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Network flux analysis: Impact of ^{13}C-substrates on metabolism in *Arabidopsis thaliana* cell suspension cultures

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Abstract

The aim of this study was to test the assumption that ^{13}C-enrichment of respiratory substrate does not perturb metabolism. Cell suspension cultures of *Arabidopsis thaliana* were grown in MS medium containing unlabelled glucose (with ^{13}C at natural abundance), 100% [1-^{13}C]glucose, 100% [U-$^{13}C_6$]glucose or 10% [U-$^{13}C_6$]glucose plus 90% unlabelled glucose. There was no significant difference in the metabolism of [U-^{14}C]glucose between the cultures. Similarly, the pattern of $^{14}CO_2$ release from specifically labelled [^{14}C]-substrates was unaffected. Principal component analysis of ^{13}C-decoupled ^{1}H NMR metabolite fingerprints of cell extracts was unable to discriminate between the different culture conditions. It is concluded that ^{13}C-enrichment of the growth substrate has no effect on flux through the central pathways of carbon metabolism in higher plants. This conclusion supports the implicit assumption in metabolic flux analysis that steady-state ^{13}C-labelling does not perturb fluxes through the reactions of the metabolic network it seeks to quantify.
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Keywords: *Arabidopsis thaliana*; Brassicaceae; [^{13}C]glucose; Steady-state labelling; Metabolic flux analysis; Kinetic isotope effects; [^{14}C]glucose metabolism; ^{1}H NMR; Metabolite fingerprinting

1. Introduction

Steady-state labelling with ^{13}C-labelled substrates is a powerful technique for quantifying fluxes in the metabolic networks of micro-organisms (Blank et al., 2005; Fuhrer et al., 2005), and it is also finding an increasing number of applications in the analysis of the more complex compartmented networks found in plants (Kruger et al., 2003; Ratcliffe and Shachar-Hill, 2006). The method hinges on measuring the redistribution of ^{13}C through the network and it is axiomatic that the use of non-tracer amounts of the isotope should have no significant effect on the underlying metabolic fluxes. However, isotopes have well-established effects on the rates of biochemical reactions and the isotopic composition of a substrate can affect the rate at which it is metabolised by an enzyme (Kohen and Limbach, 2006). These kinetic isotope effects are detectable in the measurable differences in ^{13}C enrichment in organic material (Tcherkez and Farquhar, 2005), providing information about a diverse range of physiological processes, including mode of photosynthesis (Crayn et al., 2004), water-use efficiency (Masle et al., 2005) and substrate utilisation (Gleixner et al., 1998; Tcherkez et al., 2003) in plants, as well as diet in heterotrophic organisms (Fuller et al., 2006). Nevertheless, such isotope effects are typically only a few parts per thousand and are generally regarded as being unlikely to have a direct effect on estimates of flux through metabolic networks (Cornish-Bowden, 2004).

Despite these considerations, there is at least one instance – *Paracoccus denitrificans*, a facultative chemolithotroph from the alpha subdivision of the proteobacteria – in which ^{13}C-enrichment of respiratory substrate has been reported to have a profound effect on flux through the network of central carbon metabolism (Dunstan et al., 1990a,b). This

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conclusion was based on three lines of evidence. First, the fractional ^{13}C-enrichments of metabolites formed directly from pyruvate in cells grown on [1-^{13}C]glucose differed from those found in cells grown on [6-^{13}C]glucose (Dunstan et al., 1990b). These labelling patterns yielded different estimates of flux of carbon through the Entner–Doudoroff, pentose phosphate and glucuronic acid cycle (G6) pathways. In cells grown on [1-^{13}C]glucose, about 4% of the glucose was metabolised through the G6 pathway, just over 22% through the oxidative pentose phosphate pathway and the remaining 74% via the Entner–Doudoroff pathway; whereas in cells grown on [6-^{13}C]glucose over 43–50% was metabolised through the G6 pathway with the remainder being metabolised through the Entner–Doudoroff and pentose phosphate pathways, which could not be distinguished with this precursor. Secondly, cells grown on different ^{13}C-enriched media differed in their metabolite composition (Dunstan et al., 1990b). Glucose 6-P and mannitol 6-P were the only hexose derivatives detected in cells grown on natural abundance glucose; whereas only gluconate, 1,4-gluconolactone and 1,5-gluconolactone were detected in cells grown on 92% [1-^{13}C]glucose; and glucuronate, galacturonate and glucuronolactone were the only sugar derivatives detected in cells grown on [6-^{13}C]glucose. Lastly, the rate of $^{14}CO_2$ release from specifically-labelled glucose fed to cells grown on either [1-^{13}C]glucose or [6-^{13}C]glucose differed substantially from each other and from the rate observed for similar batches of cells grown on un-enriched glucose (Dunstan et al., 1990a).

