Metabolomic characteristics of Catharanthus roseus plants in time and space

Qifang, P.

Chapter 7

[13]C-isotope labeling experiments to study metabolism in *Catharanthus roseus*

Qifang Pan[1,2], Natali Rianika Mustafa [1], Robert Verpoorte [1], Young Hae Choi [1]

[1] Natural Products Laboratory, Institute of Biology, Leiden University, Sylviusweg 72, 2333 BE Leiden, The Netherlands

[2] Plant Biotechnology Research Center, SJTU-Cornell Institute of Sustainable Agriculture and Biotechnology, Fudan-SJTU-Nottingham Plant Biotechnology R&D Center, School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, PR China

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, e.g. to protect the plant against herbivore 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 culture medium. In a time course the amount and position of ^{13}C incorporation into the metabolites were analysed by ^{1}H-NMR and ^{1}H-^{13}C 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 fluxes analysis.

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 performance of a specific pathway in a plant's development and its interaction with the environment, e.g. defense against herbivores or microorganisms (Ratcliffe and Shachar-Hill, 2006). 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, e.g. [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 (Szyperski, 1998; Mollney *et al.*, 1999). 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 is 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), 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 (Ratcliffe and Shachar-Hill, 2005). 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 is 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 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 levels of primary metabolites in plants is many fold 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 have been applied for both pathway elucidation and systemwide flux quantification. By feeding [1-^{13}C] glucose to a cell culture of *C. roseus* with ^{13}C NMR spectroscopy (Contin et al, 1998) 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 (Mustafa *et al.*, 2009). 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 (Sriram *et al.* 2007; Schuhr *et al.* 2003). Antonio *et al.* (2013) used plant cell suspension cultures of *C. roseus* to study the the changes in fluxes after elicitation with jasmonate. The incorporation of fully labeled pyruvate was measured by GC-MS and UPLC-MS. The elicitation was found to disturb various metabolic pathways, as could be concluded from the differences in 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 system than cell cultures or hairy root cultures which only have one 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 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 (Lundstrom *et al.*, 2007; Mustafa *et al.*, 2009; Contin *et al.*, 1998) (Fig. 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.

Fig. 1 Label pattern of TCA cycle, amino acids, loganic acids, chlorogenic acid and 4-*O*-caffeoylquinic acid based on [1-^{13}C] glucose feeding. Carbons and filled cycles in red indicate ^{13}C enriched positions in the first round of the TCA cycle. The superscript number in front of carbons indicates the round in which ^{13}C incorporated into the metabolite.

Materials and methods

Plant material and in-vitro culture

Catharanthus 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 min and 15 min (respectively), and subsequently washed three times with sterile distilled water. Sterilized seeds were germinated on solid MS medium (basal culture medium by Murashige and Skoog, 1962) with 1% non-labeled glucose. Finally, 54 seedlings were obtained, sub-cultured 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 ℃.

Chemicals

Murashige & Skoog (MS) medium (including vitamins) and gelrite (strength: 550 ~ 850 g/cm2) 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).

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 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 h, 6 h, 24 h, and 72 h 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 (5 replicates per sample).

NMR analysis

^{1}H-NMR spectra were recorded in CH_3OH-d_4 using a Bruker DMX 600

MHz spectrometer, whilst the coherence order selective gradient heteronuclear single quantum coherence (HSQC) spectra were recorded in CH_3OH-d_4 by 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 (Kim *et al.*, 2010). INEPT transfer delays were optimized for a heteronuclear coupling of 145 Hz and a relaxation delay of 1.5 s was applied. Data was 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 2D Fourier transformation and a sine bell shaped window function shifted by π/2 in both dimensions was applied. 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 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.

Results and discussion

Comparison of growth and metabolism of C. roseus plants grown in the solid culture 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 (Fig. 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 (Fig. 2A and 2B). Moreover, the soil plantlets grew higher than those grown in MS medium (Fig. 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.

