Plant Biotechnology 31, 511–518 (2014)
DOI: 10.5511/plantbiotechnology.14.1008a

Original Paper

Revisiting anabasine biosynthesis in tobacco hairy roots expressing plant lysine decarboxylase gene by using ^{15}N-labeled lysine

Somnuk Bunsupa[1], Kana Komastsu[1], Ryo Nakabayashi[2], Kazuki Saito[1,2], Mami Yamazaki[1,*]

[1]Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Chiba 260-8675, Japan; [2]RIKEN Center for Sustainable Resource Science, Yokohama, Kanagawa 230-0045, Japan
*E-mail: mamiy@faculty.chiba-u.jp Tel & Fax: +81-43-226-2932

Received September 10, 2014; accepted October 8, 2014 (Edited by T. Shoji)

Abstract Anabasine is an alkaloid found in a small number of *Nicotiana* species. The components of the anabasine biosynthetic pathway have yet to be identified. Here, we report the reinvestigation of biosynthetic pathways of anabasine and related tobacco alkaloids in genetically engineered cells. Hairy roots of *N. tabacum* harboring a lysine/ornithine decarboxylase gene from *Lupinus angustifolius* (La-*L*/*ODC*) were fed with labeled [ε-^{15}N]- or [α-^{15}N]-L-lysine. Relative to the unfed control, feeding of labeled ^{15}N-L-lysine greatly enhanced anabasine levels 13.5-fold in La-*L*/*ODC*-expressing line compared to 5.3-fold in the control line, suggesting that both LDC activity and substrate supplied are important factors for the efficient production of anabasine. *GUS*-expressing line showed preferential incorporation of [ε-^{15}N]-L-lysine into anabasine, indicating the main biosynthetic pathway of Δ^1-piperideine intermediate in tobacco is asymmetrically processes. In contrast, the expression of La-*L*/*ODC* showed the symmetric labeling of ^{15}N atom into anabasine, implying the occurrence of free cadaverine, which is produced by La-L/ODC enzyme, during the biosynthesis of Δ^1-piperideine intermediate. No considerable incorporation of ^{15}N into other tobacco alkaloids such as, nicotine, anatabine, and anatalline, was detected. Detailed analysis using ultra-high resolution mass spectrometry indicated that two ^{15}N atoms were incorporated into anabasine in La-*L*/*ODC*-expressing lines after feeding [ε-^{15}N]- or [α-^{15}N]-L-lysine. Our results not only provide information insight into the biosynthesis of anabasine but also suggest an alternative route for the production of anabasine by genetic engineering.

Key words: Anabasine, tobacco alkaloids, Lys-derived alkaloids, lysine/ornithine decarboxylase.

Introduction

Alkaloids, a group of nitrogen-containing specialized metabolites, represent a highly diverse group of plant metabolites that are distributed throughout the plant kingdom (Facchini 2001). Nicotine and tropane alkaloids are the two important classes of specialized metabolites that are mainly found in solanaceous plants (Eich 2008). These metabolites play an important role as defensive compounds against herbivores and are also used by humans for medical purposes (Steppuhn et al. 2004). Nicotine, the most abundant alkaloid found in tobacco accounts for about 95% of its total alkaloids contents (Hashimoto and Yamada 1994; Saitoh et al. 1985). However, some species, such as *Nicotiana glauca* accumulate anabasine, a pyridine-piperidine alkaloid, as a major compound (Saitoh et al. 1985; Sisson and Severson 1990). Nicotine is one of the most toxic drugs of abuse while anabasine, similar in both structure and effects to nicotine, is considerably more toxic to human (Furer et al. 2011).

The biochemistry and physiology of tobacco alkaloids has been extensively studied for more than half a century. The biosynthesis of nicotine and related alkaloids in tobacco has been recently reviewed by Dewey and Xie (2013). While nicotine is composed of two rings; a pyridine ring and a pyrrolidine ring, anabasine is composed of a pyridine ring and a piperidine ring (Shoji and Hashimoto 2008). The pyrrolidine ring of nicotine is derived from putrescine, which in turn is produced by the decarboxylation of ornithine or arginine by ornithine decarboxylase (ODC) and arginine decarboxylase (ADC), respectively (Figure 1). Putrescine is then *N*-methylated and oxidatively deaminated by putrescine *N*-methyltransferase (PMT) and *N*-methylputrescine oxidase (MPO), respectively, to 4-aminobutanol, which undergoes spontaneous cyclization to generate 1-methyl-Δ^1-prrolinium cation, which is then coupled

This article can be found at http://www.jspcmb.jp/
Published online January 14, 2015