The unexpected sensitivity of *Paracoccus* metabolism to the positional ^{13}C-labelling of glucose challenges the assumption that kinetic isotope effects can be ignored when studying flux through the metabolic network. Since this assumption underpins the application of all steady-state stable isotope strategies for metabolic flux analysis, the degree to which the response of *Paracoccus* is a general phenomenon needs to be established. Here we assess the influence of the ^{13}C-isotopic composition of the respiratory substrate on flux through the network in plants in two ways: first, by comparing metabolism through the dominant pathways of carbohydrate utilisation in cell suspension cultures of *Arabidopsis thaliana* grown heterotrophically in media containing glucose of differing ^{13}C-enrichment as the sole carbon source; and secondly by analyzing the metabolite fingerprint of cell extracts using a nuclear magnetic resonance (NMR)-based metabolomic approach. These experiments provide compelling and reassuring evidence that metabolic activity of the *Arabidopsis* metabolic network is unaffected by the use of non-tracer amounts of ^{13}C-label.

2. Results

2.1. Influence of ^{13}C isotopic enrichment on carbohydrate metabolism in Arabidopsis cells

To investigate the influence of ^{13}C on the central pathways of carbon metabolism, we examined the metabolism of [U-^{14}C]glucose by cells that had been sub-cultured into a glucose growth medium containing unlabelled glucose (with ^{13}C at natural abundance), 100% [1-^{13}C]glucose, 100% [U-^{13}C]glucose or 10% [U-^{13}C] glucose plus 90% unlabelled glucose, and then incubated in darkness for 5 d prior to analysis. These experiments were conducted on two separate sets of cultures grown three months apart. Similar results were obtained on both occasions, although data for only one of the two studies are presented (Table 1). Following incubation in [U-^{14}C]glucose for 4 h, radioactivity was recovered in CO_2 as well as both ethanol-soluble and ethanol-insoluble substances. Treatment of the ethanol-insoluble sample with amylase and protease released label that fractionated into neutral, basic and acidic components during ion-exchange chromatography. The neutral and basic fractions arise from starch and protein, respectively, while the acidic fraction and insoluble residue are attributed to cell wall components. The ethanol-soluble sample also contained appreciable label that partitioned into acidic components, which include organic acids and phosphate esters, and basic components, mainly amino acids, although the majority of the label was recovered in the neutral fraction following ion-exchange chromatography. Comparison of the label recovered in the isolated compounds with that in the unfractionated ethanol-soluble and ethanol-insoluble fractions established that these analyses were achieved without serious loss: $98.5 \pm 1.5\%$ (mean $\pm$ SE, $n = 14$) of the ^{14}C present in each sample was accounted for in the final fractions. Analysis of the neutral components of the ethanol-soluble sample by TLC revealed that almost all label in this fraction was in unmetabolised glucose. Using this approach it was not possible to achieve sufficient resolution to quantify the amount of label in other sugars such as sucrose and fructose. Overall, the redistribution of radiolabel following incubation with [U-^{14}C]glucose was similar to that reported previously in a wide range of plant material including cell cultures (Fernie et al., 2001; Klein and Stitt, 1998), storage organs (Bindon and Botha, 2002; Hill and ap Rees, 1995; Macrae et al., 1992; Malone et al., 2006; Urbanczyk-Wochniak et al., 2003) and other non-photosynthesising tissues (Averill et al., 1998; Hargreaves and ap Rees, 1988; Scott and Kruger, 1995). This comparison suggests that the *Arabidopsis* cell cultures used in this study are entirely unexceptional in their metabolic activities.

