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Expression of deacetylvindoline-4-*O*-acetyltransferase in *Catharanthus roseus* hairy roots

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Abstract

Madagascar periwinkle [*Catharanthus roseus* (L.) G Don] is a pantropical plant of horticultural value that produces the powerful anticancer drugs vinblastine and vincristine that are derived from the dimerization of the monoterpenoid indole alkaloids (MIAs), vindoline and catharanthine. The present study describes the genetic engineering and expression of the terminal step of vindoline biosynthesis, deacetylvindoline-4-*O*-acetyltransferase (DAT) in *Catharanthus roseus* hairy root cultures. Biochemical analyses showed that several hairy root lines expressed high levels of DAT enzyme activity compared to control hairy root cultures expressing β-gulucuronidase activity (GUS) activity. Metabolite analysis using high performance liquid chromotagraphy established that hairy root extracts had an altered alkaloid profile with respect to hörhammericine accumulation in DAT expressing lines in comparison to control lines. Further analyses of one hairy root culture expressing high DAT activity suggested that DAT expression and accumulation of hörhammericine (**9**) were related. It is concluded that expression of DAT in hairy roots altered their MIA profile and suggests that further expression of vindoline pathway genes could lead to significant changes in alkaloid profiles. Evidence is provided that hörhammericine (**9**) accumulates via a DAT interaction with the root specific minovincinine-19-*O*-acetyltransferase (MAT) that inhibits the MAT mediated conversion of hörhammericine (**9**) into 19-*O*-acetyl-hörhammericine (**12**).

Keywords: Periwinkle; *Catharanthus roseus*; Metabolic engineering; Monoterpenoid indole alkaloids; Apocynaceae; Hairy roots; Protein–protein interactions; Deacetylvindoline-4-*O*-acetyltransferase mediated inhibition of minovincinine-19-*O*-acetyltransferase

1. Introduction

Catharanthus roseus (L.) G. Don (Madagascar periwinkle) is a tropical plant that belongs to the family Apocynaceae. The presence of the therapeutically valuable alkaloids vinblastine and vincristine in *C. roseus* has justified many biotechnological studies to increase their production. The low yields of these dimeric monoterpenoid indole alkaloids (MIAs) have also prompted extensive efforts to develop inexpensive and efficient chemical syntheses, but their chemical complexity has made this task difficult and MIAs continue to be obtained from biological sources for commercial uses (Hughes et al., 2004 Laflamme et al., 2001). The possibility for inexpensive production of these bis-indole alkaloids by cell and tissue cultures has led to the use of *C. roseus* as an experimental system for plant metabolic engineering (Hughes et al., 2004).

Hairy root cultures represent an alternative to cell suspension cultures for production of secondary metabolites (Rijhwani and Shanks, 1998). The advantages of hairy roots in comparison to cell culture systems include their apparent genotypic and biochemical stability, morphological differentiation and growth in hormone-free media (Rijhwani and Shanks, 1998; Rodriguez et al., 2003). Several environmental conditions have been shown to influence the production of MIAs in *C. roseus* cultures (Moreno-Valenzuela et al., 1998), including the composition of the culture medium, light treatment and temperature conditions.

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C. roseus cell suspension and hairy root cultures do not produce the two bisindole alkaloids due to their inability to make the vindoline (**7**) (Fig. 1) part of dimeric MIAs, while they do accumulate the catharanthine as well as the tabersonine (**1**) precursor of vindoline (**7**) (Hong et al., 2006; Rodriguez et al., 2003). While tabersonine (**1**) is converted into vindoline (**7**) by six enzymatic steps in the above ground plant parts (Fig. 1), hairy roots and cell cultures convert tabersonine (**1**) to the oxygenated derivatives, lochnericine (**8**) and horhammericine (**9**) (Shanks et al., 1998). The accumulation of these root-derived MIAs has also been shown to increase in response to treatment with