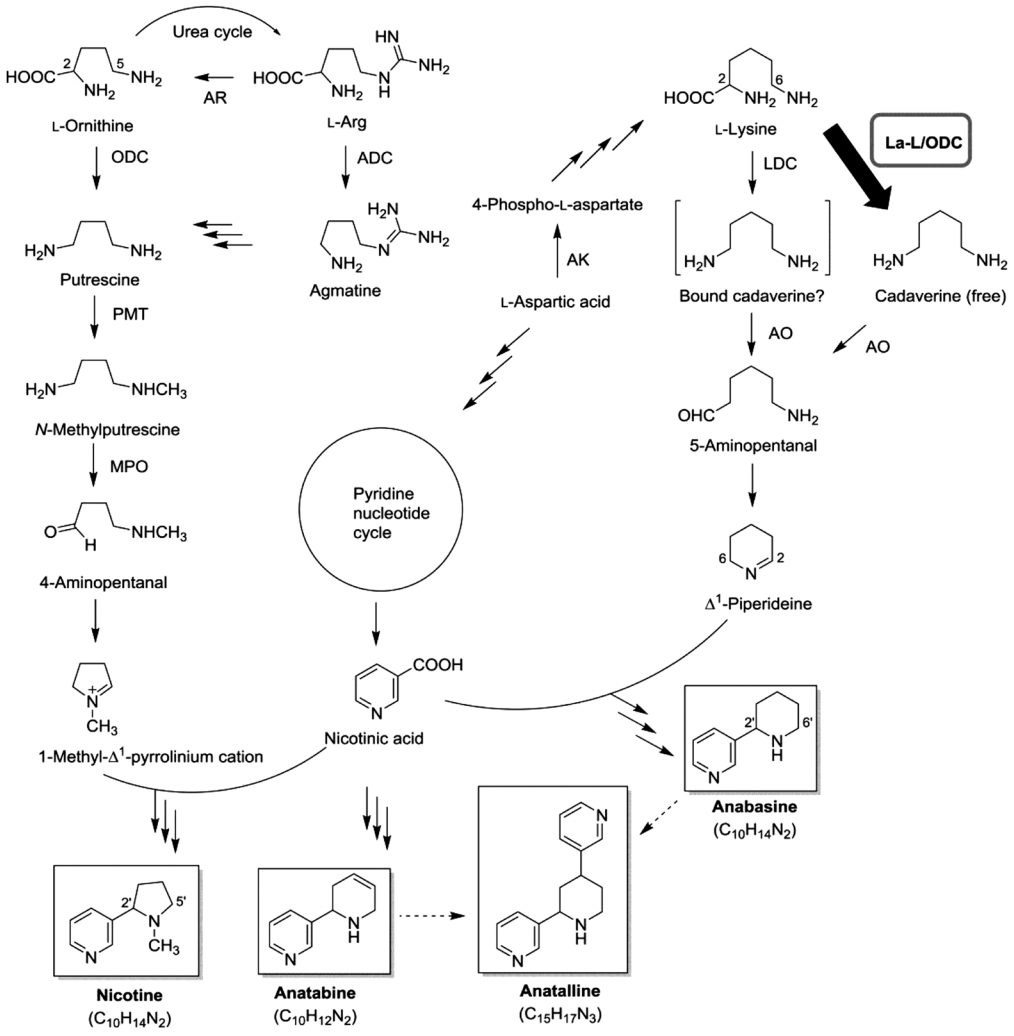

Figure 1. Biosynthetic pathway of tobacco alkaloids. ODC, ornithine decarboxylase; ADC, arginine decarboxylase; LDC, lysine decarboxylase; PMT, putrescine *N*-methyltransferase; MPO, methyl putrescine oxidase; AO, amine oxidase; AK, aspartate kinase; AR, arginase. Multiple arrows indicate more than one reaction. Broken lines represent undefined reactions.

with a pyridine ring derived from nicotinic acid to form nicotine (Figure 1). On the other hand, the piperidine ring of anabasine is derived from cadaverine which is formed through decarboxylation of lysine by lysine decarboxylase (LDC) (Figure 1). Cadaverine is then oxidized by amine oxidase (AO) and spontaneously cyclized to form Δ^1-piperideine ring. Further coupling of the Δ^1-piperideine ring with the pyridine ring similar to the one during nicotine biosynthesis generates anabasine (Figure 1) (Dewey and Xie 2013 and references therein; Shoji and Hashimoto 2008).

It has been shown that the incorporation of labeled [2-^{14}C]-lysine cannot proceed via free cadaverine, because the ^{14}C atom of [2-^{14}C] lysine was incorporated into the C-2′ not to C-6′ of the piperidine ring of anabasine (Figure 1) (Leete 1956; Leete and Siegfried 1957; Leistner and Spenser 1973). A similar result was obtained when a mixture of [2-^{14}C]-lysine and [α-^{15}N]- or [ε-^{15}N]-L-lysine was fed to excised *N. glauca* roots. In this case, ^{14}C was incorporated at C-2′ of anabasine and ^{15}N from only [ε-^{15}N]-L-lysine was incorporated into anabasine (Leete et al. 1964). However, genes encoding the enzymes for the production of anabasine have yet to be identified in tobacco. It has been suggested that the decarboxylation step is catalyzed by single bi-functional enzyme for both ODC and LDC reactions in tobacco (DeBoer et al. 2013; Lee and Cho 2001). A berberine bridge enzyme-like protein (BBL) has been reported to be involved in the final condensation reaction to form anabasine (Kajikawa et al. 2009).

Recently, we have identified a bi-functional enzyme, lysine/ornithine decarboxylase (La-L/ODC), from quinolizidine alkaloid (QA)-producing *Lupinus angustifolius* (Bunsupa et al. 2012a). La-L/ODC catalyzes the decarboxylation of L-lysine to cadaverine, which is the first step in biosynthesis of QAs (Bunsupa et al. 2012a). For QA biosynthesis, an oxidative deamination of cadaverine gives rise to 5-aminopentanal which is further cyclized into Δ^1-piperideine, a plausible intermediate for QAs production (Bunsupa et al.

2012b). Unlike biosynthesis of anabasine in tobacco, lysine is incorporated into the QAs via the symmetrical intermediate cadaverine (Leeper et al. 1981; Leistner and Spenser 1973).

