Altered nitrogen metabolism associated with de-differentiated suspension cultures derived from root cultures of *Datura stramonium* studied by heteronuclear multiple bond coherence (HMBC) NMR spectroscopy

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Abstract

De-differentiation of transformed root cultures of *Datura stramonium* has previously been shown to cause a loss of tropane alkaloid synthetic capacity. This indicates a marked shift in physiological status, notably in the flux of primary metabolites into tropane alkaloids. Nitrogen metabolism in transformed root cultures of *D. stramonium* (an alkaloid-producing system) and de-differentiated suspension cultures derived therefrom (a non-producing system) have been compared using Nuclear Magnetic Resonance (NMR) spectroscopy. ¹⁵N-Labelled precursors [(¹⁵NH₄)₂SO₄ and K¹⁵NO₃] were fed and their incorporation into nitrogenous metabolites studied using Heteronuclear Multiple Bond Coherence (HMBC) NMR spectroscopy. In both cultures, the same amino acids were resolved in the HMBC spectra. However, marked differences were found in the intensity of labelling of a range of nitrogenous compounds. In differentiated root cultures, cross-peaks corresponding to secondary metabolites, such as tropine, were observed, whereas these were absent in the de-differentiated cultures. By contrast, N-acetylputrescine and γ-aminobutyric acid (GABA) accumulated in the de-differentiated cultures to a much larger extent than in the root cultures. It can therefore be suggested that the loss of alkaloid biosynthesis was compensated by the diversion of putrescine metabolism away from the tropane pathway and toward the synthesis of GABA via N-acetylputrescine.

Key words: *Datura stramonium*, HMBC NMR, nitrogen metabolism, NMR, transformed cultures (root), transformed cultures (de-differentiated), tropane alkaloids.

Introduction

When transformed root cultures are treated with exogenous phytohormones, they rapidly lose their capacity to accumulate alkaloids and this is associated with a de-differentiation to form a dispersed suspension culture (Rhodes et al., 1989; Robins et al., 1991b). A number of altered physiological parameters have been identified as associated with the modified phenotype, in particular, a loss of key enzymes of the biosynthetic pathway (Robins et al., 1991a, b) and an increase in γ-aminobutyric acid (GABA) (Ford et al., 1996a). However, not all the pathway
is lost: dispersed *Datura stramonium* cultures retain the ability to biotransform tropinone to tropine and simple tropine esters (Ford et al., 1996b). Furthermore, root cultures regenerated from dispersed cultures recover their biosynthetic capacity, indicating that this phenomenon is at the phenotypic level and not due to gene deletion (Rhodes et al., 1989). In *D. stramonium* cultures exposed to phytohormones, an early event is the suppression of several pathway enzymes, notably putrescine *N*-methyltransferase (PMT) (Robins et al., 1991a, b). During subsequent sub-cultures, this enzyme is absent from the cultures, indicating that the loss of biosynthetic capacity is due to repression of this critical activity. This changed physiological state precedes the altered phenotype of the cultures. By *in vivo* $^{15}$N NMR, Ford et al. (1996a) observed the alteration in N-metabolism during the first few hours following phytohormone application and showed a correlated increase in GABA. This increase was found not to be associated with either a perturbed intracellular pH or a state of hypoxia.

Thus, it appears that this change in phenotype is associated with major alterations in metabolism, notably a reversible decoupling of alkaloid biosynthetic capacity and associated alterations in the levels of other N-metabolites. In order to understand better the differences in N-metabolism between the two states of the *D. stramonium* cultures, N-metabolites present in organized and 6-month-old de-differentiated suspension cultures have been compared by NMR. $^{15}$N NMR has been shown to be an effective technique to follow the metabolism of nitrogenous metabolites (van Heerden et al., 1996; Amâncio and Santos, 1992), including *Nicotiana* and *Datura* alkaloids in cell cultures (Ford et al., 1994). However, direct 1D $^{15}$N NMR suffers from a poor resolution of the α-amino acids, which largely show coincident resonances. To overcome this problem, 2D NMR techniques of heteronuclear decoupling, particularly HMBC and HMQC sequences, have proved a powerful method to identify nitrogenous molecules in plant cell culture extracts (Shachar-Hill et al., 1996). 2D NMR combines the advantages of the resolution of bidimensional NMR with the sensitivity of $^{1}$H NMR by the association of 2D dispersion and reverse gradient detection. Moreover, the HMBC sequence allows the $^{15}$N-bonded labile protons to be observed via $^{2}$J or $^{3}$J couplings. By this approach, it has been possible to examine the nitrogen metabolism of alkaloids in non-productive suspension cultures of *Nicotiana plumbaginifolia* (Mesnard et al., 2000). In this study it was shown that the primary/secondary interface at the initiation of the pyrrolidine alkaloid pathway is active and hence that the deficiency in alkaloid accumulation occurs after the formation of putrescine, and that a number of putrescine-derived metabolites accumulated.