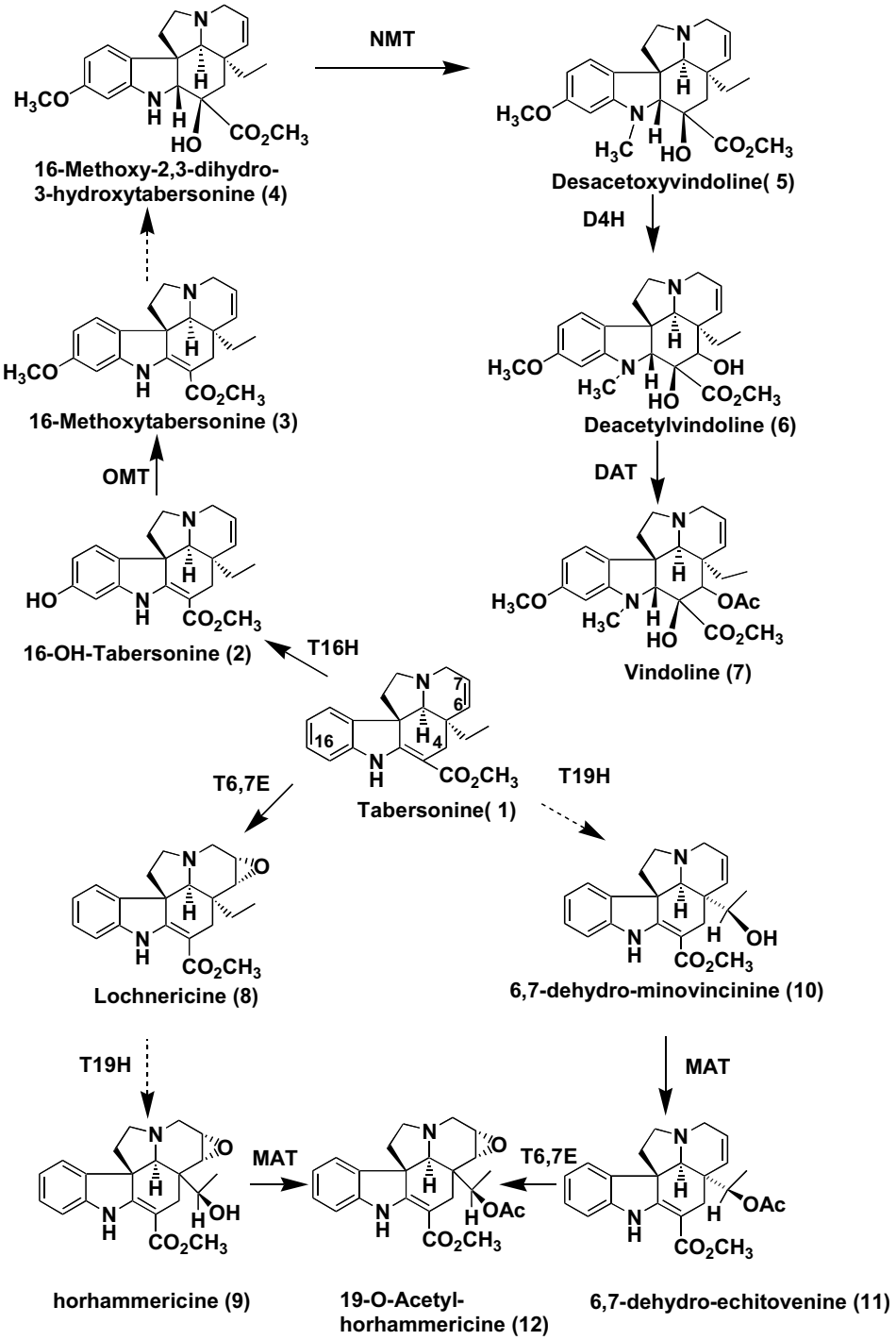

Fig. 1. Biosynthesis of tabersonine derived metabolites in *C. roseus* organs [adapted from Rodriguez et al. (2003)]. In the aerial parts, tabersonine (**1**) is converted to vindoline (**7**) via six enzymatic steps. In the roots, tabersonine (**1**) is converted to various oxidized tabersonine intermediates leading to the formation of 19-*O*-acetyl-hörhammericine. The abbreviations stand for tabersonine 16-hydroxylase (T16H); 16-hydroxytabersonine-16-*O*-methyltransferase (16-OMT); *N*-methyltransferase (NMT); deacetoxyvindoline-4-hydroxylase (D4H); deacetylvindoline 4-*O*-acetyltransferase (DAT); tabersonine-6,7-epoxidase (T6,7E); tabersonine 19 hydroxylase (T19H), minovincinine-19-*O*-acetyltransferase (MAT). Dotted arrows describe uncharacterized enzyme reactions.

jasmonic acid, with the coordinate decrease of tabersonine (**1**) levels (Morgan and Shanks, 1999; Rijhwani and Shanks, 1998). The jasmonic acid activated conversion of tabersonine (**1**) to lochnericine (**8**) and hörhammericine (**9**) in hairy root cultures is affected by inhibitors of oxygenases and has been reported (Morgan and Shanks, 1999; Rodriguez et al., 2003). While the exact pathway and enzymes responsible for the biosynthesis of tabersonine (**1**) and its metabolism in roots are presently poorly characterized, the oxidation reactions provide a competitive pathway that may prevent the biosynthesis of vindoline (**7**) by diverting away the flux of tabersonine (**1**) (Rodriguez et al., 2003). In addition, the late steps of vindoline (**7**) biosynthesis have been studied in detail and require specialized cell types, idioblast and laticifer cells that are located in stems and leaves, for part of their biosynthesis (St-Pierre et al., 1999). Root cultures lack these cell types and are therefore incapable of producing vinblastine and vincristine (Vaszquez-Flota et al., 2002). For these reasons the metabolic engineering of vindoline (**7**) production in hairy roots will probably require expression of the last six enzymatic steps and the removal of the oxidative reactions for diverting tabersonine (**1**) into lochnericine (**8**) and hörhammericine (**9**).

Since suitable plant transformation and whole plant regeneration protocols have yet to be firmly established for this plant, tissue culture systems (cell suspension and hairy root cultures) alone have been useful for genetic engineering of *C. roseus* tissues (Hughes et al., 2004). The present study describes the expression of the last step in vindoline (**7**) biosynthesis, deacetylvindoline-4-*O*-acetyltransferase (DAT), in hairy root cultures as an initial step to express the complete set of six pathway genes responsible for the conversion of tabersonine (**1**) to vindoline (**7**) (Fig. 1). Enzymatic profiling identified several hairy root lines that expressed high DAT activities compared to non-expressing control lines. Furthermore, high performance liquid chromatography of MIAs extracted from transformed hairy roots revealed that DAT expressing lines had an altered MIA profile. Further analysis of a highly active line, DAT7, revealed that hörhammericine (**9**) accumulation increased with DAT activity and with root maturation. Additional studies provided evidence that hörhammericine (**9**) accumulates via a DAT interaction with MAT that inhibits the MAT mediated conversion of hörhammericine (**9**) into 19-*O*-acetyl-hörhammericine (**12**). The results obtained provide new unsuspected insights into the problems to be encountered for metabolic engineering of vindoline (**7**) biosynthesis in hairy root cultures.

2. Results and discussion

2.1. Generation of transgenic *N. tabacum* plants and *C. roseus* hairy root lines

Leaves of *in vitro* grown *N. tabacum* were infected with *Agrobacterium tumefasciens* LBA 4404 harboring the binary plasmids *pBI121/DAT* and *pBI121/GUS*. The plasmid *pBI121/DAT* contains the coding region of the wild-type *C. roseus DAT* gene under the control of the CaMV 35 S promoter. It also contains a marker for kanamycin selection. The plasmid *pBI121/GUS* permits expression of the GUS reporter gene under the control of the CaMV 35 S promoter and also contains a kanamycin selection marker. Following co-cultivation of wounded leaves with *Agrobacterium* harboring either plasmid, these were maintained on solid selection media containing 100 mg/l kanamycin and 400 mg/l of cefatoxime (as described in Experimental). After several weeks of cultivation, 15 independent *pBI/GUS* and *pBI/DAT* transformed calli were transferred to shoot meristem generating medium. Of the 20 green shoots adapted to kanamycin selection media, three DAT lines and three GUS lines were transferred and maintained in Magenta boxes.

