spd and Spm, respectively. The assay mixture for SPDs contained 3 mM Put, 0.2 mM dSAM, 100 mM Tris-HCl buffer, pH 9.0, and the enzyme in a total volume of 200 μL. The reaction was performed at 37°C for 1 h and stopped by the addition of 200 μL of 65 mM borate-KOH buffer, pH 10.5, followed with 1 mL of 2 N NaOH and 10 μL of benzoylchloride. The products were separated with an HP 1100 HPLC system. SPMS was assayed similarly to SPDs by replacing Put with Spd in the assay mixture.

Western Blotting, RNA Extraction, and Northern Analysis

Protein samples were electrophoretically resolved, transferred to nitrocellulose membranes, and incubated with an antisera raised in rabbit against the hybrid protein MBP-ADC (Primikirios and Roubelakis-Angelakis, 2001). Total RNA was extracted as described by Logemann et al. (1987) and quantified by spectroscopy and ethidium bromide staining. RNA was denaturated, electrohoresed, and transferred to Gene Screen membranes (NEL Life Science Products, Boston) according to Sambrook et al. (1989) that were screened with the specific probes below.

A 1,047-bp tobacco adc cDNA clone obtained from suspension cell cultures (K. Primikirios and K. Roubelakis-Angelakis, unpublished data) was used as adc probe. Two degenerate oligonucleotide primers corresponding to two conserved regions in the aligned sequences of oat (Avena sativa); Bell and Malmberg, 1990) and tomato (Lycoopersicon esculentum; Rastogi et al., 1993) ADC proteins (forward primer, CNTGARGCNNGTNCAARCC; reverse primer, GNCNCCRANAGITRITG) were used to obtain the 1,047-bp product, which was cloned and sequenced. A 2,112-bp grapevine (Vitis vinifera) adc cDNA fragment (Primikirios and Roubelakis-Angelakis, 1999) with 91% nucleotide identity to the tobacco adc was also used for hybridization revealing exactly the same RNA patterns, suggesting the high specificity of probes. A full-length tomato odc DNA (Alabadi and Carbonell, 1998), with deduced amino acid sequence 89.8% identical to that of tobacco, was used as odc probe. Furthermore, a grapevine 829-bp odc fragment (accession no. AY174164) revealed the same transcripts with the tomato probe. Finally, a tobacco samdc PCR-derived fragment (A.J. Michael, Institute of Food Research, Norwich, UK) and a 945-bp Nicotiana olyestris spds reverse transcription-PCR fragment (Flashimoto et al., 1998) were also used as probes. The tobacco samdc was obtained from Agrobacterium rhizogenes-transformed hairy-root cultures. Two degenerate oligonucleotide primers, sense 5’-TT(T/C)GAAG(A/G)(T/C)GNGTTT(A/G)TG(A/G)AA-3’ and antisense 5’-TCN-GGNGTNA(T/G)(A/G)GTNGAT(G/T/G)(A/G)NG-3’ representing, respectively, the amino acid sequences FEG(F/T/P)EEK and T(M/I/L)HTPE, were used to obtain the 723-bp PCR product, which was cloned and sequenced (Taylor et al., 1992). PCR conditions, cloning, and sequencing were described by Michael et al. (1996).

Statistical Analysis

Statistical analysis of data concerning endogenous PAs, activities, Arg and Orn levels, and the ratio mRNA/ribRNAs was performed with one-way ANOVA to reveal any statistically significant differences (P < 0.01 or P < 0.05) that occurred. Data were logarithmically transformed to homogenize variances when necessary (Bartlett’s test). If F values were significant, pairwise comparisons of means were made by Tukey’s multiple comparisons test (P < 0.01). Data that contained two groups were compared using t tests. All experiments were carried out three times with similar results and included a minimum of three replicates per sample. Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes.

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Polyamine Synthesis in Tobacco Plants


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