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Chapter 5

^{13}C-Isotope-Labeling Experiments to Study Metabolism in *Catharanthus roseus*

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Additional information is available at the end of the chapter

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Abstract

Plant metabolism is a complex network. Pathways are correlated and affect each other. Secondary metabolic pathways in plant cells are regulated strictly, and upon an intra- or extra-stimuli (e.g. stress), the metabolic fluxes will change as a response on the stimuli, for example, to protect the plant against herbivores or against microbial infections. ^{13}C-isotope-labeling experiment has been performed on cell cultures and hairy roots of *Catharanthus roseus* to measure fluxes through some pathways. However, due to the complexity of the total metabolic network in an intact plant, no experiments have yet been carried on *C. roseus* plants. In this study, [1-^{13}C] glucose was first applied to *C. roseus* seedlings grown in Murashige and Skoog's (MS) medium. In a time course, the amount and position of ^{13}C incorporation into the metabolites were analyzed by proton nuclear magnetic resonance (^{1}H NMR) and ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) NMR. The results show that the fed ^{13}C-isotope was efficiently incorporated into and recycled in metabolism of the intact *C. roseus* plant. The *C. roseus* plants seem to be a good system for metabolic flux analysis.

Keywords: ^{13}C-isotope labeling, *Catharanthus roseus*, intact plant, metabolic fluxes, NMR, HSQC

1. Introduction

Metabolic flux analysis (MFA) aims at the quantitation of the carbon flow through a metabolic network by measuring the enrichment and position of labels in the various measurable metabolites after feeding a labeled precursor *in vivo* or *in vitro*. Though now common in microorganisms, in plants, with their complex secondary metabolic pathways; MFA is so far mostly focused on primary metabolism. In fact, each metabolic flux reflects the function and

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performance of a specific pathway in a plant's development and its interaction with the environment, for example, defense against herbivores or microorganisms [1]. Consequently, metabolic fluxes represent the fourth dimension of a living organism. There are three dimensions of space, which form the phenotype, but the dynamics, the fluxes, represent life. Flux analysis based on ^{13}C-isotope-labeling experiments (^{13}CLE) has been established as an effective method for determining the flux distribution through the compartmented pathways of primary metabolism in plant cells. The ^{13}C isotope is not radioactive, thus convenient to be used to label the metabolites in the pathways. Usually, a specifically ^{13}C-isotope-labeled substrate, for example, [1-^{13}C] glucose, is used in a CLE study. After feeding, this labeled material is distributed over the various metabolic pathways. At various time points, the distribution of the label over the various measurable metabolites is measured by using different NMR or MS instruments [2, 3]. By NMR, the position of the label as well as the enrichment on every position in a molecule can be measured. By MS, the overall enrichment of a molecule can be determined, whereas the position can only to some extent be determined by analysis of the fragments.

There are two strategies for ^{13}C MFA: one is dynamic-labeling strategy (time course experiments), and the other is steady-state-labeling strategy. The dynamic-labeling strategy has an advantage in studying small partial networks and it is particularly effective for the analysis of secondary metabolism [1]. In this approach, a specific labeled advanced precursor of a pathway is pulse fed, and after a given time the incorporation is measured in the products of the pathway involved. In a steady-state-labeling strategy, the organisms are permanently growing in a medium containing a very early substrate for primary metabolism (e.g., a labeled sugar of pyruvate) and the diffusion of the label through all pathways is monitored by measuring the incorporation and position of the label in all measurable metabolites. This approach is usually utilized in studies of central carbon metabolism. In fact, the limiting factor in flux analyses in plants is the detection limits for the various metabolites, as the level of primary metabolites in plants is many folds higher than of secondary metabolites, the dynamic range analytical tools hamper the analysis of minor compounds. Therefore, often specific and selective extraction methods are used for the dynamic approach, whereas for the steady-state approach one uses the more general metabolomics analytical protocols.

In *Catharanthus roseus*, ^{13}C label has been applied for both pathway elucidation and system-wide flux quantification. By feeding [1-^{13}C] glucose to a cell culture of *C. roseus* with ^{13}C NMR spectroscopy [4], the biosynthetic pathway of secologanin was elucidated from which secologanin was found to originate from the triose-phosphate pathway. Salicylic acid biosynthesis was uncovered in *C. roseus* cell cultures by a retrobiosynthetic study based on ^{13}C feeding experiments [5]. Flux quantification in central carbon metabolism of *C. roseus* hairy roots by ^{13}C-labeling-based flux analysis, and quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in cell cultures of *C. roseus* were also reported [6, 7]. Antonio et al. (2013) used plant cell suspension cultures of *C. roseus* to study the changes in fluxes after elicitation with jasmonate. The incorporation of fully labeled pyruvate was measured by gas chromatography-mass spectrometry (GC-MS) and ultra performance liquid chromatography (UPLC)-MS. The elicitation was found to disturb various metabolic pathways, as could be concluded from the differences in the incorporation of labels. Up to now, ^{13}CLE-based MFA has not been implemented on intact *C. roseus* plants. The major reason is that intact plants are a more complex metabolic