In this study, to clearly define anabasine biosynthetic pathway in tobacco, we conducted an isotope tracer experiment by feeding labeled [ε-^{15}N]- or [α-^{15}N]-L-lysine to tobacco hairy roots expressing La-*L*/*ODC* (Bunsupa et al., 2012a). The incorporation of ^{15}N-L-lysine into tobacco alkaloids; nicotine, anatabine, anabasine, and anatalline was analyzed by mass spectrometry (MS). We found that anabasine was mainly produced via an asymmetric intermediate, most likely via bond-cadaverine. Detailed analysis using ultra-high resolution mass spectrometry suggests an alternative route for the production of anabasine in La-*L*/*ODC*-expressing lines, in which two heterocyclic rings of anabasine are derived from lysine.

Materials and methods

Chemicals

All chemicals and solvents used were of analytical, high-performance liquid chromatography (HPLC), or liquid chromatography (LC)-MS grade, unless otherwise specified. [α-^{15}N]-L-Lysine and [ε-^{15}N]-L-lysine were purchased from Cambridge Isotope Laboratories, Inc. (MA, USA). Nicotine was purchased from Nacalai Tesque (Kyoto, Japan). (R,S) Anabasine was purchased from Alfa Aesar (MA, USA). (R,S) Anatabine and (R,S) anatalline were purchased from Toronto Research Chemicals (ON, Canada).

Plant materials and transformation

The transgenic tobacco (*N. tabacum* cv Petit Havana line SR1) hairy roots expressing either La-*L*/*ODC* or bacterial β-glucuronidase (*GUS*) as a control, were established as previously described (Bunsupa et al. 2012a). The selected transformed tobacco hairy roots were maintained in Gamborg B5 medium supplemented with 2% sucrose (growth medium) at 25°C on an orbital shaker (54 rpm) in dark and subcultured every 2 weeks.

Feeding experiments with labeled precursors

After subculturing for 2 weeks, the hairy roots were cut into ca. 1 cm pieces. Six pieces were transferred to 20 ml of growth medium in a 100-ml Erlenmeyer flask. Nine flasks were set up from the same parent transgenic line. Cultures were incubated at 25°C on an orbital shaker (54 rpm) in the dark. To feed labeled precursors, the growth medium in the flasks was replaced after 1 week of incubation with a new medium supplemented with, 1 mM [α-^{15}N]-L-lysine, 1 mM [ε-^{15}N]-L-lysine, or mock (no addition of lysine). A set of three flasks was used for each feeding treatment. One week later, all cultures were harvested onto paper towels, frozen in liquid nitrogen, homogenized, and stored at −80°C.

Measurement of alkaloids and ^{15}N incorporation

Alkaloids were extracted from homogenized frozen hairy root tissue as described (Bunsupa et al. 2012a). Quantitative analysis of tobacco alkaloids was performed by using LC (Agilent HPLC 1100 series, Agilent technologies)-diode array (DAD) detection-electrospray ionization (ESI)/quadrupole (Q) (quadrupole LC/MS 6120, Agilent technologies)-MS using the same column and chromatographic conditions as described for HPLC/DAD (Bunsupa et al. 2012a). A 10-μl aliquot was analyzed and the alkaloids were identified by comparing against the standard compounds. The incorporation of ^{15}N from ^{15}N-labeled lysine was confirmed by LC-Fourier transform ion cyclotron resonance (FTICR)-MS for accurate estimation of *m*/*z* values of isotopic ions. The theoretical ion pattern of anabasine was calculated using SmartFormula in DataAnalysis 4.0 (Bruker Daltonik GmbH, Germany) with the following limiting conditions: <1 mDa; $C_{0-50}H_{0-100}N_{0-5}O_{0-50}$; charge, 1, as described (Nakabayashi et al. 2013).

Results

The effect of exogenous feeding of labeled [α-^{15}N]- and [ε-^{15}N]-L-lysine on the production of tobacco alkaloids

The alkaloid levels of the transgenic tobacco lines were analyzed by LC-Q-MS. Sum of the peak intensity values of labeled and unlabeled metabolites were used to calculate the levels of tobacco alkaloids. The La-*L*/*ODC*-expressing lines, on an average had 1.6 times higher anabasine contents compared to the control *GUS*-expressing lines. However, no marked changes in the levels of other tobacco alkaloids levels were observed between the two lines (Figure 2A–D). Feeding of [ε-^{15}N]- or [α-^{15}N]-L-lysine to La-*L*/*ODC*-expressing lines increased average of anabasine, anatabine, anatalline, and nicotine content by 13.5, 4.3, 4.5, and 1.4 times, respectively, relative to their corresponding levels in unfed lines (Figure 2A–D). A similar increase in the levels of all other tobacco alkaloids except anabasine was observed in the *GUS*-expressing control lines after incubation with ^{15}N-L-lysine. Feeding of ^{15}N-L-lysine to the *GUS*-expressing control lines increased the average anabasine content only 5.3 times over the unfed control (Figure 2A–D). The addition of labeled ^{15}N-L-lysine increased the ratio of anabasine to nicotine from 0.06 to 0.23 in *GUS*-expressing lines, and from 0.08 to 0.82 in La-*L*/*ODC*- expressing lines (Figure 2A–D). These results suggest that both substrate availability and LDC activity are important factors for the efficient production of anabasine in tobacco hairy roots.