Leaves of *C. roseus* were infected with *Agrobacterium rhizogenesis* R1000 carrying the plasmids *pBI121/GUS* and *pBI121/DAT*. Of the 30 pBI/GUS hairy roots excised, only 15 grew significantly on solid selection media containing 100 mg/l kanamycin and 400 mg/l of cefatoxime. Of these, only eight adapted to long term maintenance on liquid media. Of the 50 pBI/DAT hairy roots excised, 33 showed strong growth on solid selection media containing antibiotics. As reported by Bhadra et al. (1993) the most difficult step is the adaptation of *C. roseus* hairy roots to liquid media. While thirty pBI/DAT hairy root lines grew well on solid selection media containing antibiotics, only 10 of these lines could be maintained in liquid media. There was significant variability in the morphology and growth patterns in individual root clones (data not shown), in spite of continuous cultivation of these pBI/DAT hairy root lines in liquid media for the past 12 months (Bhadra et al., 1993). Similar variable growth morphology was also observed by Batra et al. (2004) who speculated that the variation was due to separate transformation events, although there is no experimental evidence for this suggestion. The initial generation of DAT expressing hairy roots in MS medium prior to transfer in half strength B_5 medium may have also contributed to the differences observed in the type of branching and lateral root growth of the transgenic lines. This is suggested by subsequent experiments that generation of *C. roseus* hairy roots directly on half strength B_5 medium produced very uniform hairy root cultures that could be more easily transferred to liquid medium after only 6–8 weeks of initiating the transformation (data not shown).

An additional *C. roseus* hairy root line R/J1, generated in 1993 as described in Vazquez-Flota et al., 1994; was also used for comparative analysis.

2.2. Expression of deactylvindoline-4-*O*-acetyltransferase in leaves of *N. tabacum* and *C. roseus* hairy roots

The *dat* gene was expressed and tested for activity in tobacco since this plant does not have this enzyme activity.

While several *DAT* and *GUS* expressing transgenic tobaccos were produced, the leaves of only three plants were assayed for the presence of DAT enzyme activities and these were compared with the activities occurring in negative control *GUS* expressing tobacco and in positive control *C. roseus* leaves. The results showed that *DAT* transformed tobacco produced variable but low levels of DAT activity, depending on the tobacco plant assayed compared to the GUS expressing tobacco that did not have any activity. While the specific activity of DAT in transformed tobacco was at least 10-fold lower than the levels of enzyme found in *C. roseus* leaf extracts (85 pmol/min/mg protein), these studies suggested that the vector pBI121/DAT should produce a functionally active DAT in *C. roseus* hairy roots.

DAT enzyme activities were assayed in extracts obtained from 10 independently *DAT* transformed *C. roseus* hairy root lines in comparison to the activities found in *C. roseus* leaves, in a GUS expressing hairy root culture and from the well-established hairy root line RJ/1 (Fig. 2, upper panel). All lines were harvested in triplicate for enzyme assay. Significant variation in DAT activity was observed in independently transformed DAT expressing hairy roots (specific activities between 1 and 200 pmol/min/mg of protein) compared with *C. roseus* leaves that displayed a specific activity of 100 pmol/min/mg protein. In contrast, neither GUS expressing hairy roots nor line RJ/1 expressed any detectable DAT activity. For example, lines DAT3 and 7 contain DAT activities that were 100 and 200 times higher than the control hairy root lines whereas other *dat* expressing lines were 20 times more active than those of control lines. In contrast, the high DAT expressing line 7 expresses 2-fold higher DAT activity than enzyme extracted from *C. roseus* leaves (Fig. 2, upper panel). These results suggest that overexpression of *dat* in hairy roots of *C. roseus* can produce high enzymatic activity of DAT and this raised the question whether this modification could alter the patterns of MIAs produced in DAT expressing hairy root.

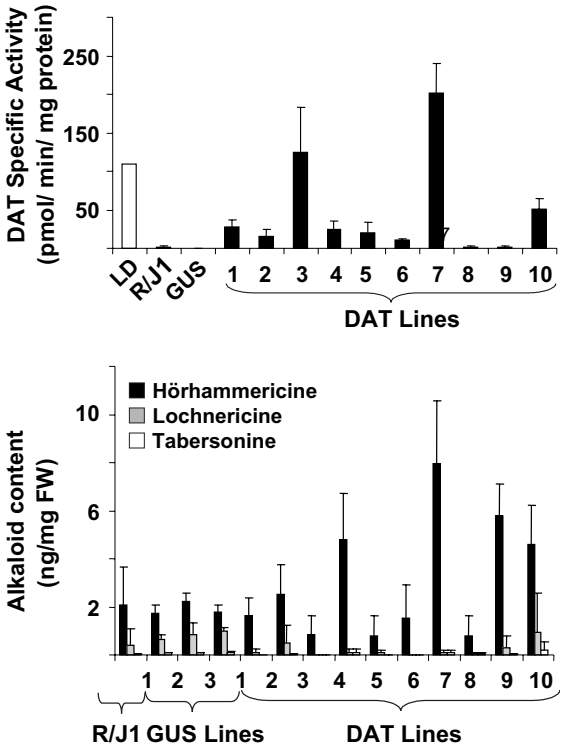

Fig. 2. Upper panel: relative DAT enzyme activities found in *C. roseus* (cv. Little delicata) leaves (L.D.), hairy roots (R/J1), GUS expressing hairy roots (GUS) and in Lines DAT1-DAT10 transformed with the DAT gene. In the case of lines DAT1-10, three sets of roots were extracted and assayed for DAT activity for each line in order to generate the standard errors displayed in the figure. Lower panel: hörhammericine (**9**), lochnericine (**8**) and tabersonine (**1**) accumulation in hairy root cultures of *C. roseus*: DAT expressing lines labeled 1-10; GUS, *C. roseus* hairy roots expressing *gus*; GUS, *C. roseus* hairy roots expressing *gus*; R/J1, *C. roseus* hairy roots. Three separate sets of roots were extracted for MIAs to provide a measure of the variability of alkaloid profiles found in each hairy root line.