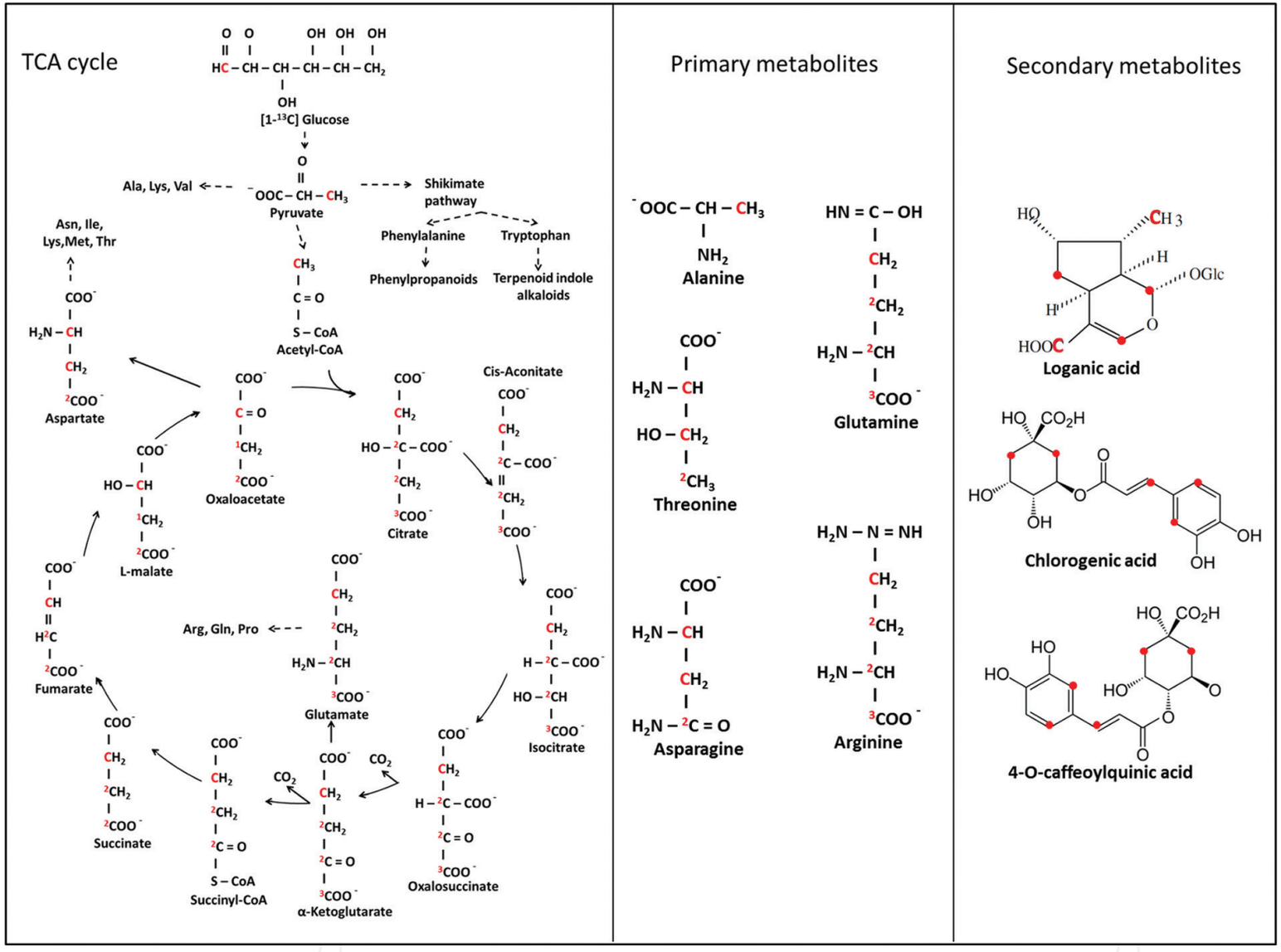


Figure 1. Label pattern of TCA cycle, amino acids, loganic acids, chlorogenic acid, and 4-*O*-caffeoyl quinic acid based on [1-^{13}C] glucose feeding. Carbons and filled cycles in red indicate ^{13}C-enriched positions.

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system than cell cultures or hairy root cultures which only have a few different cell types. For example, previous research showed that some valuable TIAs (e.g., vindoline, vinblastine, and vincristine) can only be produced in leaves of *C. roseus*, not in cell cultures and hairy roots, due to the tissue- and cell-specific organization of terpenoid indole alkanoid (TIA) biosynthesis. So, a more detailed understanding of carbon flux distribution in the complex metabolic networks of intact *C. roseus* plants is a prerequisite for progress in metabolic engineering of TIA production in order to feed the rapidly growing market demands of these important TIAs.

In this study, the fate of [1-^{13}C] glucose fed to the intact *C. roseus* plants via the root system was analyzed in considerable detail. Labeling patterns of targeted metabolites were deduced from previous publications [4, 5, 8] (**Figure 1**), and confirmed by the current experiment. By tracing the label in some of the detected primary and secondary metabolites through a time course, we have information about the ^{13}C incorporation status of these compounds and thus in the metabolic fluxes in the *C. roseus* plant metabolism and the channeling of carbon into the MIA biosynthesis. Also, the metabolic changes after elicitation were measured in this model.

2. Materials and methods

2.1. Plant material and in vitro culture

C. roseus seeds (Pacifica Cherry Red cultivar) were purchased from PanAmerican Seed Company (USA). The seeds were surface sterilized in 75% of ethanol (v/v) and 10% of NaClO (v/v) for 1 and 15 min (respectively), and subsequently washed three times with sterile distilled water. Sterilized seeds were germinated on solid MS [9] basal medium with 1% non-labeled glucose. Finally, 54 seedlings were obtained, subcultured every 2 weeks in the same MS solid medium, and used before flowering for the labeling experiments. After 10 weeks, 19 plants as control were transferred to glass tubes and reared (each) with 5 ml of 10 g/l non-labeled glucose solution, whereas the other 35 plants were placed in separate glass tubes containing 5 ml of 10 g/l [1-^{13}C] glucose solution. The plant cultures were grown in a climate chamber under a 16-h light and 8-h dark photoperiod at 25 ± 2°C.

2.2. Chemicals

Murashige & Skoog medium (including vitamins) and gelrite (strength: 550–850 g/cm^2) were purchased from Duchefa Biochemie. D (+)-Glucose (>99.0%) was obtained from Fluka Chemie (Buchs, Germany), whereas [1-^{13}C]-D-glucose (>99.0%, with > 99% atom 1-^{13}C) was from Campro Scientific (Veenendaal, The Netherlands). Jasmonic acid (JA) was from Sigma-Aldrich Chemie (Steinheim, Germany).

2.3. Jasmonic acid elicitation

A stock solution of 10 mg/ml of JA 40% EtOH was prepared, filter sterilized, and used for elicitation. After 5 days of submerging the plant roots with 5 ml of [1-^{13}C] glucose solution (1%, w/v), 11 μl of the JA stock solution was aseptically spiked into each tube. The control samples received only the same volume of 40% EtOH. The plants were harvested at 0, 6, 24, and 72 h

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after treatment; young leaves, old leaves, stems, and roots of *C. roseus* plants were harvested separately, immediately frozen and ground in liquid nitrogen into powder and freeze-dried for 72 h before NMR extraction (five replicates per sample).

2.4. NMR analysis

^{1}H NMR spectra were recorded in CH_3OH-d_4 using a Bruker DMX 600-MHz spectrometer, while the coherence-order selective gradient heteronuclear single quantum coherence (HSQC) spectra were recorded in CH_3OH-d_4 using a Bruker AV 500-MHz spectrometer. HSQC spectra were recorded for a data matrix of 256 × 2048 points covering 30182.7 × 7812.5 Hz with 64 scans for each increment [10]. INEPT transfer delays were optimized for a heteronuclear coupling of 145 Hz and a relaxation delay of 1.5 s was applied. Data were linear predicted in F_1 to 512 × 2048 using 32 coefficients and then zero-filled to 2048 × 2048 points prior to echo-anti-echo-type two-dimensional (2D) Fourier transformation and a sine bell-shaped window function shifted by $\pi/2$ in both dimensions was applied. One-dimensional (1D) projection along the F_1-axis was extracted using the build-in positive projection tool of Topspin (version 2.1, Bruker Biospin).

The signal intensity of carbons at certain positions of a given metabolite was obtained from peak height in the ^{13}C-dimension spectra abstracted from the 2D HSQC spectra. The signal height of CH_3OH-d_4 was selected as the standard and set as 1 in both labeled and non-labeled samples. The other signals were normalized and expressed relative to this signal. ^{13}C signal intensity ratio was calculated by comparison of normalized ^{13}C signal heights between ^{13}C-labeled and non-labeled samples.

3. Results and discussion

3.1. Comparison of growth and metabolism of *C. roseus* plants grown in the solid MS medium versus soil

Two batches of *C. roseus* seeds (each containing of 10 seeds) were germinated, one batch in soil and another one in solid MS medium with glucose. They were kept in the same condition of light and temperature. The height, the size, and the leaf pairs of the plants from seedling until flowering were monitored and recorded regularly to determine the growth state of plants.

After 10–12 days, seeds in both batches germinated and produced their first pair of leaves. In the first 3 weeks after germination, there were no significant differences of height, leaf pairs, and leaf size between plantlets grown in MS medium and in the soil (**Figure 2**). However, in the following days, the plantlets in MS medium provided one more pair of leaves than those in soil did, but the leaf size was much smaller than that of plantlets grown in the soil (**Figure 2A** and **B**). Moreover, the soil plantlets grew higher than those grown in MS medium (**Figure 2C**). Plantlets in MS medium entered flowering time around 100 days after sowing, whereas those in soil flowered at 75 days. The plantlets grown in soil had a higher growth rate and a larger biomass than those grown in MS medium.