The incorporation of ^{15}N from labeled [α-^{15}N]- and [ε- ^{15}N]-L-lysine into tobacco alkaloids analyzed by LC-Q-MS

The isotope enrichment factors (EF) of tobacco alkaloids

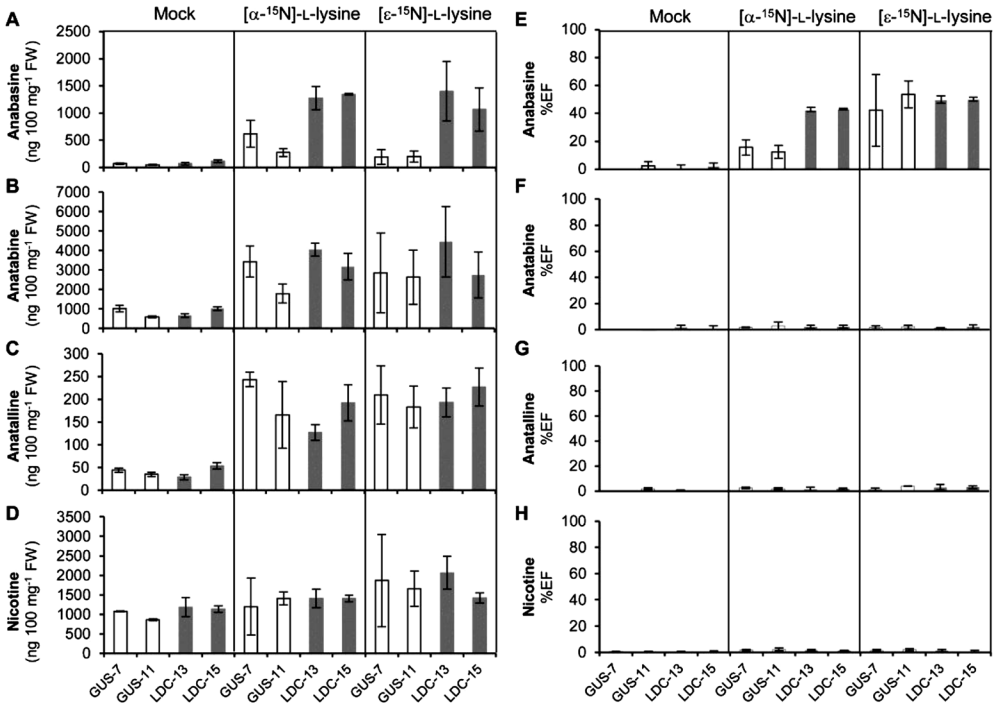

Figure 2. Profiles of tobacco alkaloids and percent isotope enrichment factor (%EF) in tobacco hairy roots expressing La-*L*/*ODC* (*LDC*) or *GUS*-expressing lines fed with labeled ^{15}N-L-lysine. Data were obtained by LC-Q-MS. (A–D) The contents of each tobacco alkaloid in individual lines. (E–H) %EF of feeding experiments on each alkaloid. Each bar represents the mean±SD (experimental replicate; $n=3$). FW, fresh weight.

synthesized from labeled ^{15}N-L-lysine were calculated from the intensity distribution of LC-Q-MS isotopic mass spectral data. The peak intensity values for M and M+1, where M is molecular weight of unlabeled metabolite (monoisotopic), were corrected by subtracting the natural abundance of corresponding peak (Campbell 1974). These corrected peak intensities were used to calculate the %EF value using following formula: %EF=[(intensity of M+1)/(sum of intensities of M and M+1)]×100.

When [α-^{15}N]- and [ε-^{15}N]-L-lysine were fed to La-*L*/*ODC*- and *GUS*-expressing lines, only anabasine was labeled (Figure 2 E–H). La-*L*/*ODC*-expressing lines showed similar enrichment of ^{15}N in anabasine by both α- and ε-labeled ^{15}N-L-lysine (Figure 2E). In *GUS*-expressing lines, on the other hand, enrichment of ^{15}N in anabasine was about 3.5 times higher by [ε-^{15}N]-L-lysine than by [α-^{15}N]-L-lysine (Figure 2E). These results imply that anabasine in tobacco is synthesized via an asymmetric intermediate, most likely protein-bound cadaverine. In contrast, in La-*L*/*ODC*-expressing lines anabasine is synthesized via a symmetric free cadaverine.

The incorporation of ^{15}N into anabasine in La-L/ODC expressing hairy roots analyzed by LC-FTICR-MS

Since we did not have sufficient plant tissue for further analysis, independent experimental samples were used for the analysis by LC-FTICR-MS. This method offers the best performance with respect to ultra-high resolution [1,000,000 full-width at half-maximum (fwhm) at 7.0 T] and mass accuracy (1 ppm) (Nakabayashi et al. 2013). Anabasine and nicotine are structural isomers of each other, and are chemically similar (both are $C_{10}H_{12}N_2$). These two compounds had same retention time under our LC-FTICR-MS analytical condition. Thus, these data must be carefully interpreted particularly the total contents of anabasine and nicotine in each analyzed sample as indicated by the percentage of anabasine in the sum of anabasine and nicotine (Figures 3 and 4). Since the LC-Q-MS data showed no significant enrichment of nicotine by ^{15}N in both transgenic lines under the set experimental conditions (Figure 2H), observed %EF of ^{15}N is practically due to anabasine. A comparative analysis of the observed and theoretical masses was performed in the region of M, M+1, and M+2 ion peaks to identify the specific elemental compositions (Figure 3). Isotopic patterns of anabasine in the M+1 region confirmed the incorporation of labeled ^{15}N-L-lysine in different ratios in fed conditions of both La-*L*/*ODC*- and *GUS*-expressing lines, as observed in the analysis of LC-Q-MS. Again, no considerable incorporation of ^{15}N-L-lysine into of others tobacco alkaloids was found. The isotopic pattern at the region of M+2 of anabasine also showed a clear incorporation pattern of labeled ^{15}N-L-lysine (Figure 4). In addition, incorporation of two ^{15}N atoms into anabasine was observed from both [α-^{15}N]- and [ε-^{15}N]-L-lysine in La-*L*/*ODC*-expressing