In a preliminary report, it has been shown that the HMBC NMR approach allows a number of compounds, including alkaloids, to be resolved in *D. stramonium* cultures (Fliniaux et al., 2001). In the present paper, this technique was used to identify further metabolites and to examine the dynamic changes in N-metabolism during root de-differentiation.

**Materials and methods**

**Plant material and growth conditions**

Transformed roots of *D. stramonium* were initiated from leaf-disc infection with a wild strain of *Agrobacterium rhizogenes* 15834 (Bensaddek et al., 2001). These cultures were grown in 100 ml liquid FMO medium [that is, FMD medium (Mesnard et al., 2001) without plant growth regulators] in 300 ml Erlenmeyer flasks and were maintained in the dark at 25 °C on a rotary shaker at 110 rpm. Cell suspensions of *D. stramonium* were initiated from transformed roots grown in FMD medium and were maintained in liquid FMD medium in the same environmental conditions as the transformed roots.

**Feeding experiments**

Feeding experiments were performed in 100 ml flasks, with each flask containing 3.75 g roots or 3.75 g de-differentiated cells in 25 ml of sterile modified FMO or modified FMD liquid medium (with no nitrogen source), respectively, and each culture was grown with $^{15}$N-labelled or unlabelled (NH$_4$)$_2$SO$_4$ and KNO$_3$ at 1.0 and 2.5 mM, respectively. At 2, 4, 6, 10, 14, and 21 d, each culture was harvested and separated from the medium by filtration. Roots and cells were extracted with 30 ml 70% ethanol at room temperature for 1 h. After filtration, the crude extracts were evaporated to dryness under reduced pressure and each residue was dissolved in 0.5 ml D$_2$O/H$_2$O (1.9 v/v, pH 5.0 ± 0.2). The culture media and washings were pooled, freeze-dried and each residue was dissolved in 0.6 ml of the same D$_2$O/H$_2$O solution (1.9 v/v, pH 5.0 ± 0.2) before NMR analysis.

**NMR analysis**

1D $^{1}$H-decoupled $^{15}$N NMR spectra were recorded at 300 K on a Bruker DMX 300 spectrometer, operating at 30.42 MHz using an 8 mm broad-band probe head. Spectra were accumulated using a 90° pulse angle, a recycle time of 10 s and an acquisition time of 1.07 s, for a spectral width of 15 kHz for 32 K data points. Before Fourier transform, a zero filling to 64 K was applied, and a line broadening of 1 Hz was used to improve the spectral signal-to-noise ratio.

2D spectra were recorded at 300 K on a Bruker DMX 500 spectrometer operating at 500.13 MHz for $^{1}$H and 50.68 MHz for $^{15}$N, using a 5 mm broad-band inverse gradient probe. A pre-saturation during the 2 s relaxation delay was used to suppress the water signal. The transfer delay used was 5.55 ms, corresponding to 1/(2T$_{1N}$), and detection in the indirect dimension used the phase-sensitive States-TPPI scheme. An additional delay of 100 ms just before the acquisition was used to allow for the evolution of long-range couplings. Typically, 4096 data points with 64 scans per increment and 256 increments were used. The required acquisition time was 0.41 s. The spectral width was 5 kHz in the $^{1}$H dimension and 7.6 kHz in the $^{15}$N dimension. Free induction decays were zero filled and filtered using a shift sine bell in both dimensions before Fourier transform. All chemical shifts were referenced to TMSP-d$_4$ and NH$_4$Cl signals at δ=0 ppm for $^{1}$H and $^{15}$N, respectively.