Metabolic differences between the plants grown in soil and MS medium were observed by ^{1}H NMR (**Figure 3**). The ^{1}H NMR spectra showed that qualitatively metabolites of plants grown

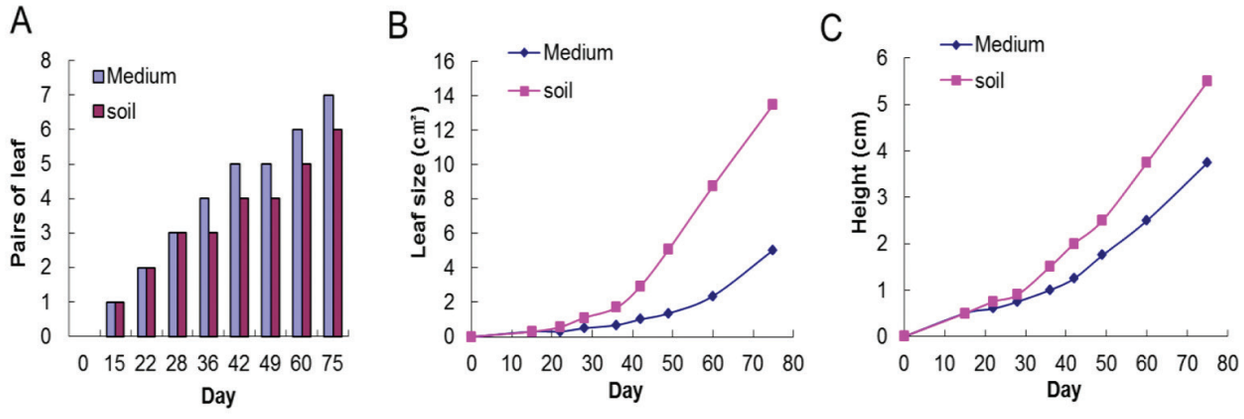


Figure 2. Comparison of the number of leaf pairs (A), leaf size (B), and height (C) of *Catharanthus roseus* plants grown in MS medium and soil during the development stage.

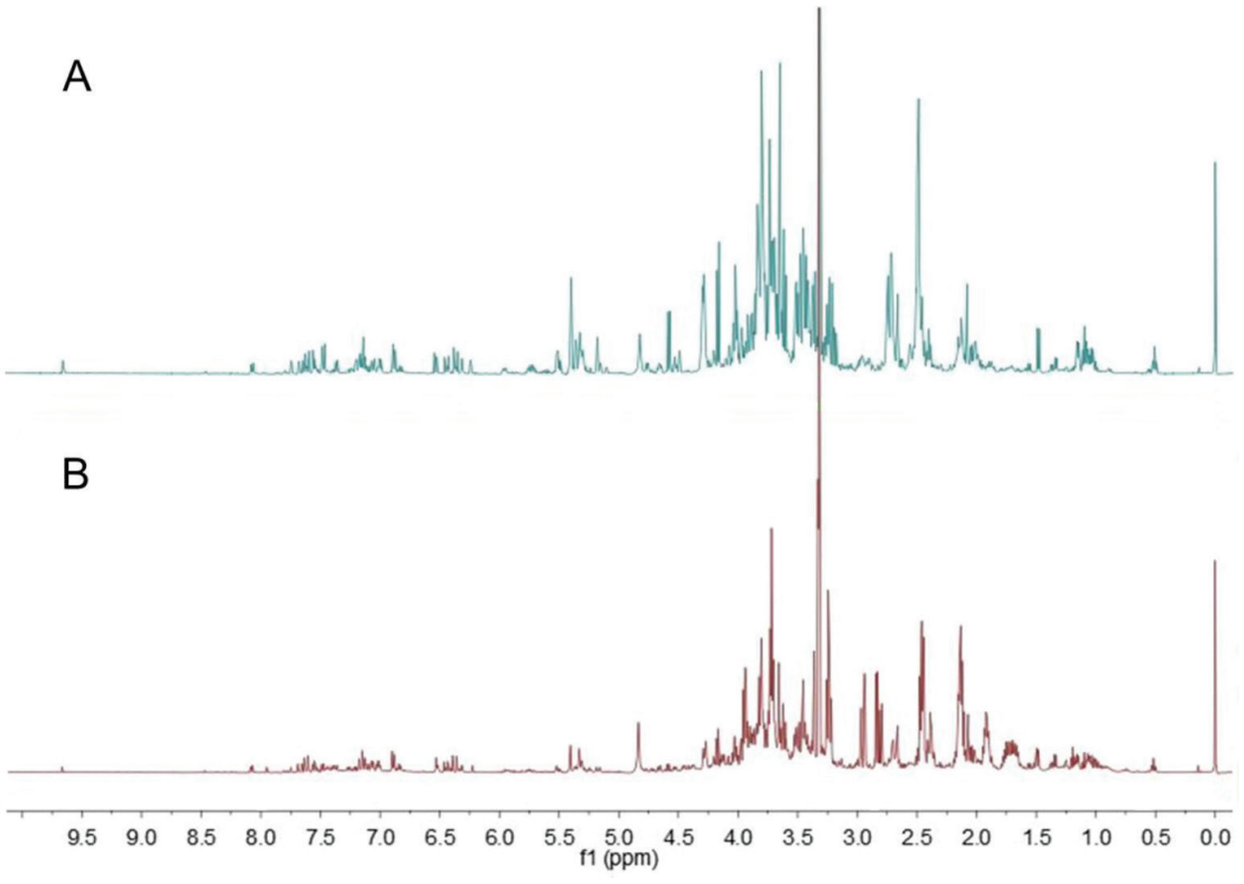


Figure 3. ^{1}H NMR spectrum of the crude extracts of *Catharanthus roseus* plants grown in soil (A) and MS medium (B).

in soil or MS medium were similar, but the levels varied (**Table 1**). Plants grown in soil produced higher levels of organic acids and sugars (malate, fumaric acid, glucose, and sucrose) than those grown in MS medium, indicating a low function/reduced level of carbon fixation in the leaves of the MS-grown plants. Also, secondary metabolites (such as secologanin, vindoline, quercetin, and kaempherol) were found in higher levels in soil-grown plants than the

Compounds	Signal intensity		Signal intensity ratio (S/M)
	Soil (S)	Medium (M)	
Vindoline	0.42	0.22	1.91
Threonine	0.27	0.34	0.79
Lactic acid	0.14	0.22	0.64
Alanine	0.54	0.51	1.06
Arginine	0.82	4.27	0.19
Quinic acid	0.21	0.30	0.70
Glutamate	1.25	2.14	0.58
Glutamine	2.94	7.08	0.42
Malate	5.96	1.82	3.27
Asparagine	0.03	0.45	0.07
β-glucose	0.67	0.13	5.15
α-glucose	0.42	0.08	5.25
Sucrose	1.34	0.39	3.44
Chlorogenic acid	0.12	0.11	1.09
Fumaric acid	0.10	0.07	1.43
Catharanthine	0.20	0.17	1.18
4-*O*-Caffeoyl quinic acid	0.15	0.12	1.25
Quercetin-3-*O*-glucoside	0.10	0.04	2.50
Kaempherol	0.13	0.08	1.63
Secologanin	0.13	0.03	4.33

Table 1. Comparison of metabolite levels in *Catharanthus roseus* plants grown in soil and MS medium, based on ^{1}H NMR.

plants grown in MS medium. On the other hand, plants cultured in MS medium displayed significantly higher levels of arginine, glutamine, and asparagine but relatively low level of glucose and sucrose. The levels of threonine, glutamate, quinic acid, and lactic acid were also higher in plants grown in MS medium than those in soil.

Some groups of metabolites have a close correlation with plant growth and biomass, such as the tricarboxylic acid cycle (TCA) intermediates succinate, citrate, or malate, as well as amino acids [11]. Both glutamine and asparagine are the major compounds for nitrogen fixing, transport, and storage in plants [12]. With the much more abundant nitrogen source in the medium than in the soil, the high levels of the amino acids in the medium grown plants could be explained. Meyer et al. [11] reported a negative correlation to the plant biomass with glutamine, which is in line with our findings. Sucrose starvation may lead to the presence of a large excess of asparagine in plant cells [13]. In the present study, the plants cultured on solid

MS medium require an aseptic jar with a cap, which limits the space to grow, and also affects air exchange, CO_2 availability, and accumulation of volatiles in the head space if compared with plants grown in soil. Despite the uptake of carbohydrates from the medium through the roots, the growth was less than the plants grown in soil which are dependent of carbon fixation by leaves. The limited availability of CO_2 in the sterile closed containers may thus be a reason for lower biomass production.