The detailed pattern of metabolism in the different cultures was analysed in two ways. First, we used multiple analysis of variance (MANOVA) to compare the distribution of radioactivity following chemical fractionation within and between 3 and 5 independent replicate cultures of cells grown in each of the four culture conditions (Table 2). These comparisons were made on both the absolute amount of radioactivity recovered in each fraction and on the ^{14}C recovered in each fraction expressed as a percentage of the ^{14}C metabolised by the specific culture. The latter analysis was conducted because the large proportion of applied radioactivity that remained as

Table 1
Metabolism of [U-^{14}C]glucose by cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose

Fraction	Radioactivity in specified fraction in cells grown in:			
	Natural abundance glucose	100% [1-^{13}C]glucose	10% [U-^{13}C]glucose	100% [U-^{13}C]glucose
Ethanol soluble	1,638,218 ± 13,584	1,672,756 ± 18,781	1,623,326 ± 5628	1,662,745 ± 24,405
Neutral	1,502,711 ± 55,799	1,576,897 ± 78,125	1,656,113 ± 12,297	1,621,332 ± 10,582
Acidic	30,128 ± 696	30,852 ± 707	30,233 ± 938	31697 ± 1513
	(32.6 ± 1.5)	(32.2 ± 0.3)	(35.9 ± 1.7)	(34.1 ± 0.4)
Basic	13,307 ± 744	13,750 ± 1549	12,399 ± 1260	14,446 ± 2021
	(14.3 ± 0.5)	(14.1 ± 1.3)	(14.7 ± 1.2)	(15.4 ± 1.5)
Glu/Asp	3081 ± 185	3598 ± 700	2523 ± 194	3284 ± 662
	(3.3 ± 0.3)	(3.7 ± 0.7)	(3.0 ± 0.1)	(3.5 ± 0.6)
Others	9971 ± 525	10,050 ± 811	8547 ± 978	11,480 ± 1001
	(10.7 ± 0.27)	(10.3 ± 0.6)	(10.0 ± 0.5)	(12.3 ± 0.7)
Ethanol insoluble	39,084 ± 4386	42063 ± 530	32,518 ± 3290	34,892 ± 1604
	(41.3 ± 2.3)	(43.4 ± 1.5)	(38.3 ± 1.7)	(37.7 ± 2.2)
Neutral	33,173 ± 4012	34,973 ± 2494	24,591 ± 2922	27,002 ± 1690
	(35.1 ± 2.7)	(36.1 ± 3.0)	(28.9 ± 1.7)	(29.3 ± 2.6)
Basic	2396 ± 346	2001 ± 106	1626 ± 156	2371 ± 518
	(2.5 ± 0.2)	(2.1 ± 0.2)	(1.9 ± 0.1)	(2.5 ± 0.4)
Acidic	1798 ± 226	1897 ± 130	1295 ± 143	1793 ± 374
	(1.9 ± 0.2)	(2.0 ± 0.1)	(1.5 ± 0.1)	(1.9 ± 0.3)
Residue	3228 ± 316	3495 ± 242	2731 ± 330	3518 ± 397
	(3.4 ± 0.2)	(3.6 ± 0.3)	(3.2 ± 0.3)	(3.8 ± 0.3)
Carbon dioxide	10,786 ± 510	10,067 ± 51	9468 ± 1108	11,909 ± 1102
	(11.7 ± 0.8)	(10.4 ± 0.4)	(11.1 ± 0.8)	(12.8 ± 0.7)
Total ^{14}C metabolised (dpm)	93,305 ± 5825	97,107 ± 2167	84,618 ± 5687	92,944 ± 4739
Total recovery of ^{14}C (%)	(93.4 ± 3.0)	(102.1 ± 0.9)	(101.6 ± 0.5)	(100.4 ± 0.2)

Samples from 5-day-old cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose were incubated with 37 kBq [U-^{14}C]glucose for 4 h prior to extraction in formic acid/ethanol and subsequent metabolic fractionation. The amount of radioactivity in each fraction is presented as dpm, and is also expressed in parentheses as a percentage of total ^{14}C metabolised (taken as the sum of radioactivity in carbon dioxide, the ethanol-insoluble fraction, and the acidic and basic components of the ethanol-soluble fraction in each cell culture). Each value is the mean ± SE from three independent cultures except for measurements from cells grown in natural abundance glucose for which five cultures were used. Total recovery is derived from the sum of radioactivity in the terminal fractions obtained from each sample expressed as a percentage of ^{14}C applied to the flask.

unmetabolised [U-^{14}C]glucose could potentially distort analysis of the distribution of absolute quantities of radiolabel between fractions. Irrespective of the form in which the data were expressed, MANOVA comparison failed to reveal any significant difference, by a range of different statistical criteria, in the pattern of metabolism of [U-^{14}C]glucose between the cultures grown in the differing ^{13}C-enriched media (Table 2).