2.3. Accumulation of tabersonine (*1*), lochnericine (*8*) and hörhammericine (*9*) in hairy roots

Hairy roots (RJ/1, lines GUS1 to GUS3, and lines DAT1 to DAT10) were cultivated over a 4-week period and triplicate cultures of each line were harvested in preparation for alkaloid extraction and analysis by HPLC. Root tissues from all DAT transformed lines accumulated similar levels of tabersonine (**1**) and lochnericine (**8**) compared to the levels found in control hairy root lines, varying between 0 to 0.2 ng/mg fresh weight of tissue for tabersonine (**1**) and 0 to 1.0 ng/mg fresh weight of tissue for lochnericine (**8**) (Fig. 2, lower panel). In contrast the hörhammericine (**9**) levels varied significantly among the hairy root lines compared to both the GUS expressing and RJ/1 line. For example, line DAT7 exhibited four times higher levels of hörhammericine (**9**) compared to either control line while other DAT lines accumulated lower levels of this alkaloid (Fig. 2, lower panel). The important increase in accumulation of hörhammericine (**9**), a non *O*-acetylated MIA, in line DAT7 is difficult to explain since this pattern of accumulation does not appear to be correlated with the variations in DAT specific enzyme activities observed between different DAT transformed lines (Figs. 2 and 3) and it is known that hörhammericine (**9**) is a substrate for MAT but not for DAT (Laflamme et al. (2001).

2.4. Expression of DAT activity and accumulation of hörhammericine (*9*) are correlated with root maturation in hairy root line DAT7

In order to further analyze the high DAT activities and increased hörhammericine (**9**) accumulation of hairy root line DAT7, individual hairy roots were harvested from a 4-week-old culture. Each hairy root was divided into 0.5–1 cm sections that were combined to generate four separate developmental stages from young root tips to the more mature parts of the root (Fig. 3). Each of the four develop-

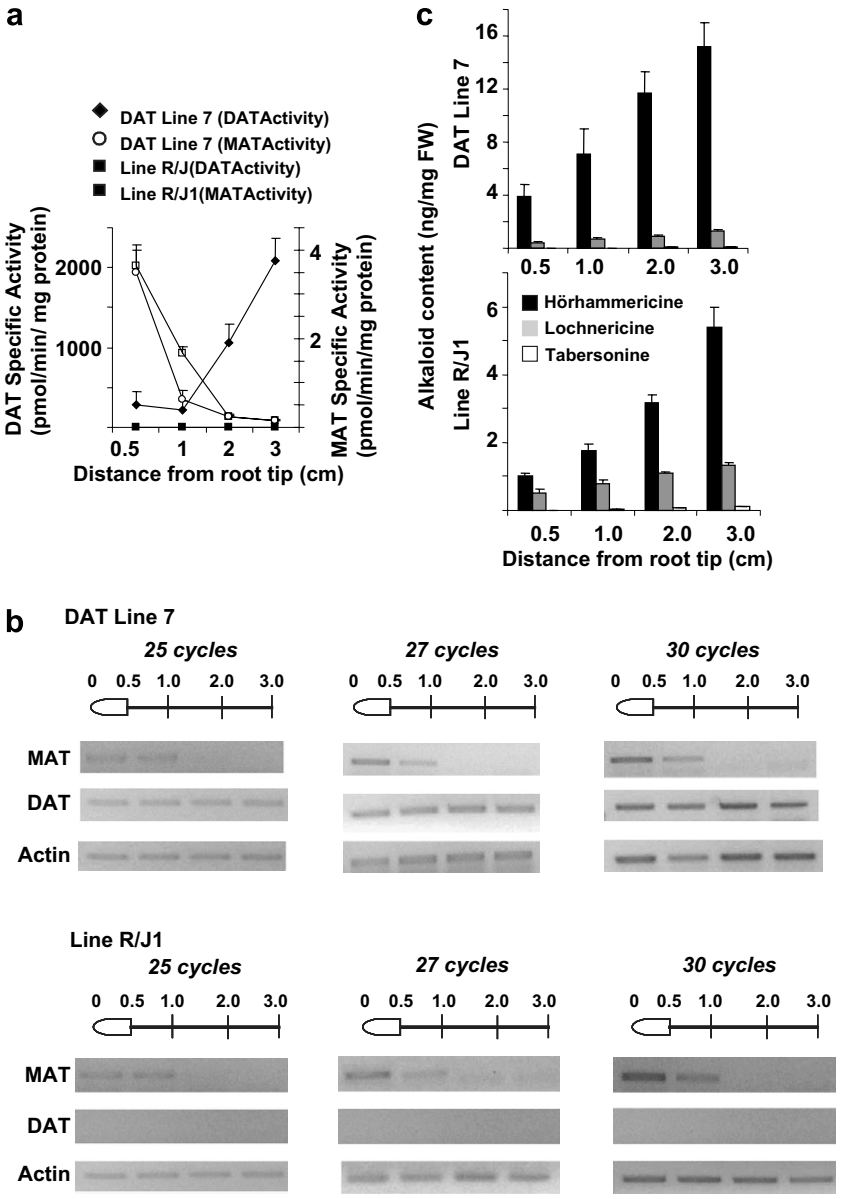

Fig. 3. Enzyme, mRNA and MIA profiles in different parts of *C. roseus* hairy roots in Lines DAT7 and R/J1, respectively. (a) DAT and MAT enzyme activities were assayed in extracts from sequential 0.5 cm sections of hairy root tissues harvested from the root tip to more mature parts of the root. DAT enzyme assays were incubated for 15 or 30 min, while MAT assays were incubated for 60 min. Sections from each stage of development were harvested in triplicate and each lot was extracted and assayed separately for DAT and MAT activities in order to generate the standard errors displayed in the figure. (b) The relative expression of MAT and DAT transcripts isolated from different hairy root tissue sections were monitored by RT-PCR (25, 27 and 30 cycles). The schematic diagram shows where the sections were obtained in relation to the root tip. (c) Contents of tabersonine (**1**), lochnericine (**8**) and hörhammericine (**9**) in extracts of different hairy root tissue sections as determined by UPLC. Sections from each stage of development were harvested, extracted and analysed by UPLC in triplicate to generate the standard errors displayed in the figure.