3.2. [1-^{13}C] glucose feeding experiment and JA elicitation on *C. roseus* plantlets

Samples from different organs (upper and lower leaves, stems, and roots) were measured by proton and carbon NMR. After feeding the plants with [1-^{13}C] glucose for 5 days, the incorporation of ^{13}C label was found in some primary and secondary metabolites detected in all organs of the *C. roseus* plantlets. ^{13}C signals of some primary and secondary metabolites were assigned based on the "in-house" database and some references [14, 15], and confirmed in Chapter 4. Totally, 14 amino acids, nine organic acids, two carbohydrates, six phenylpropanoids, five TIAs, two terpenoids, and three other compounds were identified. Among them, only the metabolites from which characteristic signals were clearly visible and non-overlapping in both ^{1}H and ^{13}C NMR spectra were quantified (**Figure 4**). Those include some primary metabolites such as amino acids (threonine, alanine, asparagine, aspartate, glutamine, glutamate, and arginine) and malic acid (**Figure 4A**), as well as some secondary metabolites such as phenylpropanoids (chlorogenic acid and 4-*O*-caffeoyl quinic acid), terpenoids (loganic acid and secologanin), and TIA (vindoline) (**Figure 4C**).

Figure 5 shows the ^{13}C-dimension HSQC spectra and ^{1}H NMR spectra of the non-labeled sample and the ^{13}C-enriched sample determined in CH_3OH-d_4. As expected, the superimposed ^{1}H-NMR spectra of leaves (**Figure 5**) and stems (data not shown) did not show any significant difference in proton signal intensity of the metabolites for the control and the ^{13}C-enriched sample. Production of these sugars caused a decrease in the levels of glucose and sucrose in roots but did not affect the metabolite levels in other organs. Except this, there was no significant change in metabolite levels of the plants fed with labeled- and non-labeled glucose solution (**Table 2**). This information is necessary to confirm that the ^{13}C signals of the spectra of enriched samples are due to incorporation of label, and not because of higher levels of production of the metabolites. Superimposed ^{13}C-dimension HSQC spectrum showed that the enriched sample had a much higher intensity of ^{13}C signals than the non-labeled one. The results indicate that the [1-^{13}C] glucose fed *C. roseus* plants grew normally, and incorporated the labeled sugar into its metabolic network. Previous work with *Arabidopsis* supports that ^{13}C feeding does not in itself distort the fluxes through the metabolic network in a plant [16].

3.3. ^{13}C incorporation into primary and secondary metabolites

The signals in the HSQC spectra of the enriched samples were identified (**Figure 4**). The carbon position of ^{13}C incorporation into a metabolite was investigated by calculating ^{13}C signal intensity ratios between the same carbons of the metabolite in labeled and non-labeled samples (**Table 3**).

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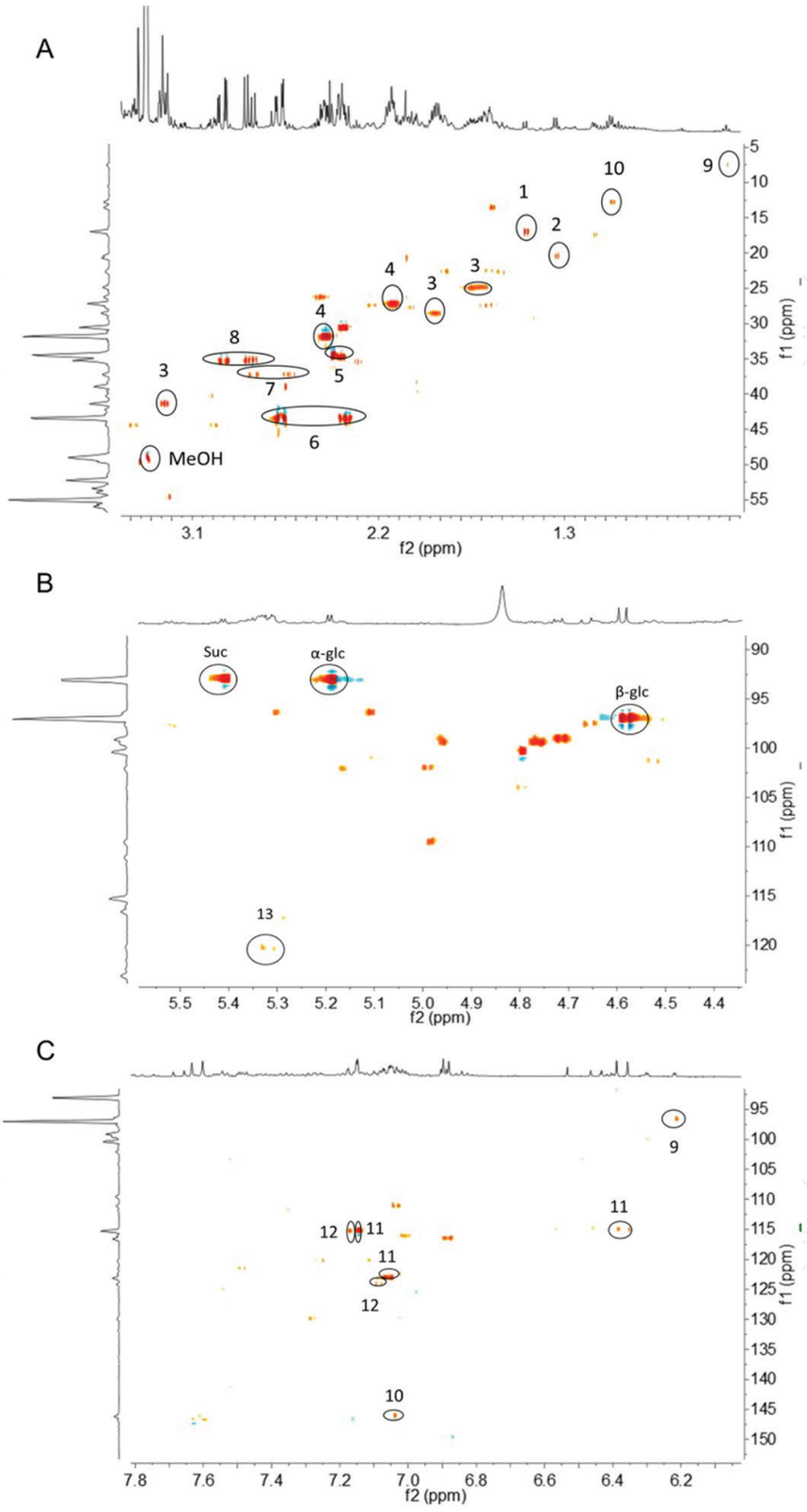


Figure 4. 2-D [^{13}C, ^{1}H] HSQC spectrum of CH_3OH-d_4 extract of *Catharanthus roseus* leaves. A, spectrum region displaying amino acid resonances; B, spectrum region displaying sugar resonances; C, spectrum region displaying aromatic resonances. 1, alanine; 2, threonine; 3, arginine; 4, glutamine; 5, glutamate; 6, malate; 7, aspartate; 8, asparagine; 9, vindoline; 10, loganic acid; 11, chlorogenic acid; 12, 4-*O*-caffeoyl quinic acid.