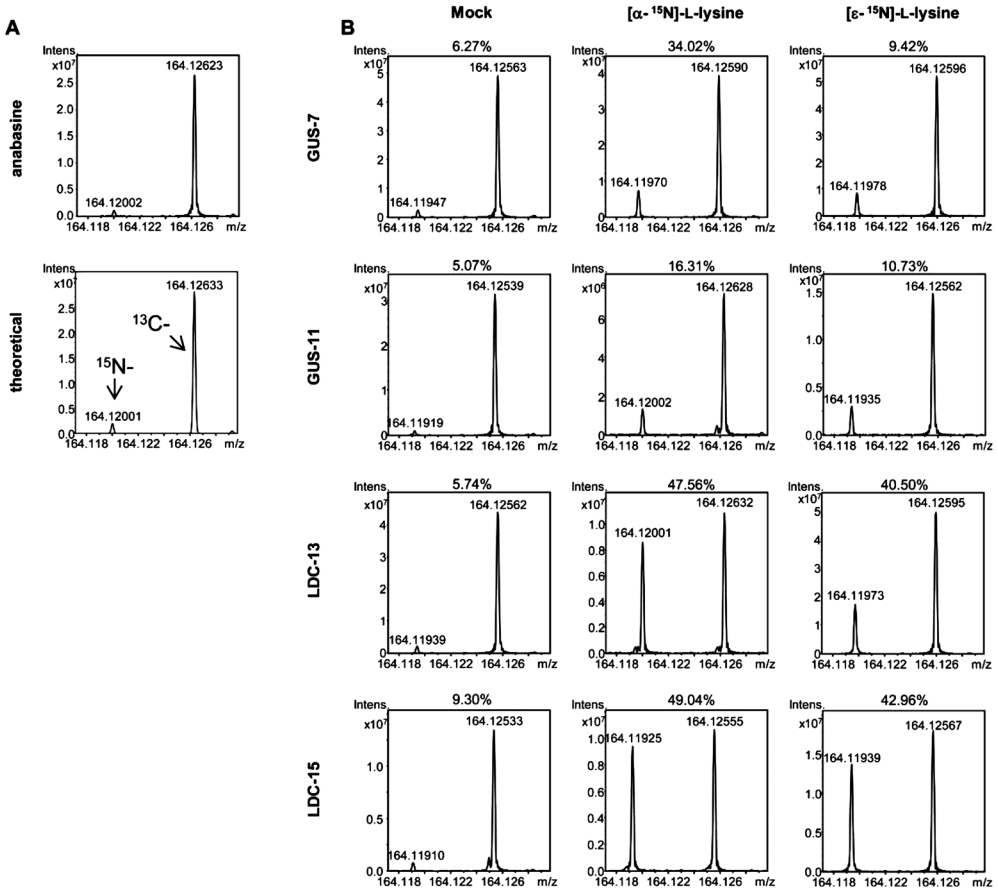

Figure 3. Isotopic patterns in the region of M +1 peak of anabasine obtained by LC-FTICR-MS. (A) Standard and theoretical spectra of anabasine. (B) ^{15}N- and ^{13}C-substituted ions in the region M +1 from different feeding experiments. The ratio of peak intensities of ^{15}N- to ^{13}C-substituted ions was used to compare the incorporation pattern from different experimental conditions. The peak resolution of ^{15}N- and ^{13}C-substituted ions was approximately 770,000 fwhm. The percentage on the top of each graph represents the amount of anabasine as a percent of both anabasine and nicotine eluting at the same retention time.

lines (Figure 4). These results indicate an alternative pathway(s) for the production of anabasine from lysine in tobacco hairy roots expressing La-*L*/*ODC* gene.

Discussion

The results presented here suggest that LDC is the rate-limiting enzyme in anabasine biosynthesis. It is also evident that the supply of L-lysine, the substrate of LDC, is also important for the efficient production of anabasine. Our results are in agreement with the enhanced cadaverine and anabasine levels observed after feeding lysine to hairy roots of *N. tabacum* expressing a bacterial LDC gene (Fecker et al. 1993). It has been shown that the accumulation of cadaverine and anabasine could be further enhanced by expressing bacterial LDC fused to a chloroplast signal peptides in plant cells (Herminghaus 1996). Although the La-L/ODC used in this study was targeted to chloroplasts, the La-*L*/*ODC*-expressing line produced only 2 times higher anabasine compared to the control line (Figure 2) (Bunsupa, et al. 2012a). Thus, it is necessary to confirm whether La-L/ODC is targeted to leucoplasts in tobacco hairy roots. Overall, the low levels of anabasine in tobacco hairy roots are probably due not only to the low levels of LDC activity but also the amount of substrate supplied. Additionally, directing the target protein to the compartment(s) where the substrate is localized is important to increase the production of anabasine.