mental stages was extracted for MAT and DAT enzyme assays (Fig. 3a), for MIA analysis (Fig. 3a) MAT and DAT gene expression (Fig. 3b). The enzyme activity of MAT was highest in the root tip (3.5 pkat/mg protein) and it decreased rapidly in developmentally older root sections for both hairy root lines (Fig. 3a). These results correlated with previous studies by Laflamme et al. (2001), where *MAT* gene expression was localized to the root cortex and epidermal tissues of the root tip. The lack of DAT enzyme activity in line RJ/1 (Fig. 3a) was expected since previous studies localized DAT expression only to laticifers and idioblasts of aerial tissues (St-Pierre et al., 1999). In contrast, DAT enzyme activity in line DAT7 increased significantly with root maturity (Fig. 3a), perhaps as a result of expression of *DAT* driven by the constitutive CaMV 35 S promoter. These effects of the CaMV 35 S promotor were confirmed by histochemical staining of control GUS expressing hairy roots. The distribution of GUS obtained in the stained tissue of stably transformed roots (data not shown) suggests a similar distribution of DAT enzyme activity driven by the CaMV 35 promoter (Fig. 3a). This is also consistent with previous histochemical localization

studies of GUS activity confirming that the CaMV 35 S promoter is active in the root cortex and confers comparable levels of GUS expression and activity in transgenic root tissue (Battraw and Hall, 1990). RT-PCR analysis to measure relative *DAT* mRNA levels showed consistent expression profiles (Fig. 3b) with the enzyme results (Fig. 3a). The expression of *DAT* transcripts throughout the length of the root in line DAT7 appeared similar. In contrast, *MAT* expression was preferentially distributed to younger root tissue (root tip-1 cm) whereas it declined with root maturity (the section 3 cm from the root tip) (Fig. 3b). The results for MAT expression in lines DAT7 and RJ/1 were consistent with those obtained by northern blot analysis of hairy root tissue line RJ1 obtained by Laflamme et al. (2001) and correlated with MAT activities observed in both hairy root lines (Fig. 3a).

MIA analyses clearly showed an increasing accumulation of hörhammericine (**9**) with root maturity in both hairy root cultures, but hörhammericine (**9**) levels were much higher in line DAT7 (Fig. 3a). For example RJ/1 accumulated 5.5 ng of hörhammericine (**9**)/mg fresh weight of tissue in developmentally older hairy root sections (3.0 cm) whereas line DAT7 accumulated three times more of this MIA. In contrast, the tabersonine (**1**) content in line DAT7 was similar to that found in RJ/1, varying between 0.01 to 0.1 ng/mg fresh weight of tissue in the root tip to older section 3.0 cm from the root tip (Fig. 3a). Similarly, there was little variation observed in lochnericine (**8**) levels in different one cm-sections in either of the hairy root lines. The increased accumulation of hörhammericine (**9**) in line DAT7 is clearly correlated with the increase in DAT enzyme with root maturation (Fig. 3), but it is difficult to explain how expression of this gene in hairy roots could lead to these results.

2.5. Expression of DAT activity in C. roseus hairy roots interferes with MAT activity and leads to the accumulation of hörhammericine (9)

Southern blot analyses have suggested that DAT and MAT occur in *C. roseus* as single-copy genes, with the root-specific MAT sharing 63% nucleic acid and 78% amino acid identities with the above-ground specific *DAT* gene (Laflamme et al., 2001). In vitro kinetic studies with proteins produced in *E. coli* showed that rDAT only accepted deacetylvindoline (**6**) as a substrate, while rMAT did accept minovincinine, hörhammericine (**9**) and deacetylvindoline (**6**) as substrates (Laflamme et al., 2001). In addition, the catalytic efficiencies and turnover rates of rMAT were very poor compared to those of rDAT whose turnover rate for acetyl CoA and DAV was approximately 240- and 10,000-fold greater than that of rMAT (Laflamme et al., 2001). In order to address the unusual effects of DAT on the accumulation of hörhammericine (**9**), rDAT and rMAT were produced in *E. coli* and their respective enzyme activities were compared. Remarkably, incubation of MAT with varying amounts of pure rDAT inhibited MAT activity in a dose dependent manner (Fig. 4a). Similarly, incubation of DAT with varying amounts of crude rMAT inhibited DAT activity in a dose dependent manner (Fig. 4b). In contrast, when either experiment was repeated with varying levels of bovine serum albumin, this protein did not inhibit the activities of either rDAT or rMAT

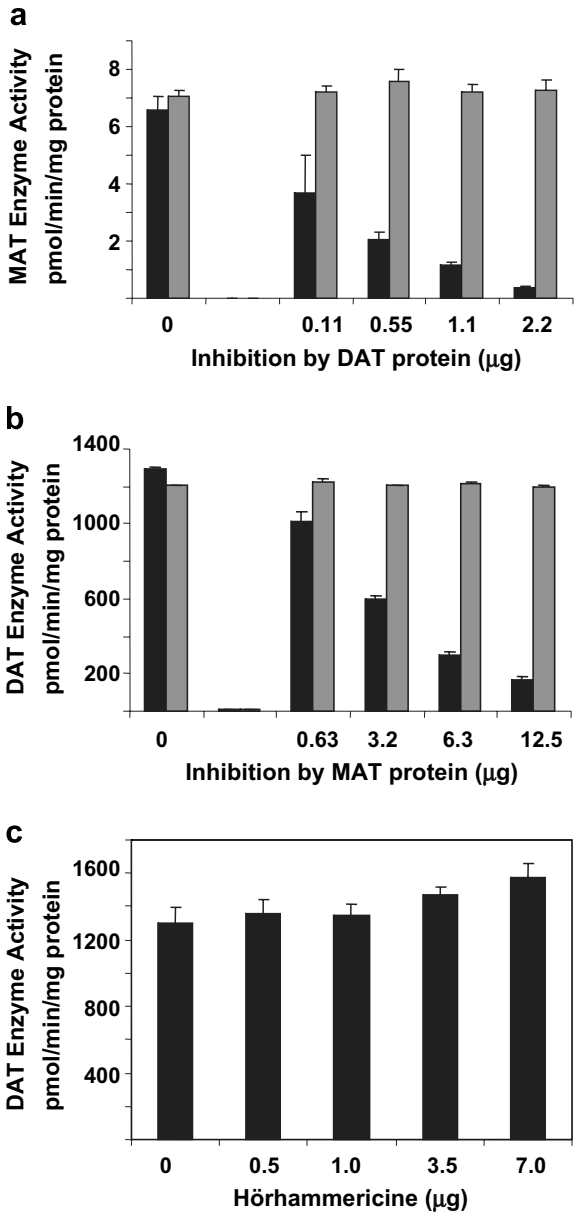

Fig. 4. (a) Inhibition of rMAT enzyme activity by adding different amounts of affinity purified rDAT protein to the incubation mixture (black columns) compared with the lack of inhibition observed (grey columns) when assayed in the presence of another protein (bovine serum albumin). The empty lane 2 reflects the lack of enzyme activity of rDAT with hörhammericine (**9**) as a substrate. Enzyme assays were done in triplicate. (b) Inhibition of rDAT enzyme activity by adding different amounts of unpurified rMAT protein to the incubation mixture(black columns) compared with the lack of inhibition observed (grey columns) when assayed in the presence of another protein (bovine serum albumin). The empty lane 2 reflects the small levels of enzyme activity of rMAT observed (less than 0.5% of the level of rDAT activity) with deacetylvindoline (**6**) as a substrate.