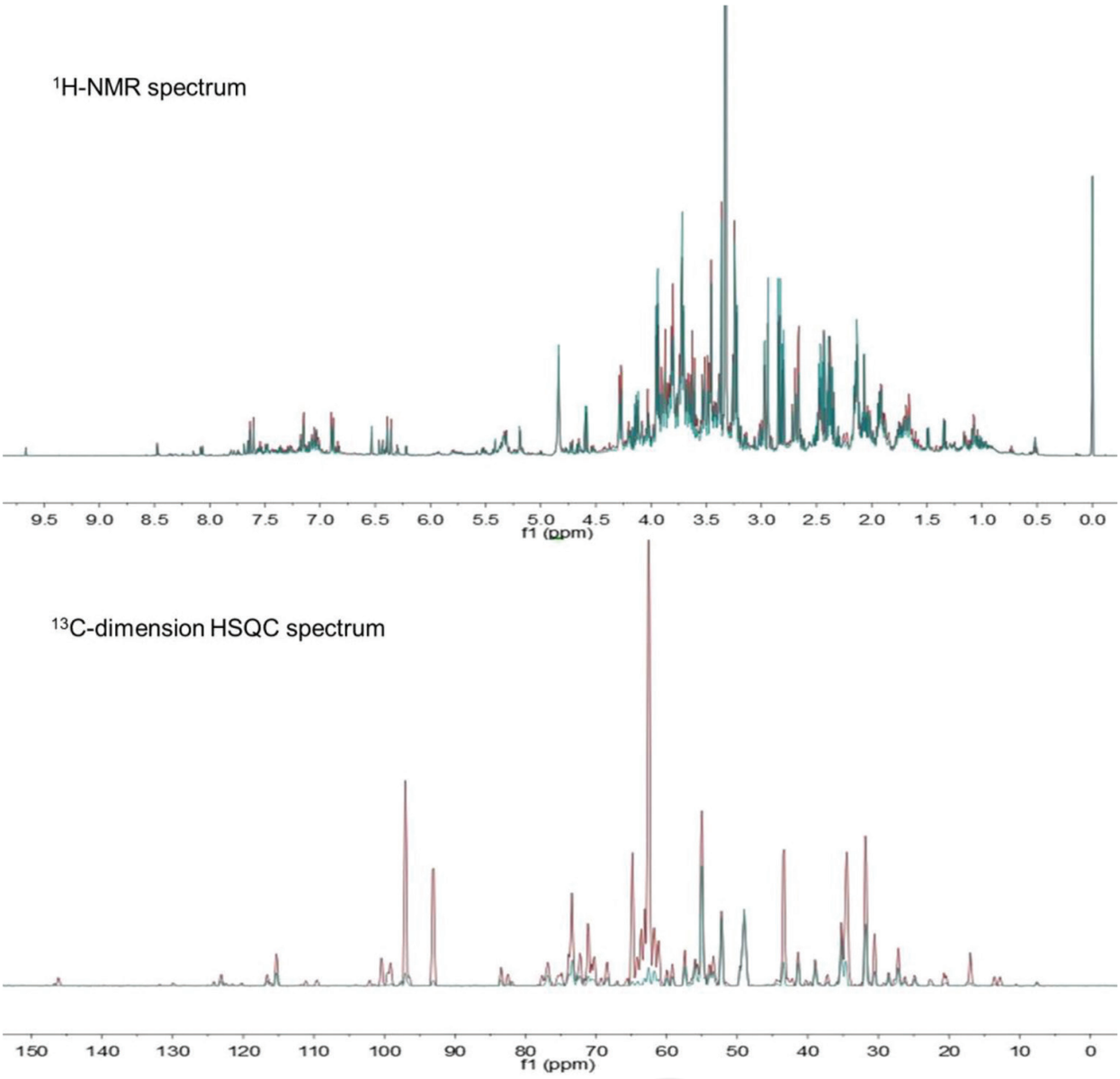


Figure 5. Superimposed ^{1}H NMR spectra and ^{13}C-dimension HSQC spectrum of labeled and non-labeled *Catharanthus roseus* plants. Spectra in green were non-labeled plants sample, spectra in red were ^{13}C-labeled plants sample.

Among amino acids, the signals corresponding to C at δ 16.98, C-3 of alanine, exhibited a high ^{13}C relative enrichment ratio. Glycolysis introduces the C-1 or C-6 of glucose into alanine C-3 [8]. Carbon signals at δ 20.47 of threonine and at δ 37.21 of aspartate also showed a relatively high labeling. The carbons of arginine and asparagine were apparently less labeled.

Glutamate (C-3 at δ 27.74, C-4 at δ 34.44, and C-5 at δ 55.67) and glutamine (C-3 at δ 27.11, C-4 at δ 31.83, and C-5 at δ 55.02) showed clear high ^{13}C incorporation. The relative enrichment ratios of C-3 and C-2 of glutamine were lower than that of C-4, which indicate the entry of a diluting flux of C4 compounds into the TCA cycle [17]. For glutamate, however, C-4 had a lower relative enrichment ratio than C-3 and C-2. Non-symmetrical enrichment ratios of

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Compounds	Ratio of metabolite levels in labeled and non-labeled samples, (L0/C0), based on ^{1}H NMR			
	Upper leaf	Lower leaf	Stem	Root
β-Glucose	1.04	0.76	1.01	0.42
α-Glucose	0.97	0.67	1.03	0.39
Sucrose	0.95	0.62	0.82	0.65
Threonine	1.25	0.97	0.88	1.05
Alanine	1.12	0.92	0.96	0.82
Arginine	1.16	0.75	0.82	0.74
Glutamate	1.13	0.95	0.89	0.70
Glutamine	0.78	0.60	0.89	0.81
Aspartate	1.28	0.83	0.87	1.22
Asparagine	0.88	0.85	0.77	0.93
Malic acid	1.63	0.85	0.98	0.95
Fumaric acid	0.90	0.77	0.67	nd
Vindoline	1.07	0.89	1.20	nd
Chlorogenic acid	1.54	0.85	0.75	nd
4-*O*-caffeoyl quinic acid	1.16	0.90	1.00	nd
Quercetin	1.50	1.33	nd	nd
Kaempferol	1.30	1.00	nd	nd
Secologanin	1.50	1.00	nd	nd
Loganic acid	1.10	1.00	0.94	0.89

nd, not detected.

Table 2. Comparison of metabolite levels in different organs between labeled and non-labeled *Catharanthus roseus* plants.

C-2 and C-3 imply that there might be a form of channeling that converts oxoglutarate C-4 to oxaloacetate C-2 or C-3 [18].

In plant cells, the labeling of amino acids alanine, glutamate, and aspartate is found to reflect that of the corresponding α-oxoacids: pyruvate, α-oxoglutarate, and oxaloacetate, respectively [19]. The organic acid malate showed a sixfold increased intensity for the carbon signal at δ 43.40.

Besides primary metabolites, secondary metabolites also exhibited clear ^{13}C incorporation. Two phenylpropanoids, chlorogenic acid and its isomer 4-*O*-caffeoyl quinic acid, have an increased ^{13}C intensity of C-6. Incorporation of ^{13}C could be observed for C-3 and C-10 of loganic acid. These results are in agreement with the prediction shown in **Figure 1**. The signal

Compound	Chemical Shift		Peak height		Relative intensity to CH_3OH-d_4		Relative enrichment ratio (*X*/*Y*)
	H	C	Control	Labeled	Control (*Y*)	Labeled (*X*)	
Glucose	4.58	97.04	3.2E+07	1.3E+08	0.17	2.89	17.31
Alanine	1.49	16.98	8.0E+06	2.1E+07	0.04	0.47	11.26
Glutamine	2.05	27.11	4.6E+07	3.9E+07	0.23	0.88	3.75
	2.38	31.83	1.6E+08	1.7E+08	0.80	3.72	4.63
	3.71	55.02	3.0E+08	2.3E+08	1.56	5.12	3.29
Glutamate	2.14	27.74	2.1E+06	7.0E+06	0.01	0.16	14.81
	2.46	34.44	6.3E+07	1.5E+08	0.32	3.36	10.45
	3.72	55.67	9.8E+06	3.9E+07	0.05	0.86	17.14
Arginine	1.72	24.9	1.7E+07	6.7E+06	0.09	0.15	1.77
	1.92	28.53	2.1E+07	8.1E+06	0.11	0.18	1.68
	3.24	41.38	5.9E+07	2.1E+07	0.30	0.47	1.56
Aspartate	2.64	37.21	1.0E+07	7.0E+06	0.05	0.16	3.05
Asparagine	2.83,2.96	35.23	1.2E+08	4.0E+07	0.62	0.90	1.44
	3.96	52.21	1.7E+08	4.7E+07	0.89	1.05	1.19
Threonine	1.34	20.47	8.8E+06	6.2E+06	0.05	0.14	3.08
Malate	2.35,2.72	43.4	6.0E+07	8.5E+07	0.31	1.91	6.21
Chlorogenic acid	7.07	123.12	1.2E+07	7.2E+06	0.06	0.16	2.53
4-*O*-Caffeoyl quinic acid	7.09	124.16	1.7E+06	2.5E+06	0.01	0.06	6.63
Loganic acid	1.07	12.69	2.5E+06	5.6E+06	0.01	0.13	9.96
	7.03	146.1	6.5E+05	5.2E+06	0.00	0.12	34.49
Vindoline	0.49	7.43	6.0E+06	2.4E+06	0.03	0.05	1.76

Table 3. The chemical shifts, peak height, and relative enrichment ratio of the same carbon signals in metabolites in labeled and non-labeled *Catharanthus roseus* plants.

corresponding to C-18 of vindoline in the labeled sample was twofold higher than in the spectrum of the control.