**GC analysis of tropane alkaloids**

Tropane alkaloids were quantified by GC. Samples extracted as described above were taken to dryness, dissolved in EtAc (50 µl) and...
follow the uptake of 15N-labelled salts by the cultures, 1D-35N NMR spectra of media were recorded. The chemical shifts of NH₄⁺ and NO₃⁻ salts were found at 0 ppm and 354.5 ppm, respectively (data not shown). As can be seen in Fig. 1, for both cultures of *D. stramonium* the amount of NH₄⁺ rapidly decreased, whereas the amount of NO₃⁻ decreased more slowly. After 2 d, around 25% of the NH₄⁺ was left in both the transformed roots and in the de-differentiated cell cultures, but NH₄⁺ uptake was complete between 4 d and 6 d. Nitrate consumption was slower, about 40% still being present after 5 d and total assimilation taking about 15 d for both *D. stramonium* cultures. These kinetics are in broad agreement with previous work reporting ammonium and nitrate assimilation by cultures, notably that NH₄⁺ is more rapidly absorbed than NO₃⁻ (Lang and Kaiser, 1994; Gerendas et al., 1997; Bown and Shelp, 1997). No new peak was observed in the medium (data not shown), indicating that no significant amount of 15N-labelled metabolite was released into the medium by either culture. Furthermore, no peaks were observed in the media from unlabelled cultures (data not shown).

**15N NMR analysis of media**

To observe any released 15N-labelled products and to follow the uptake of 15N-labelled salts by the cultures, 1D-15N NMR spectra of media were recorded. The chemical shifts of NH₄⁺ and NO₃⁻ salts were found at 0 ppm and 354.5 ppm, respectively (data not shown). As can be seen in Fig. 1, for both cultures of *D. stramonium* the amount of NH₄⁺ rapidly decreased, whereas the amount of NO₃⁻ decreased more slowly. After 2 d, around 25% of the NH₄⁺ was left in both the transformed roots and in the de-differentiated cell cultures, but NH₄⁺ uptake was complete between 4 d and 6 d. Nitrate consumption was slower, about 40% still being present after 5 d and total assimilation taking about 15 d for both *D. stramonium* cultures. These kinetics are in broad agreement with previous work reporting ammonium and nitrate assimilation by cultures, notably that NH₄⁺ is more rapidly absorbed than NO₃⁻ (Lang and Kaiser, 1994; Gerendas et al., 1997; Bown and Shelp, 1997). No new peak was observed in the medium (data not shown), indicating that no significant amount of 15N-labelled metabolite was released into the medium by either culture. Furthermore, no peaks were observed in the media from unlabelled cultures (data not shown).

**15N NMR analysis of cell extracts**

The HMBC NMR spectra recorded from extracts of both types of *D. stramonium* culture incubated in the presence of 15N-labelled salts (Fig. 2) revealed extra cross-peaks

<table>
<thead>
<tr>
<th>Culture</th>
<th>Days</th>
<th>Hyg (%)</th>
<th>Nortri (%)</th>
<th>Tro (%)</th>
<th>Tri (%)</th>
<th>AcTri (%)</th>
<th>α-TigTri (%)</th>
<th>β-TigTri (%)</th>
<th>Cusc (%)</th>
<th>Apo-atrop (%)</th>
<th>Hyo (%)</th>
<th>N.I. (%)</th>
<th>N.I. (%)</th>
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<td>5±0</td>
<td>4±0</td>
<td>56±1</td>
<td>208±10</td>
<td>26±4</td>
<td>30±2</td>
<td>38±3</td>
<td>74±8</td>
<td>1758±116</td>
<td>5±6</td>
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<td>44±5</td>
<td>21±3</td>
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<td>110±3</td>
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<td>6±4</td>
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<td>0</td>
<td>16±7</td>
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</table>

Table 1. Evolution of total alkaloid present in the cultures of *Datura stramonium* over 4–21 d

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By contrast, at best only traces of alkaloids could be detected in the extract of dispersed cultures (Table 1), as previously reported (Robins et al., 1991b). Negligible amounts were present in cultures up to 14 d and by 21 d less than 1.5% of the level in organized roots was detectable. Low levels of several unidentified compounds were present in the GC traces (data not shown).