(Fig. 4a and b). Additional studies showed that DAT enzyme activity was not inhibited by varying concentrations of hörhammericine (**9**) (Fig. 4c) and this suggests that the mechanism of accumulation of this alkaloid does not involve its binding to DAT that might prevent its conversion to 19-*O*-acetyl-hörhammericine **(12)** by MAT. Together the results suggest that inhibition of MAT appears to be caused by an interaction between the MAT and DAT proteins.

3. Concluding remarks

In summary, the present report describes the first step in the expression of late stages of vindoline (**7**) biosynthesis in hairy root cultures. While it was not surprising that expression of the terminal step in vindoline (**7**) biosynthesis was not sufficient to make vindoline (**7**) in hairy root cultures, it was surprising that a non-*O*-acetylated MIA, hörhammericine (**9**), accumulated to 4-fold higher levels in line DAT7 than in control lines. *C. roseus* hairy roots are well known to accumulate hörhammericine (**9**) rather than 19-acetoxyhörhammericine and studies with inhibitors suggested that both lochnericine (**8**) and hörhammericine (**9**) turnover and breakdown during growth (Morgan and Shanks, 1999). The direct inhibition of rMAT activity by rDAT protein provides a plausible explanation for the results observed in this study. The inhibition of rMAT activity by rDAT suggests that these proteins interact in a novel manner that remains to be investigated.

This study also illustrates the potential of future efforts to express the last six steps in vindoline (**7**) biosynthesis in order to generate transgenic vindoline (**7**) accumulating hairy roots. The results show that several unexpected factors may negatively affect efforts to achieve this objective unless reactions competing for tabersonine (**1**) can be eliminated.

4. Experimental

4.1. Construction of plant expression cassettes

Expression cassettes for *DAT* were constructed by amplifying the *DAT* cDNA from plasmid pQE30 expression vector using primers for the addition of BamHI and SacI restriction enzyme sites. A BamHI/SacI fragment containing the *DAT* cDNA was cloned into the binary vector pBI121 downstream of the CaMV 35S promoter following the initial removal of the GUS reporter gene from the vector. This construct was named pBI121/DAT.

4.2. Preparation and growth of sterile plant material

Seeds from *C. roseus* cv. Little Delicata and *Nicotiana tabacum* SR1 were surface-sterilized by treatment with a solution of 5% (w/v) NaOCl (commercial bleach, 4% active chlorine) containing a few drops of detergent and shaken for 20 min. Seeds were rinsed 3× with sterile distilled water. Sterilized seeds were germinated on solidified nutrient medium composed of MS basal salts (Murashige and Skoog), 3% sucrose, 3 g/l Gelrite. The pH of all media was adjusted to 5.8 before autoclaving. Medium (25 ml) was dispensed into each plastic Petri dish (100 mm^2). Approximately 25 seeds were used per plate and care was taken to avoid any contact between seeds. The plates were sealed with laboratory film and were grown using a 16 h photoperiod at 25 °C. Individual 14-day-old seedlings were transferred to GA-7 Magenta boxes (Sigma Chemical Company, Oakville, Canada) containing 50 ml of solidified nutrient medium composed of WPM basal salts (Woody Plant Medium), 3% sucrose, 2.6 g/l Gelrite, 2.5 g/l activated charcoal, 2 g/l IBA (indole 3-butyric acid) and 1 mM of silver thiosulfate following autoclaving (pH 5.8). Magenta boxes were sealed with laboratory film and continued to grow in the same conditions (16 h photoperiod at 25 °C). Young leaves from 6-week-old plants were harvested for hairy root induction.

4.3. Generation of transformed C. roseus hairy roots and tobacco plants

The vectors, pBI121/GUS and pBI121/DAT were transformed into *Agrobacterium rhizogenes* strain R1000 and *Agrobacterium tumefasciens* LBA 4404 by electroporation. A single transformed colony from each combination growing on solid LB kanamycin (50 mg/l) agar medium were harvested and were used to start a 50 ml culture grown at 28 °C and 200 rpm for 16 h in liquid LB/kanamycin medium. Leaves from *C. roseus* and *N. tabacum* were wounded, infected with one of each respective *Agrobacterium*/construct strain and incubated in the dark for 48 h on solid MS/knamycin (100 mg/l) medium.

Following the co-cultivation period, *C. roseus* leaves/*Agrobacterium rhizogenes* cocktails were transferred to fresh plates containing cefatoxime (400 ppm) to kill the remaining bacteria and after six weeks of cultivation, the hairy roots that were formed were excised from independently transformed leaves and selected as independent hairy root clones on liquid MS media containing kanamycin and cefatoxime. Once no further signs of contamination of *Agrobacterium* appeared, hairy root cultures were then maintained in an antibiotic free filter-sterilized solution of 50 ml half strength Gamborg's B5 salts (Gamborg et al., 1968), 2% sucrose (pH 5.8) into a 250 ml Erlenmeyer flask. The cultures were grown on rotary shakers at 25 °C at 100 rpm in the dark and transferred routinely to fresh medium every 5–6 weeks.

Following the cocultivation period, *N. tabacum* leaves/*Agrobacterium tumefasciens* cocktails were transferred to solid MS medium, composed of 3% sucrose, 3 g/l Gelrite (pH 5.8) and filter-sterilized Zeatin (1 ppm), kanamycin (100 ppm) and cefatoxin (400 ppm) following autoclaving. After 3–4 weeks, calli began to form and were transferred to a shoot meristem generating medium containing NN

basal salts (Nitsch & Nitsch), 2% sucrose, 3 g/l Gelrite (pH 5.8), kanamycin (100 ppm) and cefatoxin (400 ppm). Antibiotic resistance shoots were maintained in Magenta boxes containing 50 ml of solidified NN media (2% sucrose, 3 g/l Gelrite, pH 5.8). Magenta boxes were sealed with laboratory film and continued to grow in a 16 h photoperiod at 25 °C.