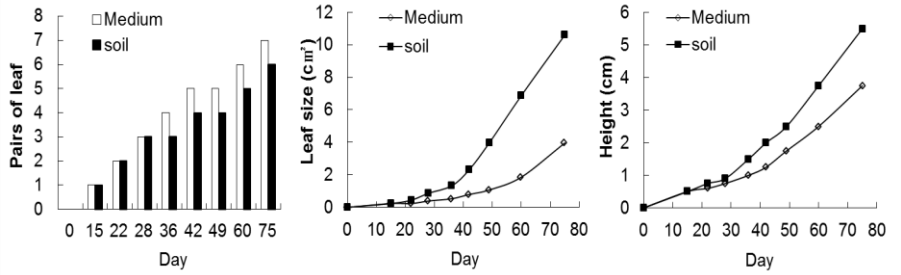

Fig. 2 Comparison of the number of leaf pairs (A), leaf size (B) and height (C) of *Catharanthus roseus* plants grown in MS medium and soil during the development stage.

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

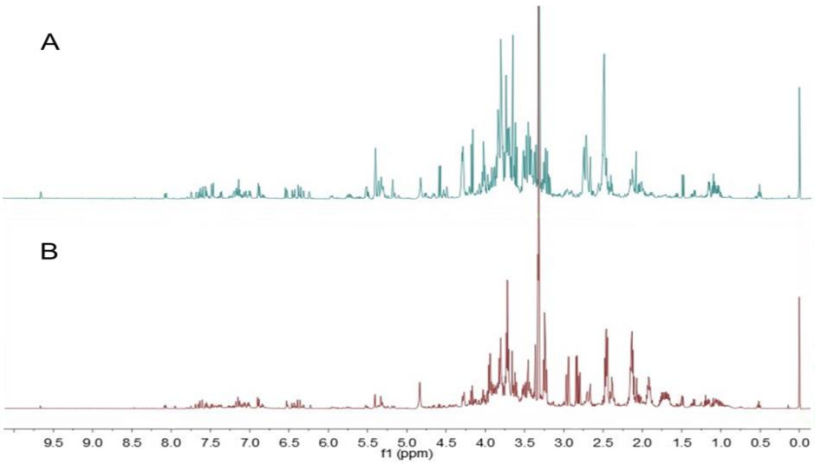

Fig. 3 ^{1}H-NMR spectrum of the crude extracts of *Catharanthus roseus* plants grown in soil (A) and MS medium (B).

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

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

Some groups of metabolites have a close correlation with plant growth and biomass, like the tricarboxylic acid cycle (TCA) cycle intermediates succinate, citrate or malate, as well as amino acids (Meyer *et al.*, 2007). Both glutamine and asparagine are the major compounds for nitrogen fixing, transport and storage in plants (Lea *et al.*, 2007). 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.*, (2007) 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 (Genix *et al.*, 1990). In the present study, the plants cultured on solid MS medium require an aseptic jar with 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.

[1-^{13}C] glucose feeding experiment and JA elicitation on Catharanthus 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 five days, 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 (Choi *et al.*, 2004; Mustafa *et al.*, 2009), and confirmed in the chapter 4. Totally 14 amino acids, 9 organic acids, 2 carbohydrates, 6 phenylpropanoids, 5 TIAs, 2 terpenoids and 3 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 (Fig. 4). Those include some primary metabolites like amino acids (threonine, alanine, asparagine, aspartate, glutamine, glutamate and arginine) and malic acid (Fig. 4A), as well as some secondary metabolites like phenylpropanoids (chlorogenic acid, 4-*O*-caffeoylquinic acid), terpenoids (loganic acid and secologanin) and TIA (vindoline) (Fig. 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 (Fig. 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 metabolites 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* support that ^{13}C feeding does not in itself distort the fluxes through the metabolic network in a plant (Kruger *et al.*, 2007).