3.4. ^{13}C incorporation in different organs

Based on ^{1}H NMR spectra, relative levels of primary and secondary metabolites in different organs were calculated by normalizing the integral of signal peaks to the internal standard (TSP). **Table 4** showed that leaves, especially upper leaves, contained higher levels of amino acids, phenylpropanoids, iridoids, and vindoline, than stems and roots. In roots, phenylpropanoids and vindoline, which are biosynthesis dependent on chloroplasts, were not detected,

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Compounds	Relative levels of metabolites			
	Upper leaf	Lower leaf	Stem	Root
β-Glucose	0.46	0.89	0.95	1.36
α-Glucose	0.34	0.56	0.62	0.83
Sucrose	0.20	0.33	0.53	0.81
Threonine	0.45	0.44	0.45	0.17
Alanine	0.45	0.46	0.29	0.17
Arginine	3.19	5.93	5.09	1.07
Glutamate	3.05	2.15	2.92	0.91
Glutamine	3.72	1.74	7.91	1.38
Aspartate	1.07	0.93	0.41	0.22
Asparagine	2.82	1.25	1.84	0.44
Malic acid	0.32	0.40	0.22	0.09
Fumaric acid	0.11	0.11	0.02	nd
Vindoline	0.28	0.09	0.05	nd
Chlorogenic acid	0.25	0.37	0.03	nd
4-*O*-caffeoyl quinic acid	0.17	0.15	0.04	nd
Quercetin	0.05	0.05	0.03	0.03
Kaempferol	0.09	0.10	0.02	nd
Secologanin	0.02	0.02	0.0006	0.0002
Loganic acid	0.07	0.14	0.11	0.05

Table 4. Relative level of metabolites in different organs of *Catharanthus roseus* based on ^{1}H NMR spectra.

whereas iridoids displayed a much lower level in roots while glucose and sucrose had relatively higher levels than in other organs.

The incorporation of ^{13}C in different organs (upper leaf, lower leaf, stem, and root) was also investigated by comparison of relative enrichment ratios in order to have a clue about the accumulation of label in different organs and its connection with transport and compartmentation of the pathways in the plants (**Table 5**). From the ^{13}C dimension of HSQC spectra of all organs, ^{13}C signals of labeled samples showed an apparently higher intensity in the amino acid and sugar areas than those of non-labeled ones (**Figure 6**), which indicated that ^{13}C-isotope was efficiently incorporated into the primary metabolism of intact *C. roseus* plants via the roots. Glucose had higher ^{13}C intensity ratio in lower leaves and roots but relatively low in upper leaves and stems, thus showing a time-dependent distribution through the plant. Glutamate and aspartate, directly derived from α-ketoglutarate and oxaloacetate of the TCA cycle, showed clear ^{13}C enrichment in all organs. So did malate, one of the bricks in the TCA cycle. Meanwhile, glutamate, aspartate, and malate all displayed

Compounds	^{13}C Chemical shift	Relative enrichment ratio (labeled:control)			
		Upper leaf	Lower leaf	Stem	Root
Alanine	16.98	9.76	32.38	118.89	84.86
Threonine	20.47	3.55	1.06	1.39	18.91
Arginine	24.9	1.89	1.12	1.67	2.99
	28.53	1.58	1.07	1.17	1.15
	41.38	1.52	0.91	0.91	1.66
Glutamine	27.11	2.80	2.34	1.89	6.38
	31.83	3.21	4.03	3.09	6.70
	55.02	2.34	1.38	2.01	8.43
Glutamate	27.74	11.79	5.39	3.36	20.65
	34.44	7.21	3.37	4.63	16.75
	55.67	15.49	2.72	5.11	25.06
Asparagine	35.23	1.21	0.75	1.09	5.67
	52.21	1.17	0.95	1.23	4.18
Aspartate	37.21	2.25	3.40	4.07	36.29
Malate	43.4	4.67	4.41	7.82	26.51
β-glc	97.04	30.96	55.55	15.01	32.79
Vindoline	7.43	2.96	nd	nd	nd
Loganic acid	12.69	7.66	3.75	4.26	23.62
	146.1	27.96	13.77	6.59	24.79
Chlorogenic acid	123.12	2.88	1.42	nd	nd
	146.8	93.69	nd	nd	nd
4-*O*-Caffeoyl quinic acid	124.16	10.35	nd	nd	nd

nd, not detected.

Table 5. Relative enrichment ratios of the carbons of some metabolites in different organs of *Catharanthus roseus* plants fed with [1-^{13}C] glucose.

the highest ^{13}C intensity ratio in roots. These results indicate that ^{13}C was efficiently incorporated and recycled in the primary metabolism of intact plants. Upper leaves had higher levels and higher relative enrichment ratios of glutamate and malate compared with lower leaves, reflecting the faster rate of TCA cycle in the upper parts for plants growing. The glutamate-derived amino acids glutamine and arginine displayed a different pattern of ^{13}C incorporation. Glutamine showed ^{13}C incorporation in all organs with the highest intensity ratio in roots and the lowest in stems, whereas arginine showed low ^{13}C incorporation in all organs, implying a low flux in its biosynthetic pathway and low usage for other pathways. The ^{13}C incorporation of aspartate-derived amino acids asparagine and threonine was also