**Results**

**Quantitative analysis of tropane alkaloids**

Cellular extracts of organized root cultures analysed by GC showed profiles of tropane alkaloids characteristic of *D. stramonium* (Robins et al., 1991a), dominated by hyoscyamine with smaller quantities of tropine and α-acetyltropine and lesser amounts of a range of other tropane derivatives (Table 1). The non-tropane alkaloids hygrine and cuscohygrine, also putrescine-derived, were present. During growth from 4–21 d, the total amount of alkaloid increased steadily. However, throughout the culture period, the profile retained more-or-less the same proportionality between the products, hyoscyamine fluctuating between 72% and 81% of the total.

By contrast, at best only traces of alkaloids could be detected in the extract of dispersed cultures (Table 1), as previously reported (Robins et al., 1991b). Negligible amounts were present in cultures up to 14 d and by 21 d less than 1.5% of the level in organized roots was detectable. Low levels of several unidentified compounds were present in the GC traces (data not shown).
compared with the experiments in the presence of unlabelled salts (data not shown). The majority of these signals can be assigned to amino acids and to nitrogenous metabolites derived therefrom. The HMBC NMR spectra from transformed root cultures also show a number of cross-peaks that can be assigned to tropane alkaloids (Fig. 2B). These peaks are absent in the spectra from dispersed cultures (Fig. 2A).

**Primary metabolites**

As can be seen from Fig. 2A, B, both types of *D. stramonium* culture show similar profiles for a number of primary metabolites. Cross-peaks corresponding to $^3J$ couplings of amido and amino groups of glutamine were found at 91.1/2.38 ppm ($^{15}N/^1H$) and at 19.8/2.05 ppm respectively. This latter cross-peak also contains a large contribution of glutamate. Other cross-peaks that can be assigned on the strength of their $^2J$ and/or $^3J$ couplings to alanine, aspartate, asparagine, serine, glycine, uridine, N-acetyl compounds, and GABA are also present in both cultures. In this respect, they resemble closely the dispersed cultures of *N. plumbaginifolia* (Mesnard et al., 2000). In addition, a pair of cross-peaks that can be assigned to choline was identified at 26.9/3.11 and 26.9/3.98 ppm. This compound was also present in the extracts of *N. plumbaginifolia* but was not previously identified. Furthermore, by reference to a pure standard, it is now possible to assign the N-acetyl cross-peaks at 105.1/1.66, 105.1/1.90, and 105.1/3.07 ppm in suspension cells to N-acetylputrescine.

Extracts were taken over a full 21 d culture period, in order that the evolution of the $^{15}$N-labelling of these different compounds in relation to $^{15}$N-uptake could be followed. While quantitative data cannot be obtained by this technique, it is possible to compare the amplitude of the cross-peaks for each compound from the different cultures, as they are measured under the same acquisition conditions. It was found that alanine, asparagine, serine, glycine, and choline all showed similar profiles of labelling, independent of the state of the culture, with an increase during the first 3–5 d and a steady loss of label as the culture matured (data not shown). However, four compounds showed marked differences both between the cultures and between each other.

Glutamine showed a sharp increase in labelling that was much more pronounced in the transformed roots and thereafter decayed rapidly, label being lost by 10 d (Fig. 3A). This is compatible with the role of glutamate/glutamine in the assimilation of N from ammonium and nitrate indirectly via the GS/GOGAT cycle and is consistent with the profiles of NH$_4^+$ depletion from the medium (Fig. 1). Uridine, in contrast, was more abundantly labelled in the suspension cultures, the label persisting almost 15 d in these cells, whereas it disappeared after about 5 d from the root cultures (Fig. 3B). Of particular interest, however, was the effect on GABA (Fig. 3C), which displayed a much stronger $^{15}$N-labelling in the suspension cultures and only retained $^{15}$N-label to the end of the 21 d culture in the dispersed cells. It is noteworthy that N-acetylputrescine showed a similar profile (Fig. 4), with the difference that this compound remained undetectable in the transformed roots.
Secondary metabolites