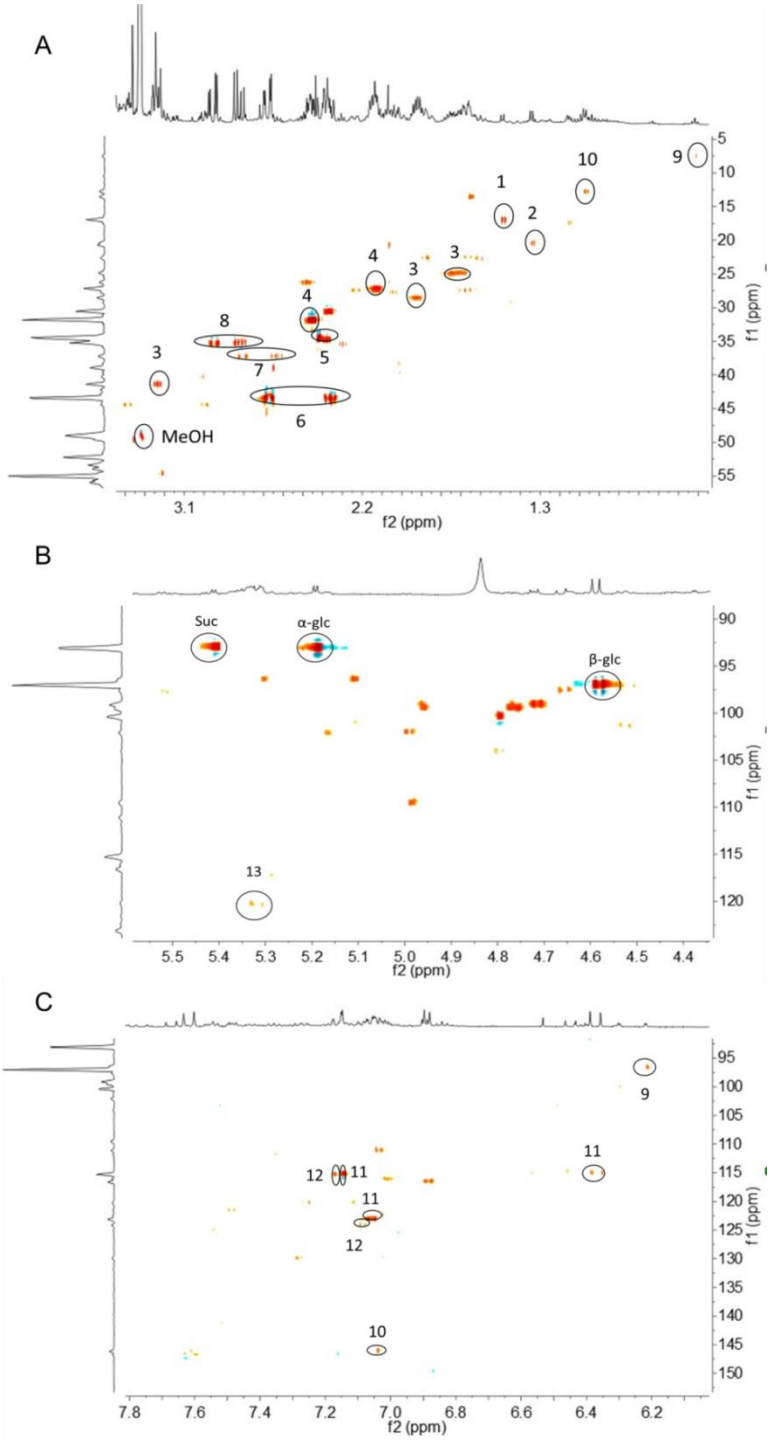
A
1
2
3
3
4
4
5
6
7
8
9
10
MeOH
f2 (ppm)
f1 (ppm)
B
Suc
α-glc
β-glc
13
f2 (ppm)
f1 (ppm)
C
9
11
12
11
11
12
10
f2 (ppm)
f1 (ppm)

Fig. 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.