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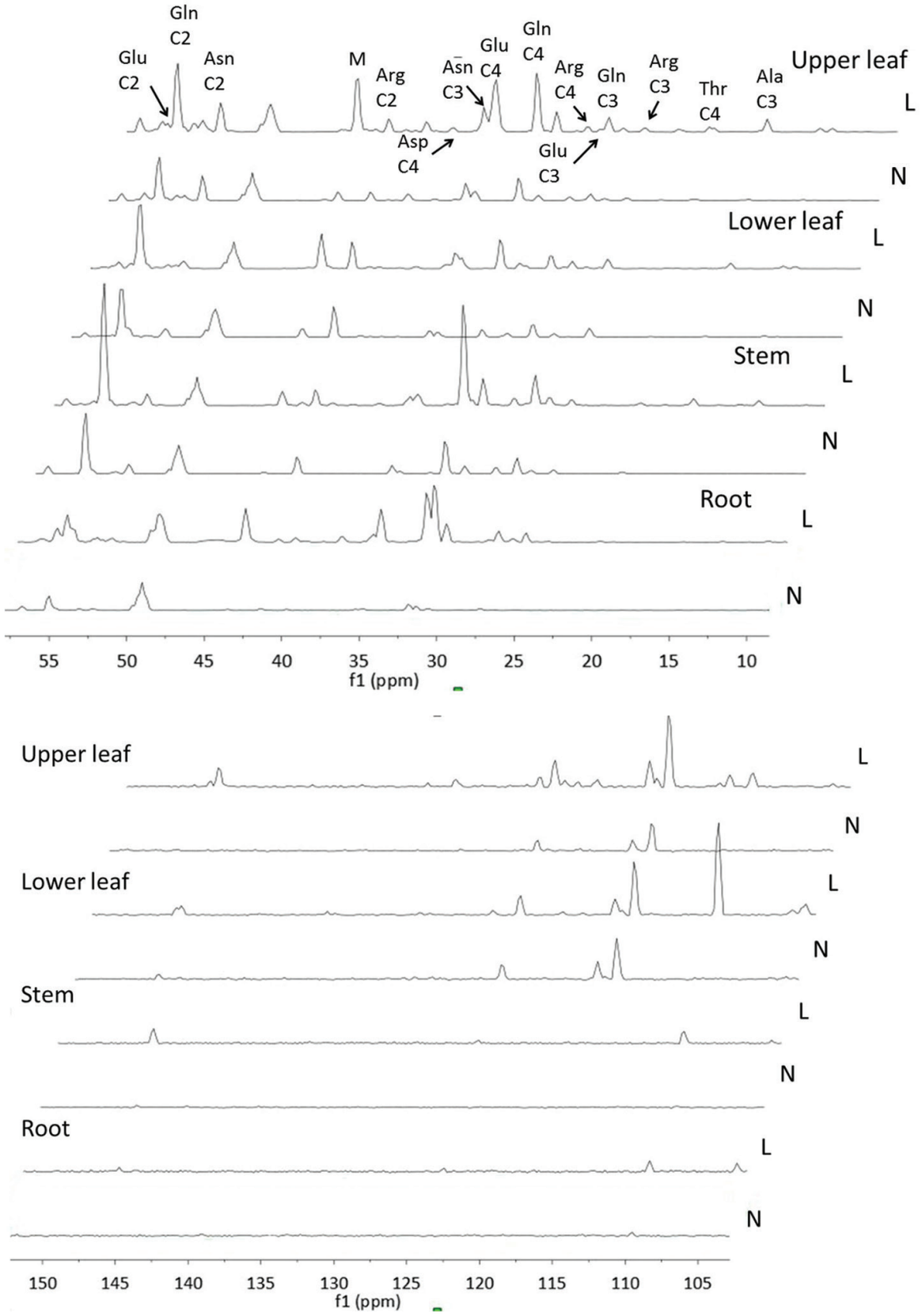


Figure 6. [13]C dimension of HSQC spectra of amino acids (δ 10–55 ppm) and secondary metabolites (δ 105–150 ppm) in different organs of *Catharanthus roseus* after feeding [1-[13]C] glucose. L, labeled samples; N, non-labeled samples; M, malate.

different. Threonine had relatively high ^{13}C incorporation in upper leaves and roots, but relatively low in lower leaves and stems, indicating a high turnover in the upper leaves. Asparagine, except for roots, displayed low ^{13}C incorporation in the other organs. Pyruvate-derived alanine exhibited the lowest relative enrichment ratio in upper leaves, while the highest was found in stems and roots.

Based on the ^{13}C dimension of HSQC spectra, leaves had more ^{13}C signals in the area of >δ 100 ppm than stems and roots (**Figure 6**), even after feeding [1-^{13}C] glucose. Upper leaves had relatively high ^{13}C incorporation for vindoline, chlorogenic acid, and 4-*O*-caffeoyl quinic acid while lower leaves only showed ^{13}C incorporation into chlorogenic acid. The levels of these phenylpropanoids in lower leaves were almost as much as in upper leaves. This means that all three compounds have a high biosynthetic rate in the upper leaves, whereas in the lower leaves the biosynthesis of chlorogenic acid is more active than of the other two compounds. In plants, lower leaves, with older age, are entering the senescence phase, among others reflected by a lower rate of both primary and secondary metabolism. Studies in previous chapters have shown that vindoline levels show an age-related decrease, consistent with previously reported results [20].

In stems and roots, no ^{13}C signals of vindoline, chlorogenic acid, and 4-*O*-caffeoyl quinic acid were detected with or without feeding [1-^{13}C] glucose. Vindoline is not found in roots due to its tissue-specific biosynthesis requiring chloroplasts for one of its biosynthetic steps [20–25]. The ^{13}C signal of loganic acid at δ 12.69 and 146.1 ppm was clearly present and showed a high relative enrichment ratio at the spectra of all organs while that of secologanin at δ 121.53 ppm was only found in the spectra of leaves. It was difficult to calculate the relative enrichment ratio of secologanin due to the signal overlapping. In roots and stems, secologanin was too low for further analysis. The high levels of loganic acid in the roots are in line with a previous study that reported that LAMT activity, which converts loganic acid into loganin (the direct substrate of secologanin), was four to eight times lower in hairy roots than that in the other organs of the plant [22].

3.5. Effect of JA elicitation on ^{13}C fluxes into metabolic pathways

JA was spiked into the labeled glucose solution at the sixth day after submerging the plant roots in the solution. The control plants were also reared in labeled glucose solution but without JA elicitation. Leaves were harvested at 0, 6, 24, and 72 h (6, 7, and 9 d of incubation with the labeled glucose solution) after elicitation and measured by ^{1}H NMR and HSQC.

For control plants, NMR spectra showed that the enrichments of malic acid and of the amino acids alanine, arginine, glutamate, glutamine, aspartate, and asparagine in the leaves were nearly identical at 6 and 9 d of incubation with the labeled glucose solution (**Figure 7**), suggesting the establishment of steady state at 6 d. However, the incorporation of label in glucose and threonine increased continuously within the measured period of 9 days. Besides, loganic acid and chlorogenic acid kept the same enrichments while vindoline and 4-*O*-caffeoyl quinic acid showed an increase of the enrichments within 9 days. Previous study with *C. roseus* hairy roots grown in the light showed that the ^{13}C label was not diluted by CO_2 fixation [7]. In tobacco plants grown on agar containing labeled glu-

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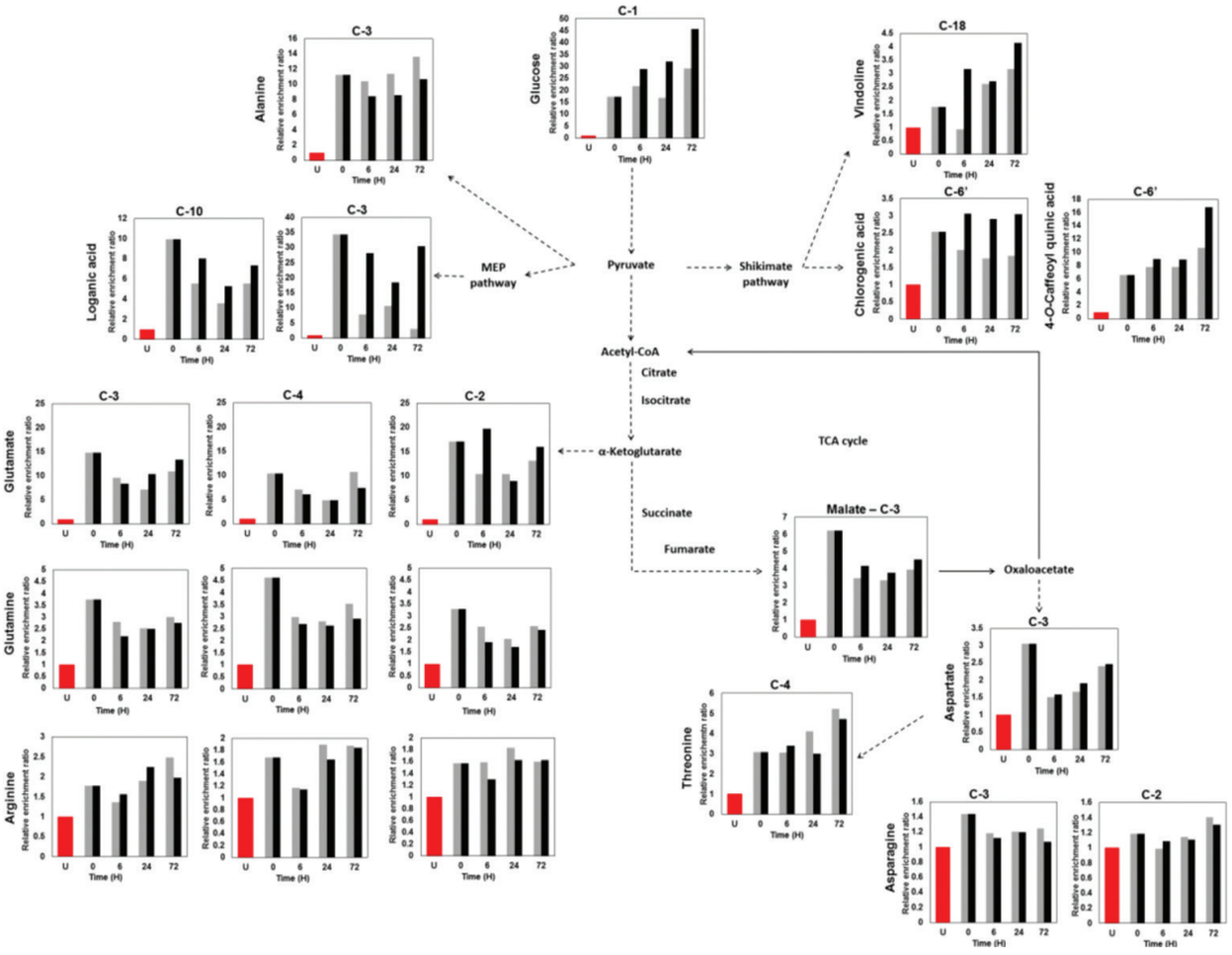