The incorporation of ^{15}N atom into anabasine from [ε-^{15}N]-L-lysine was higher than that from [α-^{15}N]-L-lysine in the control *GUS*-expressing lines, suggesting the main biosynthetic pathway of Δ^1-piperideine intermediate in tobacco is through specific loss of α-amino group rather than ε-amino group of lysine. It has been proposed that the decarboxylation and subsequent oxidation reactions of lysine are closely linked to the production of anabasine, and the reaction might proceed via the Schiff base between lysine and pyridoxol-5′-phosphate and similarly bound cadaverine (Walton 1988). Since the %EF for [α-^{15}N]-L-lysine into anabasine was not zero (as expected from the hypothetical bound-form) but ca. 14%, the partial equilibrium between the bound and free cadaverine or an

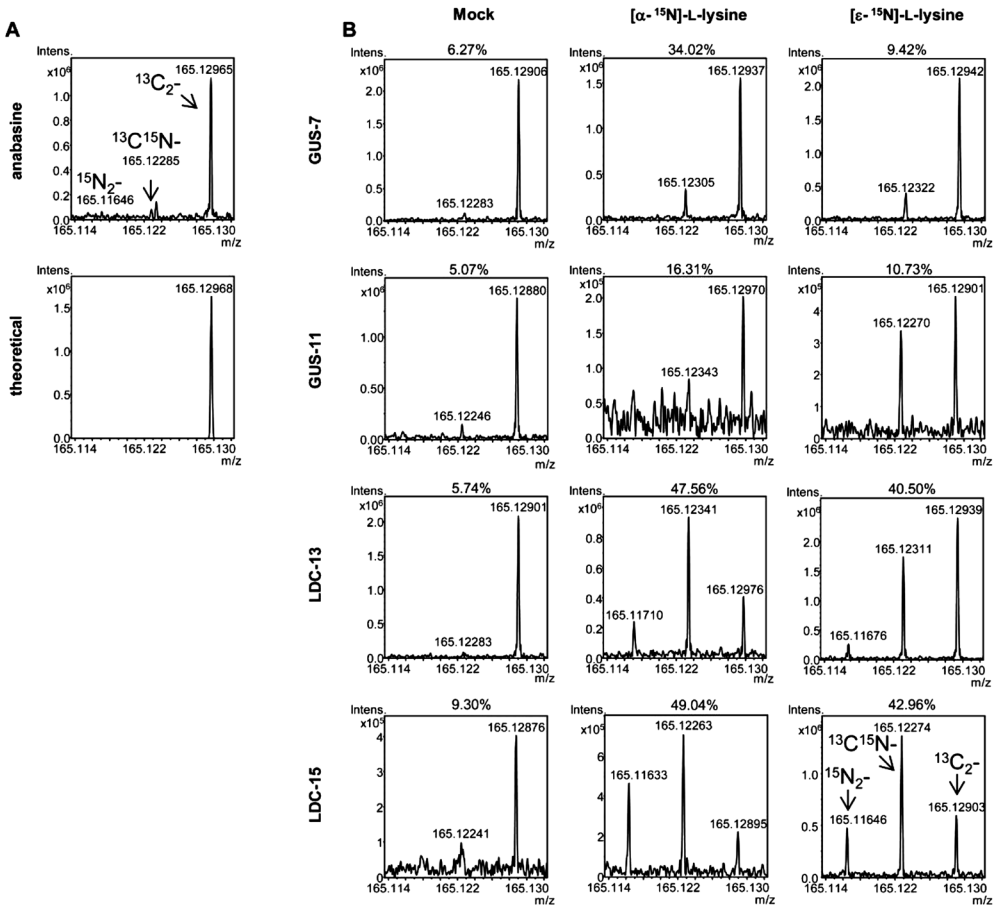

Figure 4. Isotopic patterns in the region of M +2 peak of anabasine obtained by LC-FTICR-MS. (A) Standard and theoretical spectra of anabasine. (B) $^{15}N_2$-, $^{15}N^{13}C$-, and $^{13}C_2$-substituted ions in the region M +2 from different feeding experiments. The ratios of peak intensities of $^{15}N_2$- and $^{15}N^{13}C$- to $^{13}C_2$-substituted ions were used to compare the incorporation pattern from different experimental conditions. The peak resolution of $^{15}N_2$-, $^{15}N^{13}C$-, and $^{13}C_2$-substituted ions was approximately 770,000 fwhm. The percentage on the top of each graph represents the amount of anbasine as a percent of both anabasine and nicotine eluting at the same retention time.

alternative pathway to produce free cadaverine may exist in plant cells. Overall, production of the Δ^1-piperideine intermediate may mainly be processed via substrate channeling and enzyme complexes without releasing free cadaverine to enhance reaction rates. Numerous examples of natural and synthetic complexes and potential benefits of such complexes have been reviewed recently (Zhang 2011).