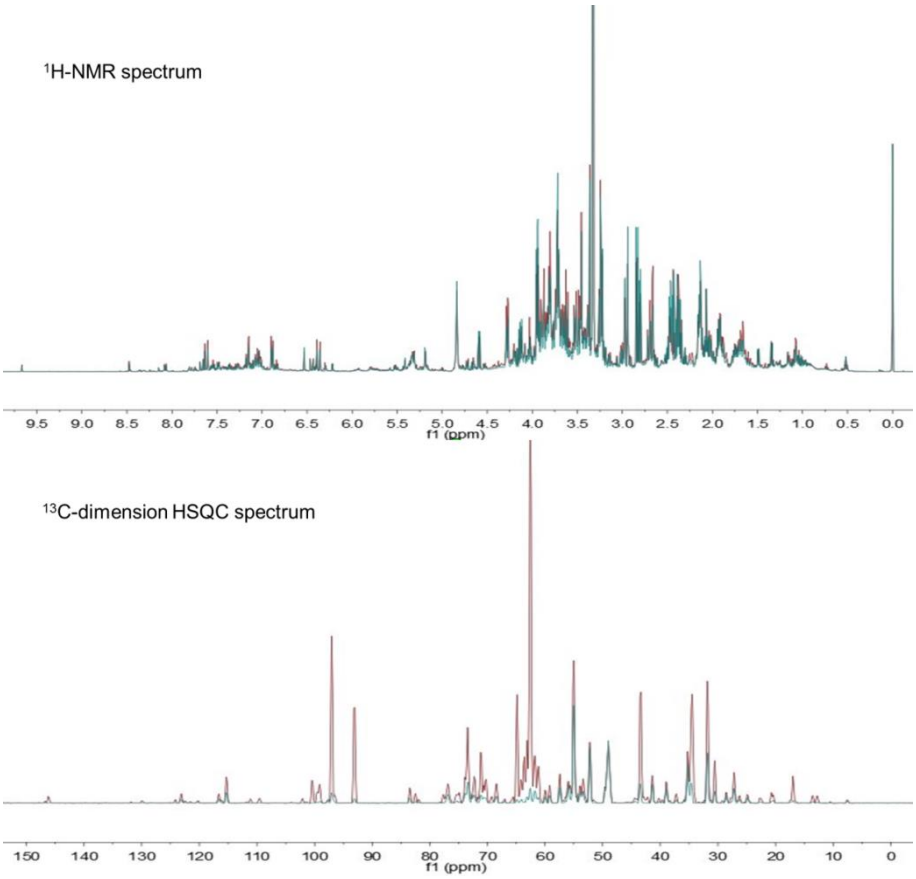

Fig. 5 Superimposed ^{1}H-NMR Spectra and ^{13}C-dimension HSQC spectrum of labeled and non-labeled *Catharanthus roseus* plants. Spectra in green were non-labeled plants sample, spectra in red were ^{13}C labeled plants sample.

Table 2. Comparison of metabolite levels in different organs between labeled and non-labeled *Catharanthus roseus* plants. *nd: not detected

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*-caffeoylquinic 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

^{13}C Incorporation into primary and secondary metabolites

The signals in the HSQC spectra of the enriched samples were identified (Fig. 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).

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 (Lundstrom *et al.*, 2007). 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.

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.

Compound	Chemical shift (δ) (ppm)		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	123.1	1.2E+07	7.2E+06	0.06	0.16	2.53
4-*O*-Caffeoyl quinic acid	7.09	124.2	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

Glutamate (C-3 at δ 27.74, C-4 at δ 34.44, 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 (Malloy *et al.*, 1988). For glutamate, however, C-4 had a lower relative enrichment ratio than C-3 and C-2. Non-symmetrical enrichment ratios of C-2 and C-3 implies that there might be a form of channeling that converts oxoglutarate C-4 to oxaloacetate C-2 or C-3 (Dieuaide-Noubhani *et al.*, 1995).

In plant cells, the labeling of amino acids alanine, glutamate, and aspartate are found to reflect that of the corresponding α-oxoacids: pyruvate, α-oxoglutarate, and oxaloacetate, respectively (Salon *et al.*, 1988). The organic acid malate showed a 6-fold 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 Fig. 1. The signal corresponding to C-18 of vindoline in the labeled sample was 2-fold higher than in the spectrum of the control.

^{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 biosynthesis dependent on chloroplasts were not detected, 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) were 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 (Fig. 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 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 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, whilst the highest was found in stems and roots.

Table 4 Relative level of metabolites in different organs of *Catharanthus roseus* based on ^{1}H-NMR spectra. *nd: not detected

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*-caffeoylquinic 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 5. Relative enrichment ratios of the carbons of some metabolites in different organs of *Catharanthus roseus* plants fed with [1-^{13}C] glucose. *nd: not detected

Compounds	**^{13}C Chemical Shift (ppm)**	**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