Figure 7. Relative enrichment ratio of primary and secondary metabolites during incubation of *Catharanthus roseus* plants with [1-^{13}C] glucose. Gray bars: JA-elicited samples; black bars: control samples; red bars (U): unlabeled samples (without incubation in [1-^{13}C] glucose solution).

cose, the metabolism was studied on a quantitative basis showing that the labeled glucose was efficiently absorbed via the root system, metabolized, and recycled [26]. Our results indicate that the *C. roseus* plant system can reach a relatively steady isotopic state with plants growing in ^{13}CLE.

JA elicitation had little effect on the level of most metabolites, except glutamate, glutamine, vindoline, and loganic acid. Although JA induced an increase of glutamate and glutamine levels (**Figure 8**), their relative enrichment ratio remained unchanged compared with the controls. At the same time, the enrichment of alanine at C-3 showed an increase without levels changing compared to the controls. Vindoline levels showed an increase and reached the highest level at 72 h (23% higher than the controls) after JA treatment (**Figure 8**). However, the relative enrichment ratio of the C-18 signal of vindoline was lower in JA-elicited samples than in the controls, especially at 6 h (**Figure 7**). The level of loganic acid decreased with time (**Figure 8**), leading to a dramatical decrease of its enrichment at both C-3 and C-10 from 6 to 72 h. The levels of chlorogenic acid and 4-*O*-caffeoyl

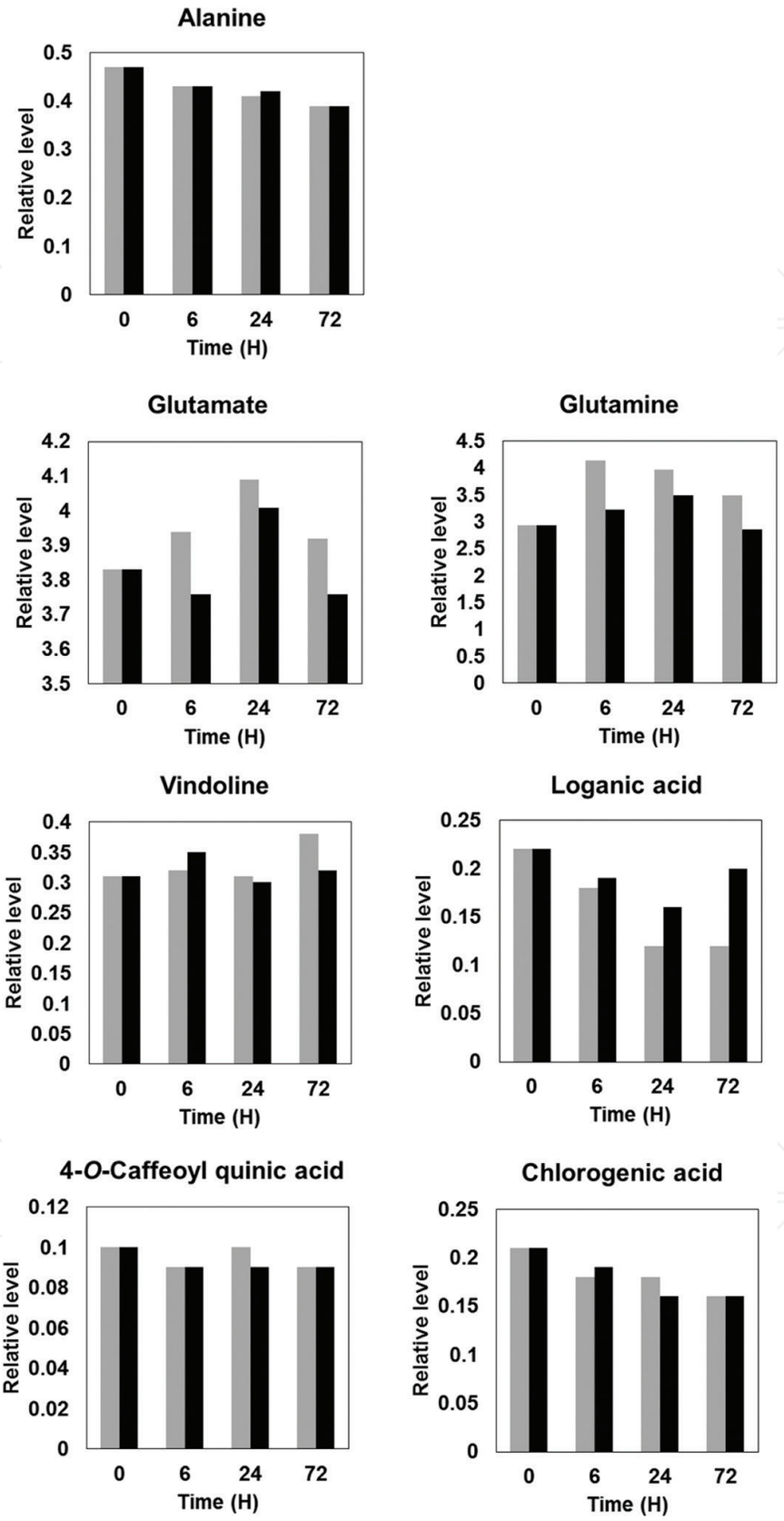


Figure 8. Relative levels of metabolites in *Catharanthus roseus* leaves after JA elicitation. Gray bars: JA-elicited samples; black bars: control samples.

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quinic acid in the time course did not change after JA elicitation (**Figure 8**), but the enrichments were lower than those of the control-labeled samples (**Figure 7**). ^{13}C fluxes to various metabolic pathways, such as glutamate and loganic acid, could be disturbed within 24 h after MeJA treatment [27].

4. Future prospects

Metabolic flux analysis is the quantification of all intracellular fluxes in an organism, which is thus an important cornerstone of metabolic engineering and systems biology. This study reports a comprehensive ^{13}C-labeling-based metabolomics of a plant system. [1-^{13}C] glucose was efficiently absorbed via the root system and recycled in the whole plant of *C. roseus*. The plant system of *C. roseus* could reach a relatively steady isotopic state in ^{13}CLE, which appears to be well qualified to study flux contributions in the biosynthesis of sink metabolites for system biology. Combined with exogenous elicitation, ^{13}C MFA appears to be a good tool to study the crosslink among pathways in the complicated plant metabolic network. The development of a comprehensive flux analysis tool for the plant system of *C. roseus* is expected to be valuable in assessing the metabolic impact of genetic or environmental changes.

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