4.4. Preparation of crude protein extracts from C. roseus hairy roots and tobacco leaves

Freshly harvested *C. roseus* hairy roots (about 500 mg fresh weight) and young leaves (1.0 g fresh weight) from transformed tobacco plants were homogenized in 3 ml of extraction buffer (0.1 M Tris–HCl buffer, pH 8.0 and 14 mM β-mercaptoethanol) using a mortal and pestle. The slurry was filtered through miracloth (Calbiochem, La Jolla, CA), the filtrate was centrifuged in an Eppendorf centrifuge for 4 min at 15,000*g* and the supernatant was desalted on a Sephadex G-25 PD-10 column (GE Healthcare, Piscataway, NJ). This crude desalted protein extract was directly used for DAT enzyme assays. Unless otherwise stated, the extractions and enzyme assays were performed in triplicate for statistical analysis.

4.5. Alkaloid extraction and analyses of C. roseus hairy roots transformed PBI121/GUS and PBI/DAT

Whole hairy roots (about 500 mg fresh weight) were harvested and used directly for alkaloid extraction. Each tissue was homogenized MeOH–H_2O (2 ml, 1:1 v/v) using a mortar and pestle and the slurry was filtered through miracloth (Calbiochem, La Jolla, CA). To the filtrate was added 0.5 ml of 10% sulfuric acid followed by extraction with an equal volume of EtoAc. After centrifugation for 5 min at 15,000*g*, the organic phase was discarded and the aqueous phase was treated with 0.25 ml of 10 N NaOH, and alkaloids were extracted with an equal volume of EtoAc. The organic phase was collected and evaporated to dryness using an SPD Speed Vac (Thermo Savant, Holbrook, New York): the residue was resuspended in MeOH (0.3 ml) and filtered through an 0.2 μm Pall filter. The filtered alkaloid extracts were analyzed by UPLC.

In addition, twenty 3.0 cm long hairy roots from DAT expressing line 11 and R/J1 hairy root lines were harvested into 0.5-cm sections from the developmentally young root tip to more mature parts of each root and were analyzed for variations in alkaloid content as described above. However, due to the small amounts of tissue used, samples were homogenized in MeOH–H_2O (400 μl, 1:1, v/v) using Kontes pellet pestle (Fisher Scientific, Canada). To the homogenate was added 100 μl of 10% sulfuric acid followed by extraction with equal volume of EtoAc. Samples were centrifuged for 5 min at 15,000*g*, and the organic phase was discarded and the aqueous phase was treated with 50 μl of 10 N NaOH, and alkaloids were extracted with an equal volume of EtoAc. The organic phase was collected and filtered as described above for analysis by UPLC.

4.6. HPLC Analysis

Alkaloid extracts were analyzed by HPLC basically as described by Uniyal et al. (2001), but the protocol was modified for chromatography using Ultra Performance Liquid Chromatography (UPLC, Waters, Milord, MA). Briefly, the solvent system was composed of solvent A [MeOH:MeCN:5 mM:NH_4oAc:Et_3N (5.8:14.2:80:0.2 by volume)] and solvent B [MeOH:MeCN:5 mM:NH_4oAc:Et_3N(23:57:20:0.2 by volume)] and solvent C [[MeOH:MeCN:5 mM:NH_4oAc:Et_3N(26:64:10:0.2 by volume)], each of which was pre-filtered through a 0.2 μm Ultipor N 66 filter (Pall Science, Mississauga, Canada). The ratio of the solvent A to B was; 0–3 min, linear gradient from 99.9:0.1 to 0.1:99.9 at the increasing flow rate from 0.047 to 0.075 ml/min; 3–5.5 min, isocratic with 0.1:99.9 at flow rate 0.075 ml/min; 5.5–6.5 min, linear gradient from 0.1:99.9 to 99.9:0.1 at the decreasing flow rate from 0.075 to 0.047 ml/min; 6.5–8.0 min, isocratic with 99.9:0.1 at flow rate 0.047 ml/min. Samples were run on Acquity UPLC BEH C_{18} 1.7 μm column (1.0 × 50 mm) (Waters, Milford, MA).

4.7. Preparation of recombinant DAT and MAT enzymes expressed in E. coli

A single colony of *E. coli* BL21 (DE3) (Invitrogen, Burlington, Canada) harboring either pQE30-MAT or pQE30-DAT, growing on LB ampicillin (LBA) (50 mg/l) agar medium, was used to inoculate LBA liquid media (3 ml), and the cells were grown for 16 h at 37 °C, shaking at 200 rpm. Bacterial cells (0.5 ml) from this stationary phase culture were used to inoculate LBA liquid medium (50 ml), and the cells were grown at 37 °C, shaking at 200 rpm until the OD_{600} absorbance was 0.5. Recombinant protein expression was then induced with addition of 2 mM isopropyl thiogalactose (IPTG), followed by incubation at 90 g for 16 h at 25 °C. After the induction, the cells were harvested by the centrifugation at 3000*g* for 10 min at 4 °C. The cell pellet was resuspended in 3 ml of 0.1 M Tris–HCl buffer (pH 8.0) containing 14 mM β-mercaptoethanol, then sonicated. The slurry was centrifuged in microfuge tubes for 5 min at 15,000*g* and the supernatant was desalted using a Sephadex G-25 PD-10 column (GE Healthcare, Piscataway, New Jersey).

The recombinant DAT and MAT proteins were purified from the crude desalted extracts using Ni-NTA Agarose resin (Qiagen, Mississauga, Canada), according to the manufacturer's procedures. Unlike affinity purified rDAT, affinity purified rMAT was not stable and no enzyme activity was detected. Enzyme assays were therefore performed using affinity purified rDAT and the crude desalted rMAT.