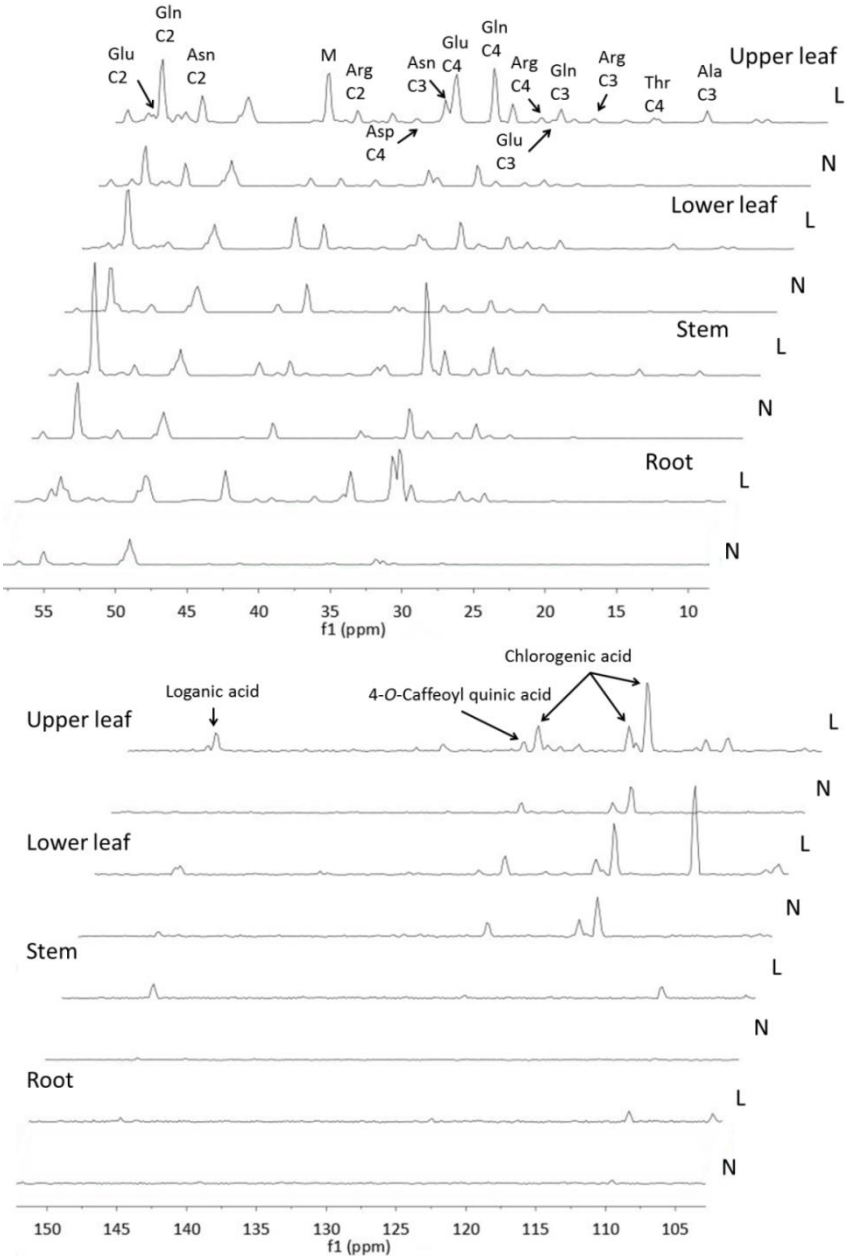

Fig. 6 ^{13}C dimension of HSQC spectra of amino acids (δ 10~55 ppm) and secondary metabolites (δ 105~150 ppm) in different organs of *Catharanthus roseus* after feeding [1-^{13}C] glucose. L, labeled samples; N, non-labeled samples; M, malate

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 (Zhou *et al.*, 2011; Murata *et al.*, 2008; Abbasi *et al.*, 2007; Shukla *et al.*, 2006; Murata and De Luca, 2005; De Luca and Cutler, 1987). 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 4 to 8 times lower in hairy roots than that in the other organs of the plant (Murata *et al.*, 2008).

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

JA was spiked into the labeled glucose solution at the 6th 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 1H-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 (Fig. 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*-caffeoylquinic 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 (Schuhr *et al.*, 2003). In tobacco plants grown on agar containing labeled glucose, the metabolism was studied on a quantitative basis showing that the labeled glucose was efficiently absorbed via the root system, metabolized and recycled (Ettenhuber *et al.*, 2005). Our results indicate that the *C. roseus* plant system can reach a relatively steady isotopic state with plants growing in ^{13}CLE.