Secondly, we compared the detailed pattern of [U-^{14}C]glucose metabolism in the different cultures using a correlative approach. If the respiratory substrate is metabolised in the same way in two different culture conditions, then the distribution of label between fractions should be the same for cultures grown under the different conditions. This can be examined by plotting the amounts of radioactivity in specific fractions in cultures from one growth medium against those for the equivalent fractions in cultures grown in a second medium. In the absence of any metabolic differences, such a plot should yield a straight line that passes through the origin with a slope of unity and have a high correlation coefficient of linear regression. Pair-wise comparison of the data obtained from cell cultures grown in each of the ^{13}C-enriched media with those grown in unlabelled glucose display this equivalence irrespective of whether the redistribution of label is analysed as absolute radioactivity or as a percentage of that

Table 2
MANOVA comparison of metabolism of [U-^{14}C]glucose by cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose

Statistical test	MANOVA statistics of metabolism of [U-^{14}C]glucose based on									
	Absolute amount of radioactivity in each fraction					Percent of ^{14}C metabolised				
	Value	*F*-ratio	df1	df2	*P*-value	Value	*F*-ratio	df1	df2	*P*-value
Pillai's trace	2.61	2.03	30	9	0.132	2.70	2.72	30	9	0.058
Wilks' Lambda	7.54×10^{-4}	1.27	30	3.61	0.467	6.63×10^{-5}	3.07	30	3.61	0.159

Analysis was conducted on the distribution of radioactivity following metabolism of [U-^{14}C]glucose by cell suspension cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose using data summarised in Table 1. Data expressed as percent of ^{14}C metabolised were arcsine transformed prior to analysis. df1 and df2 are the degrees of freedom of the numerator (hypothesis) and denominator (error) terms, respectively, in the specified statistical test.