N. glauca accumulates roughly equal proportions of nicotine and anabasine in the roots while anabasine is the major alkaloid found in the leaves (Saitoh et al. 1985). Recent studies reported that ODC from *N. glauca* (Ng-ODC) also functions as LDC to elevate levels of anabasine in response to wound-associated stress (DeBoer et al. 2013; Sinclair et al. 2004). Nicotine is derived from free putrescine which is a product of decarboxylation reaction catalyzed by ODC (Leete and Siegfried 1957). It is unlikely that this bi-functional enzyme could catalyze both reactions efficiently in the same compartment because both Ng-ODC (free) and Ng-ODC (complex) forms are needed for the production of nicotine and anabasine, respectively. If the production of Δ^1-piperideine intermediate occurs via the enzyme complexes then, the bi-functional enzyme Ng-ODC should be targeted to alternative subcellular compartments to interact with different substrates, in this case lysine or ornithine, to produce such alkaloids. Several studies have shown that anabasine levels in leaves of *N. glauca* increased several fold after wounding together with the marked increase in Ng-*ODC* transcript level in the upper leaves while only trace amounts of nicotine was detected in these tissues (DeBoer et al. 2009; Sinclair et al. 2004). Nicotine is synthesized in root tissues and is translocated to the aerial parts of the tobacco plant through xylem (Hashimoto and Yamada 2003; Shitan et al. 2009; Shoji et al. 2000). It is therefore possible that Ng-ODC mainly functions as an enzyme complex for the production of anabasine in the leaf tissues of *N. glauca*. However, this hypothesis needs further experimental validation.

In contrast to the control *GUS*-expressing lines, equal incorporation of ^{15}N from α- or ε-labeled ^{15}N-lysine into anabasine, suggests the production of Δ^1-piperideine intermediate via free symmetrical cadaverine in the

La-*L*/*ODC*-expressing transgenic lines. It has been reported that feeding of cadaverine greatly enhanced the production of anabasine in hairy root cultures of *N. rustica* (Walton 1988). The reasons why the expression of La-*L*/*ODC* in tobacco hairy roots caused the symmetric labeling could be explained by several possibilities. Since La-L/ODC involves in the first step of QA biosynthesis which proceed via free cadaverine (Leeper et al. 1981; Leistner and Spenser 1973). One possibility is that the La-L/ODC enzyme may not form complexes with endogenous tobacco enzymes, and therefore the cadaverine produced is further oxidized by endogenous oxidase enzymes for anabasine biosynthesis. On the other hand, La-L/ODC may forms complexes with endogenous tobacco enzymes but the exceed amounts of La-L/ODC may also produce free cadaverine with similar extent as bound cadaverine. To confirm those hypotheses, the tracer experiments using tobacco *L*/*ODC*-overexpressing hairy roots or plants are needed. Such experimental data will answer the question whether the properties of La-L/ODC or the increased LDC activity or both caused the symmetric labeling.

Our current knowledge of the biosynthesis of anabasine is based extensively on labeling experiments conducted for more than half a century (Dewey and Xie 2013). By using LC-FTICR-MS, which has ultra-high resolution and mass accuracy, we could not only confirm the incorporation of labeled ^{15}N-lysine into anabasine but also identified a novel pathway for the biosynthesis of anabasine from cadaverine by overexpression of *L*/*ODC*, in particular from the interpretation of the data of double ^{15}N incorporation into anabasine. It is conceivable that overproduction of cadaverine in La-*L*/*ODC*-expressing lines leads to an increase in the level of Δ^1-piperideine intermediate, resulting in the production of anabasine via a novel pathway in which both the rings of anabasine are derived from Δ^1-piperideine. Since we could not separate anabasine and nicotine in our analytical condition, it could be argued that the ε-transamination may occur as reported in the feeding of [ε-^{15}N]-lysine in *N. glauca*, in which a significant amount of ^{15}N (specific incorporation 2.5%) was found in the pyrrolidine ring of nicotine (Leete 1964). Since we did not observe incorporation of two ^{15}N atoms in *GUS*-expressing lines with higher nicotine levels (Figure 4), it is highly unlikely that double ^{15}N was incorporated into nicotine due to contamination. Our results suggest an alternative route to produce anabasine from two piperideine rings in the La-*L*/*ODC*-expressing line when a sufficient quantity of precursor is supplied. Alternatively, the excess cadaverine produced may directly or indirectly serve as a precursor for pyridine nucleotide cycle for the production of nicotinic acid by a pathway yet to be identified.

When intact *N. glauca* plants were fed with 1,5-^{14}C-cadaverine the ^{14}C label was into piperidine ring of anabasine but not pyridine ring (Leete 1958). It will be interesting to confirm using ultrahigh resolution and mass accuracy of mass spectrometry, whether *N. glauca* could produce anabasine by the same pathway as La-*L*/*ODC*-expressing lines as the intermediate, Δ^1-piperideine, is expected to accumulate to a higher level in *N. glauca*.

Collectively, our study provides a clearer understanding of alkaloid biosynthesis in tobacco, particularly of anabasine biosynthesis, and suggests an alternative route for the production of anabasine by genetic engineering. Our data could contribute to the successful molecular and biotechnological breeding of desirable traits in tobacco.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research (KAKENHI) from The Ministry of Education, Culture, Sports, Science, and Technology (MEXT).