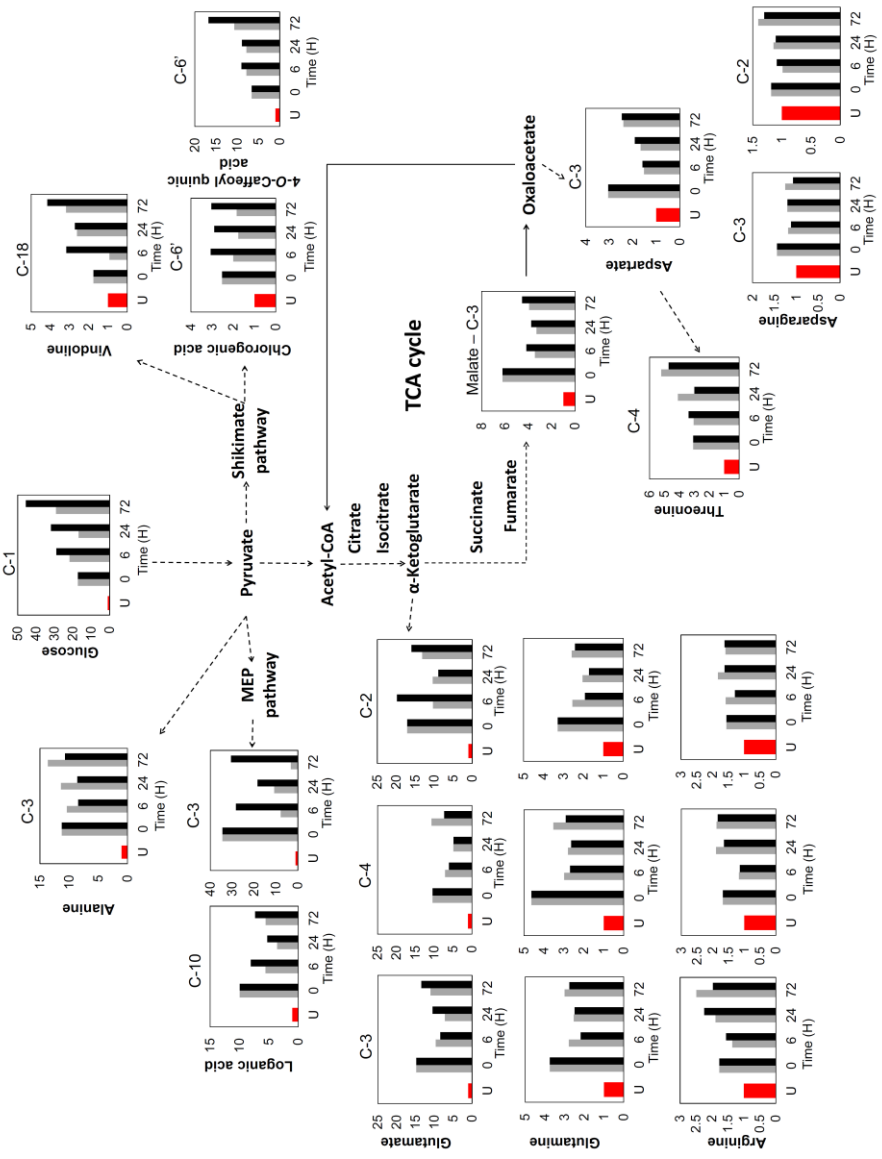

Fig. 7 Relative enrichment ratio of primary and secondary metabolites during incubation of *Catharanthus roseus* plants with [1-^{13}C] glucose. Grey bars: JA elicited samples; black bars: control samples; red bars (U): unlabeled samples (without incubation in [1-^{13}C] glucose.

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 (Fig. 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 (Fig. 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 (Fig. 7). The level of loganic acid decreased with time (Fig. 8), leading to a dramatical decrease of its enrichment at both C-3 and C-10 from 6 h to 72 h. The levels of chlorogenic acid and 4-*O*-caffeoyl quinic acid in the time course did not change after JA elicitation (Fig. 8), but the enrichments were lower than those of the control labeled samples (Fig. 7). ^{13}C fluxes to various metabolic pathways, like glutamate and loganic acid, could be disturbed within 24 h after MeJA treatment (Antonio *et al.*, 2013).