Figure 5 shows a detail of the secondary metabolite zone of the HMBC NMR spectrum of an extract from a 14-d-old *Datura stramonium* root culture. Cross-peaks at 49.3/2.32 and 49.3/2.70 ppm due to tropine are observed as early as day 2 (Fig. 2A) and increase in magnitude throughout the growth period (Fig. 6). A cross-peak at 50.6/1.85 ppm could be due to tropinone but, in view of the low abundance of this alkaloid (Table 1), it is much more likely to be due to labelling of the tropine aliphatic esters, notably α-acetyltropine. This peak follows a similar kinetics of labelling to tropine (data not shown).
Despite hyoscymine being the dominant alkaloid in the root cultures (Table 1), cross-peaks due to this product cannot be detected early in the culture cycle (Fig. 2A) and it is not until about 14 d that cross-peaks at 48.8/1.43 and 48.8/1.95 ppm that can be assigned to hyoscymine appear (Fig. 5). Since hyoscymine is present at about 35-fold and 5-fold the levels of tropine and tropine esters, respectively, over the whole culture period (Table 1), this indicates a slower or poorer relative enrichment of this pool. Ford et al. (1998) observed a similar low level of labelling of hyoscymine.

The complete absence of these cross-peaks due to alkaloids at all stages of growth of the dispersed culture (data not shown) is in agreement with the extreme paucity of tropane alkaloids, as found by GC analysis (Table 1) and as previously reported (Robins et al., 1991a, b).

These profiles are in marked contrast to those for the primary metabolites. The secondary metabolites, as they accumulate during the growth cycle, become progressively more intensely labelled with $^{15}$N. By contrast, the primary metabolites, with the sole exception of GABA in dispersed cell cultures, lose their entire $^{15}$N-label before the end of the growth cycle.

**Discussion**

HMBC $^{15}$N NMR spectroscopy has allowed the simultaneous incorporation of inorganic $^{15}$N into a wide range of primary and secondary metabolites to be followed in *D. stramonium* cultures. Critically, it has made possible a comparison of the $^{15}$N profiles in homogenic but phenotypically distinct lines. The most marked differences seen are that, in root cultures $^{15}$N is found to accumulate in tropane alkaloids, which are absent in the dispersed cells, while in the dispersed cultures $^{15}$N is found to accumulate in GABA and N-acetylputrescine to a much greater extent than in root cultures.

An accumulation of GABA during short-term labelling experiments (c. 12 h) has previously been reported for *D. stramonium* root cultures exposed to phytohormones for about 10–12 d and exhibiting initiation of de-differentiation (Ford et al., 1996a). It is now further shown that GABA persists as an important N-metabolite in long-term stable de-differentiated cultures and varies in concentration with cell growth. Although GABA accumulation is well known to be associated with stress such as hypoxia or altered intracellular pH (Carroll et al., 1994; Crawford et al., 1994), it has previously been shown that this is probably not the cause of its accumulation in de-differentiated cultures (Ford et al., 1996a). Furthermore, in this study, the dispersed cultures of *D. stramonium* show growth characteristics and stability that are inconsistent with suspension cells growing in a stressed state (data not shown).

One explanation could be that the enhanced level of GABA compensates metabolically for the lost biosynthetic capacity for tropane alkaloids. Disorganization of the root cultures causes a rapid loss of the alkaloid biosynthetic capacity (Robins et al., 1991a, b). This is apparently directly due to the loss of the enzyme PMT which, by *N*-methyllating putrescine, initiates the tropane pathway (Robins and Walton, 1993). PMT levels are greatly suppressed in the first few days after transferring root cultures to a phytohormone-containing medium (Robins et al., 1991b) and PMT is completely absent after several sequential cultures in these conditions. By contrast, other enzymes, notably ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), responsible for the formation of putrescine, remain active at levels similar to that found in root cultures (Robins et al., 1991b). Hence, the capacity to produce putrescine is preserved whereas a major putrescine-utilising capacity is lost.