4.8. DAT and MAT enzyme assays

Transformed DAT expressing *C. roseus* hairy roots (500 mg), RJ/1 hairy roots or young leaves of tobacco

(1.0 g) were homogenized in 3 ml of extraction buffer (0.1 M Tris–HCl, pH 8, 14 mM mercaptoethanol) using a mortar and pestle. The extract was filtered through miracloth and crude extract (2.5 ml) was desalted directly by PD10 column chromatography (GE Healthcare) with this extract (3.5 ml) used directly for enzyme assays. DAT and MAT enzyme activities were assayed as described previously (Laflamme et al., 2001; St-Pierre et al., 1998). All DAT assays were performed in 100 μl reaction volumes, whereas MAT assays were performed in 400 μl reaction volumes containing the desalted crude protein extract together with various substrates and cofactors related to each enzyme assay: DAT assay [30 μM deacetylvindoline (**6**) and 16.6 μM (1-^{14}C) acetyl-coenzyme A, GE Healthcare]; MAT assay [30 μM horhammericine and 16.6 μM (1-^{14}C) acetyl-coenzyme A, GE Healthcare]. MAT assays were conducted at 37 °C for 60 min and DAT assays were conducted at 37 °C for 30 min, unless otherwise mentioned. The reactions were terminated by adding 10 N NaOH 50 μl, radio-labeled alkaloids were extracted into an equal volume of EtoAc, the organic phase was harvested after separation from the aqueous phase by centrifugation and was taken to dryness by vacuum centrifugation using an SPD Speed Vac (Thermo Savant, Holbrook, New York) system. Each dried sample was dissolved in methanol 5 μl and the reaction products were separated by analytical thin layer chromatography (TLC) [Polygram Sil G/UV254 (Macherey-Nagel)]. TLC plates were developed in an EtoAc–MeOH (9:1, v/v) solvent system. The radioactivity was visualized and quantified by exposure of the TLC to a storage phosphor screen (GE Healthcare, Piscataway, NJ, USA) for 16 h and emissions were detected using a Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge ver. 3.0 (Fujifilm, Tokyo, Japan).

4.9. Inhibition of MAT activity by DAT and Inhibition of DAT activity by MAT

MAT inhibition assays were performed in a total reaction volume of 120 μl containing 62.3 μg of crude desalted protein extract (100 μl), 30 μM hörhammericine (**9**), 16.6 μM [1-^{14}C] acetyl-coenzyme A (GE Healthcare) and varying amounts of purified recombinant DAT protein (0, 0.1, 0.5, 1.0 and 2.0 μg) or in the presence of bovine serum albumin (100 μg). DAT inhibition assays were performed a total reaction volume of 120 μl containing 10 μg of the purified DAT protein (100 μl), 30 μM deacetylvindoline (**5**), 16.6 μM [1-^{14}C] acetyl-coenzyme A and varying amounts of crude desalted MAT protein extract (0, 0.1, 0.5, 1.0 and 2.0 μg) or in the presence of bovine serum albumin (100 μg). The inhibitory effect of hörhammericine (**9**) to DAT enzyme activity was determined in a total reaction volume of 120 μl containing 10 μg of purified DAT protein (100 μl), 30 μM deacetylvindoline (**6**) and 16.6 μM [1-^{14}C] acetyl-coenzyme A with the addition of varying amounts of hörhammericine (**9**) (0, 3.75, 7.5, 15 and 30 μM). All the MAT and DAT enzyme assays were conducted at 37 °C for 30 min. The reactions were terminated by the addition of 10 M NaOH (50 μl), and the radio-labeled assay products were extracted with an equal volume of EtoAc. The organic phase was harvested after the centrifugation, and was completely dried by SPD Speed Vac (Thermo Savant, Holbrook, New York) system. Each dried sample was dissolved in MeOH (5 μl) of methanol and analyzed by thin layer chromatography (TLC) using Polygram Sil G/UV254 (Macherey-Nagel, Düren, Germany). TLC plates were developed in an EtoAc–MeOH (9:1, v/v) solvent system. The radioactivity was visualized and quantified by exposure of the TLC to a storage phosphor screen (GE Healthcare) for 16 h and emissions were detected using Phosphorimager FLA-3000 (Fujifilm, Tokyo, Japan) with Multi Gauge ver. 3.0 software (Fujifilm, Tokyo, Japan).

4.10. RNA extraction and RT-PCR amplification of DAT and MAT mRNA from RJ/1 and DAT transformed C. roseus hairy root cultures

C. roesus hairy roots (50 mg) were harvested and extracted for mRNA by homogenizing samples with a Kontes pellet pestles (Fisher Scientific, Canada) in 1.5 ml Eppendorf tubes containing 1 ml of Trizol reagent. After homogenization, $ChCl_3$ (0.2 ml) was added and samples were centrifuged in an Eppendorf centrifuge for 15 min at 12,000*g* and the supernatant was recovered. Following isopropanol precipitation the pellet was dissolved in DEPC H_2O (45 μl) and 5 μl of 10× TE (10 mM Tris–HCl, 1 mM EDTA) and the RNA was treated with 10U of DNase I FPLCpure (GE Healthcare, Piscataway, New Jersey) for 30 min at 37 °C followed by phenol/$CHCl_3$ extraction and concentration by EtoH precipitation. RNA was quantified by spectrophotometry and used for RT-PCR as described by Murata and De Luca, 2005.

The mRNA levels of DAT and MAT were analyzed using gene specific oligonucleotides as follows; CrDAT-RT01 5′-GTGCGTATCCGTTGGTTTCT-3′, CrDAT-RT02 5′-CGAACTCAATTCCATCGTCA-3′, CrMAT-RT01 5′AGGATTGGGCTGCTTCTACA-3′, CrMAT-RT02 5′-CCGTAGCACATCGACAGAGA-3′. Reverse transcriptase was performed using an RNA PCR Kit (AMV) ver. 2.1 (Takara, Otsu, Japan) and the PCR reactions were carried out for 25, 27 and 30 cycles, respectively, of 15 s at 94 °C, 20 s at 57 °C and 30 s at 72 °C. ExTaq DNA polymerase (Takara, Otsu, Japan) was used in place of rTaq which was included in the kit. Amplified cDNA fragments were run on 1.5% agarose gel and visualized by ethidium bromide staining.

4.11. Protein determination

Protein was determined using the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA), which is based on the method of Bradford (1976). BSA was used as a standard.

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