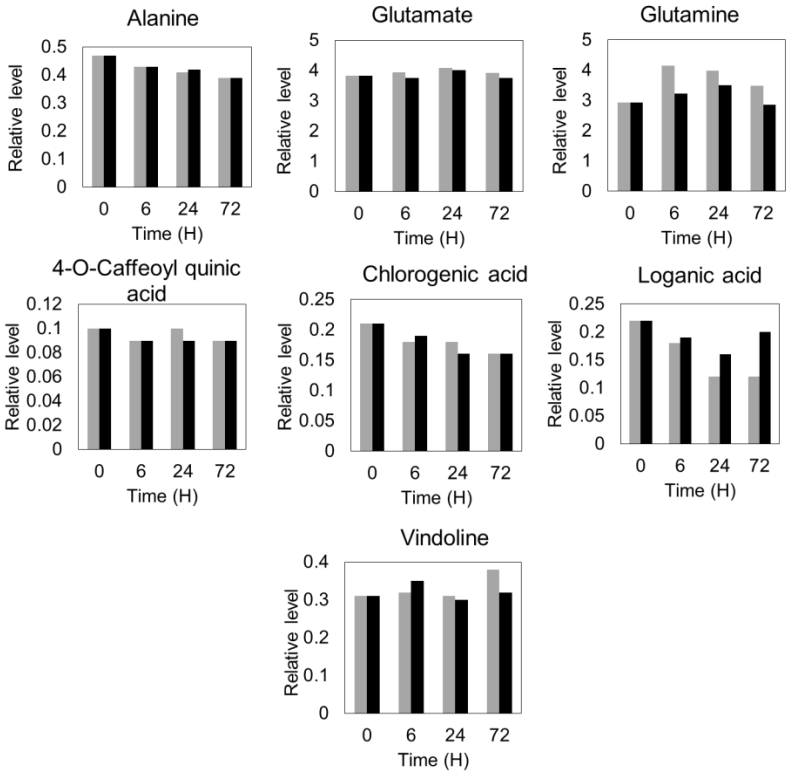

Fig. 8 Relative levels of metabolites in *Catharanthus roseus* leaves after JA elicitation. Grey bars: JA elicited samples; black bars: control samples.

Conclusion

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

Reference

Abbasi BH, Tian CL, Murch SJ, Saxena PK, Liu CZ (2007) Light-enhanced caffeic acid derivatives biosynthesis in hairy root cultures of *Echinacea purpurea*. Plant Cell Rep 26: 1367-1372.

Antonio C, Mustafa NR, Osorio S, Tohge T, Giavalisco P, Willmitzer L, Rischer H, Oksman-Caldentey KM, Verpoorte R, Fernie AR (2013) Analysis of the interface between primary and secondary metabolism in *Catharanthus roseus* cell cultures using 13C-stable isotope feeding and coupled mass spectrometry. Mol Plant 6: 581-584

Choi YH, Tapias EC, Kim HK, Lefeber AWM, Erkelens C, *et al.* (2004) Metabolic discrimination of *Catharanthus roseus* leaves infected by phytoplasma using ^{1}H-NMR spectroscopy and multivariate data analysis. Plant Physiol 135: 2398-2410.

Contin A, van der Heijden R, Lefeber AWM, Verpoorte R (1998) The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharanthus roseus* cell culture. FEBS letters 434: 413-416.

De Luca V, Cutler AJ (1987) Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus*. Plant Physiol 85: 1099-1102.

Dieuaide-Noubhani M, Raffard G, Canioni P, Pradet A, Raymond P (1995) Quantification of compartmented metabolic fluxes in maize root tips using isotope distribution from 13C-or 14C-labeled glucose. J Biol Chem 270: 13147-13159.

Ettenhuber C, Radykewicz T, Kofer W, Koop H-U, Bacher A, *et al.* (2005) Metabolic flux analysis in complex isotopolog space. Recycling of glucose in tobacco plants. Phytochemistry 66: 323-335.

Genix P, Bligny R, Martin J-B, Douce R (1990) Transient accumulation of asparagine in sycamore cells after a long period of sucrose starvation. Plant Physiol 94: 717-722.