The apparent effect of this is that putrescine catabolism is altered. Due to its key role in polyamine metabolism, putrescine levels are tightly regulated in plant cells (Tiburcio et al., 1997) and an important function is now recognized for putrescine in the regulation of plant differentiation (Walden et al., 1997). Thus, putrescine levels are low, and putrescine is rapidly metabolized: to polyamines, or to alkaloids, or to phenylpropanoid conjugates, or it undergoes degradation. The major pathway of degradation is by *N*-acetylation to *N*-acetylputrescine, followed by decarboxylation to GABA (Fig. 7). In an analysis of the changes taking place during the modification of cultures from a rooty to a dispersed phenotype, an essential role for free putrescine was demonstrated (Ford et al., 1998). Essentially, blocking putrescine formation inhibited de-differentiation and the inhibition was overcome by adding exogenous putrescine. Due to the limited resolution obtained by the 1D-$^{15}$N NMR analysis previously used, these authors (Ford et al., 1996a, 1998) were not able to resolve GABA amino-N from conjugated polyamine amino-N. However, on the strength of the conjugated polyamine amide-N signal, they were able to estimate that both metabolites were present and that labelling of conjugated polyamines diminished over a period of a few days. In the present study of long-term de-differentiated cultures, significant amounts of label could not be detected in conjugated polyamine amide-N, indicating that GABA is the principal metabolite that accumulates in these conditions.

As argued previously (Ford et al., 1996a), the accumulation of GABA appears unrelated to pH-stress-stimulated glutamate decarboxylase (GDC) activity. In the present work, it is shown that the origin of this GABA can now reasonably be assigned to putrescine metabolism. In strong support of this proposal, the spectra show cross-peaks that are unambiguously assigned to *N*-acetylputrescine and these show a metabolic profile parallel to that of GABA.
As these cross-peaks are absent in the root cultures, it can be deduced that this pathway of putrescine metabolism was much more active in the de-differentiated phenotype. GABA and N-acetylputrescine accumulated early in the culture cycle during the phase of rapid cell division and expansion but were, however, largely degraded by the end of the 21-d culture period (Fig. 4). GABA was clearly not secreted in any significant amount, as shown by the lack of any cross-peaks in the medium at 8.8/1.83 ppm (data not shown).

Hence, by implication, it could be suggested that the perturbation of metabolism in the dispersed cultures caused by the loss of PMT activity is compensated by the diversion of putrescine metabolism away from the tropane pathway and toward the synthesis of GABA. However, the situation appears to be more complex than this. Over-expressing pmt under the control of a 35S promoter in root cultures of several species can cause the accumulation of more N-methylputrescine (Moyano et al., 2002; Rothe et al., 2003), although in these cell lines neither was alkaloid accumulation enhanced nor was putrescine concentration diminished. Recently, some 35S-pmt clones of Datura metel and Hyoscyamus muticus showing enhanced alkaloid accumulation have been identified (Moyano et al., 2003). Yet these show no direct correlation between hyoscyamine accumulation and PMT activity, indicating that other transformation-related factors may be involved. Possibly, there is an indirect influence of altered phytohormone metabolism. In the short-term (28 d) 35S-pmt roots of Atropa belladonna, in showing little loss of alkaloid accumulation when treated with 1 μM indole butyric acid, did respond differently to the control roots (Rothe et al., 2003). Nevertheless, over a longer culture period, de-differentiation occurred and alkaloid production was lost (B Dräger, personal communication), indicating that constitutive expression of this putrescine-utilizing gene does not appear to protect the roots from phytohormone-induced de-differentiation. Furthermore, decreasing the Ca²⁺ concentration in the medium from 1.0 mM to 0.25 mM, leads to a drastic loss of hyoscyamine production by root cultures of D. stramonium, but only a 50% loss of pmt transcript (Piñol et al., 1999). In these conditions, the integrity of the root cultures was maintained.

Hence, it cannot at present be concluded which, if any, of the processes of de-differentiation, loss of PMT activity or the re-direction of putrescine towards N-acetylputrescine and GABA accumulation, is the primary driving force for the observed phenotypic and metabolic effects associated with the loss of alkaloid capacity. That this can be caused by phytohormone treatment, Ca²⁺ deficiency or, at least in N. sylvestris plants, by pmt down-regulation (Sato et al., 2001) indicates that alkaloid biosynthesis is subject to complex metabolic control. Further studies of the effects of gene manipulation on phenotypic change and N-metabolism may help unlock this complex system.

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References