References

Bunsupa S, Katayama K, Ikeura E, Oikawa A, Toyooka K, Saito K, Yamazaki M (2012a) Lysine decarboxylase catalyzes the first step of quinolizidine alkaloid biosynthesis and coevolved with alkaloid production in Leguminosae. *Plant Cell* 24: 1202–1206

Bunsupa S, Yamazaki M, Saito K (2012b) Quinolizidine alkaloid biosynthesis: recent advances and future prospects. *Front Plant Sci* 3: 239

Campbell IM (1974) Incorporation and dilution values—Their calculation in mass spectrally assayed stable isotope labeling experiments. *Bioorg Chem* 3: 386–397

DeBoer KD, Dalton HL, Edward FJ, Ryan SM, Hamill JD (2013) RNAi-mediated down-regulation of ornithine decarboxylase (ODC) impedes wound-stress stimulation of anabasine synthesis in *Nicotiana glauca*. *Phytochemistry* 86: 21–28

DeBoer KD, Lye JC, Aitken CD, Su AK-K, Hamill JD (2009) The A622 gene in *Nicotiana glauca* (tree tobacco): evidence for a functional role in pyridine alkaloid synthesis. *Plant Mol Biol* 69: 299–312

Dewey RE, Xie J (2013) Molecular genetics of alkaloid biosynthesis in *Nicotiana tabacum*. *Phytochemistry* 94: 10–27

Eich E (2008) *Solanaceae Convolvulaceae: Secondary Metabolites*. Springer, Berlin

Faccini PJ (2001) Alkaloid biosynthesis in plants: Biochemistry, cell biology, molecular regulation, and metabolic engineering application. *Annu Rev Plant Physiol Plant Mol Biol* 52: 29–66

Fecker LF, Rügenhagen C, Berlin J (1993) Increased production of cadaverine and anabasine in hairy root cultures of *Nicotiana tabacum* expressing a bacterial lysine decarboxylase gene. *Plant Mol Biol* 23: 11–21

Furer V, Hersch M, Silvetzki N, Breuer GS, Zevin S (2011) *Nicotiana glauca* (tree tobacco) intoxication-two cases in one family. *J Med Toxicol* 7: 47–51

Hashimoto T, Yamada Y (1994) Alkaloid biogenesis: molecular aspects. *Annu Rev Plant Physiol* 45: 257–285

Hashimoto T, Yamada Y (2003) New genes in alkaloid metabolism and transport. *Curr Opin Biotechnol* 14: 163–168

Herminghaus S, Tholl D, Rügenhagen C, Fecker LF, Leuschner C,

Berlin J (1996) Improved metabolic action of a bacterial lysine decarboxylase gene in tobacco hairy root cultures by its fusion to a *rbcS* transit peptide coding sequence. *Transgenic Res* 5: 193–201

Kajikawa M, Hirai N, Hashimoto T (2009) A PIP-family protein is required for biosynthesis of tobacco alkaloids. *Plant Mol Biol* 69: 287–298

Lee YS, Cho YD (2001) Identification of essential active-site residues in ornithine decarboxylase of *Nicotiana glutinosa* decarboxylating both L-ornithine and L-lysine. *Biochem J* 360: 657–665

Leeper FJ, Grue-Sørensen G, Spenser ID (1981) Biosynthesis of quinolizidine alkaloids. Incorporation of Δ^1-piperideine into matrine. *Can J Chem* 59: 106–115

Leete E (1956) The biogenesis of nicotine and anabasine. *J Am Chem Soc* 78: 3520–3523

Leete E (1958) The biogenesis of the *Nicotiana* alkaloids. VI. The piperidine ring of anabasine. *J Am Chem Soc* 80: 4393–4394

Leete E, Gros EG, Gilbertson TJ (1964) The Biosynthesis of anabasine. Origin of the nitrogen of the piperidine ring. *J Am Chem Soc* 86: 3907–3908

Leete E, Siegfried KJ (1957) The biogenesis of nicotine. III. Further observations on the incorporation of ornithine into the pyrrolidine ring. *J Am Chem Soc* 79: 4529–4531

Leistner E, Spenser ID (1973) Biosynthesis of the piperidine nucleus. Incorporation of chirally labeled [1-^{3}H] cadaverine. *J Am Chem Soc* 95: 4715–4725

Nakabayashi R, Sawada Y, Yamada Y, Suzuki M, Hirai MY, Sakurai T, Saito K (2013) Combination of liquid chromatography-fourier transform ion cyclotron resonance-mass spectrometry with ^{13}C-labeling for chemical assignment of sulfur-containing metabolites in onion bulbs. *Anal Chem* 85: 1310–1315

Saitoh F, Nona M, Kawashima N (1985) The alkaloid contents of sixty *Nicotiana* species. *Phytochemistry* 24: 477–480

Shitan N, Morita M, Yazaki K (2009) Identification of a nicotine transporter in leaves vacuoles of *Nicotiana tabacum*. *Plant Signal Behav* 4: 530–532

Shoji T, Hashimoto T (2008) Why does anatabine, but not nicotine, accumulate in jasmonate-elicited cultured tobacco BY-2 cells? *Plant Cell Physiol* 49: 1209–1216

Shoji T, Yamada Y, Hashimoto T (2000) Jasmonate induction of putrescine *N*-methyltransferase genes in the root of *Nicotiana sylvestris*. *Plant Cell Physiol* 41: 831–839

Sinclair SJ, Johnson R, Hamill JD (2004) Analysis of wound-induced gene expression in *Nicotiana* species with contrasting alkaloid profiles. *Funct Plant Biol* 31: 1–9

Sisson VA, Severson RF (1990) Alkaloid composition of the *Nicotiana* species. *Beitr Tabakforsch* 14: 327–339

Steppuhn A, Gase K, Krock B, Halitschke R, Baldwin IT (2004) Nicotine's defensive function in nature. *PLoS Biol* 2: 1074–1080

Walton NJ, Robins RJ, Rhodes MJC (1988) Perturbation of alkaloid production by cadaverine in hairy root cultures of *Nicotiana rustica*. *Plant Sci* 54: 125–131

Zhang Y-HP (2011) Substrate channeling and enzyme complexes for biotechnological applications. *Biotechnol Adv* 29: 715–725