Kim HK, Khan S, Wilson EG, Kricun SDP, Meissner A, *et al.* (2010) Metabolic classification of South American *Ilex* species by NMR-based metabolomics. Phytochemistry 71: 773-784.

Kruger NJ, Huddleston JE, Le Lay P, Brown ND, Ratcliffe RG (2007) Network flux analysis: Impact of 13 C-substrates on metabolism in *Arabidopsis thaliana* cell suspension cultures. Phytochemistry 68: 2176-2188.

Lea PJ, Sodek L, Parry MA, Shewry PR, Halford NG (2007) Asparagine in plants. Ann App Biol 150: 1-26.

Lundström P, Teilum K, Carstensen T, Bezsonova I, Wiesner S, *et al.* (2007) Fractional ^{13}C enrichment of isolated carbons using [1-^{13}C]-or [2-^{13}C]-glucose facilitates the accurate measurement of dynamics at backbone Cα and side-chain methyl positions in proteins. J Biomol NMR 38: 199-212.

Malloy CR, Sherry AD, Jeffrey F (1988) Evaluation of carbon flux and substrate selection through alternate pathways involving the citric acid cycle of the heart by 13C NMR spectroscopy. J Biol Chem 263: 6964-6971.

Meyer RC, Steinfath M, Lisec J, Becher M, Witucka-Wall H, *et al.* (2007) The metabolic signature related to high plant growth rate in *Arabidopsis thaliana*. Proc Nat Acad Sci 104: 4759-4764.

Möllney M, Wiechert W, Kownatzki D, de Graaf AA (1999) Bidirectional reaction steps in metabolic networks: IV. Optimal design of isotopomer labeling experiments. Biotechnol Bioeng 66: 86-103.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plantarum 15: 473-497.

Murata J, De-Luca V (2005) Localization of tabersonine 16-hydroxylase and 16-OH tabersonine-16-O-methyltransferase to leaf epidermal cells defines them as a major site of precursor biosynthesis in the vindoline pathway in *Catharanthus roseus*. Plant J 44: 581-594.

Murata J, Roepke J, Gordon H, De-Luca V (2008) The leaf epidermome of *Catharanthus roseus* reveals its biochemical specialization. Plant Cell 20: 524-542.

Mustafa NR, Kim HK, Choi YH, Erkelens C, Lefeber AW, *et al.* (2009) Biosynthesis of salicylic acid in fungus elicited *Catharanthus roseus* cells. Phytochemistry 70: 532-539.

Mustafa NR, Kim HK, Choi YH, Verpoorte R (2009) Metabolic changes of salicylic acid-elicited *Catharanthus roseus* cell suspension cultures monitored by NMR-based metabolomics. Biotechnol Let 31: 1967-1974.

Ratcliffe RG, Shachar‐Hill Y (2006) Measuring multiple fluxes through plant metabolic networks. Plant J 45: 490-511.

Salon C, Raymond P, Pradet A (1988) Quantification of carbon fluxes through the tricarboxylic acid cycle in early germinating lettuce embryos. J Biol Chem 263: 12278-12287.

Schuhr CA, Radykewicz T, Sagner S, Latzel C, Zenk MH, *et al.* (2003) Quantitative assessment of crosstalk between the two isoprenoid biosynthesis pathways in plants by NMR spectroscopy. Phytochem Rev 2: 3-16.

Shukla AK, Shasany AK, Gupta MM, Khanuja SP (2006) Transcriptome analysis in *Catharanthus roseus* leaves and roots for comparative terpenoid indole alkaloid profiles. J Exp Bot 57: 3921-3932.

Sriram G, Fulton DB, Shanks JV (2007) Flux quantification in central carbon metabolism of *Catharanthus roseus* hairy roots by 13C labeling and comprehensive bondomer balancing. Phytochemistry 68: 2243-2257.

Szyperski T (1998) C-NMR, MS and metabolic flux balancing in biotechnology research. Q Rev Biophys 31: 41-31106.

Zhou ML, Zhu XM, Shao JR, Tang YX, Wu YM (2011) Production and

metabolic engineering of bioactive substances in plant hairy root culture. Appl Microbiol Biotechnol 90: 1229-1239.