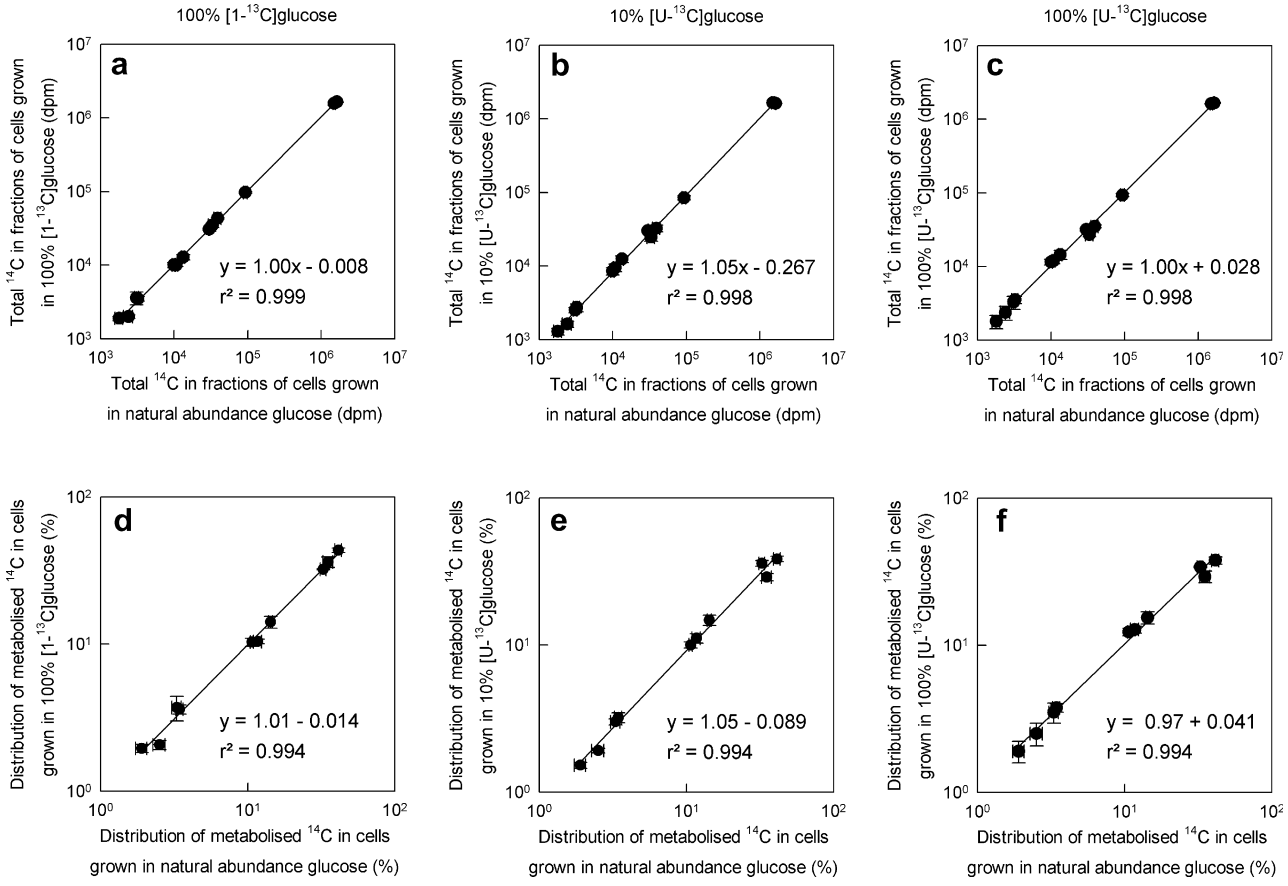

Fig. 1. Comparison of [U-^{14}C]glucose metabolism by cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose. Data summarised in Table 1 were used to compare the metabolism of [U-^{14}C]glucose by cell suspension cultures grown in glucose at natural abundance ^{13}C with those grown in media containing 100% [1-^{13}C]glucose (a, d), 10% [U-^{13}C]glucose (b, e) or 100% [U-^{13}C]glucose (c, f). Linear regression analysis was used to compare the distribution of label between chemical fractions both as absolute quantities (a–c) and as percentages of total ^{14}C metabolised (d–f). Plotted values are derived from the mean ± SE of three independent cultures grown on ^{13}C-enriched media (*y*-axis) and five independent cultures grown in natural abundance glucose (*x*-axis).

metabolised (Fig. 1). These comparisons again reveal that there is no obvious difference in the metabolism of [U-^{14}C]glucose between the cultures grown in the differing ^{13}C-enriched media.

2.2. Influence of ^{13}C isotopic enrichment on pathways of carbohydrate oxidation in Arabidopsis cells

Glycolysis and the oxidative pentose phosphate pathway are the principal routes for the initial oxidation of carbohydrates in higher plants (ap Rees, 1980). Although there is no simple method to measure flux through these two pathways accurately (Kruger and von Schaewen, 2003), the pattern of $^{14}CO_2$ production from specifically labelled glucose and gluconate may be used to provide an indication of the relative activities of the alternative routes of hexose phosphate metabolism (ap Rees, 1980; Garlick et al., 2002). Therefore, to examine the possible effects of ^{13}C-enrichment on the contribution of these pathways and other processes to carbohydrate oxidation, cells growing in medium containing unlabelled glucose, 100% [1-^{13}C]glucose, 100% [U-^{13}C]glucose or 10% [U-^{13}C] glucose plus 90% unlabelled glucose were incubated in the presence of [1-^{14}C]-, [2-^{14}C]-, [3,4-^{14}C]- and [6-^{14}C]-glucose, and [1-^{14}C]gluconate. Released $^{14}CO_2$ was monitored at 2-h intervals for 12 h and after 24 h and 48 h incubation. Similar results were obtained from two sets of cultures grown three months apart. Results are presented for only one of these two replicated experiments (Fig. 2). The total amount of radioactivity released over the 48 h incubation was between 15% and 45% of that applied, and was dependent on the nature of the labelled substrate. Following a lag of 4–6 h, the release of $^{14}CO_2$ from specifically-labelled glucose was approximately linear over the remainder of the time-course. At all sampling times, $^{14}CO_2$ was released from specific positions with metabolised glucose in the order C3,4 > C1 > C6 > C2. This pattern of release is similar to that observed in maize root tips, darkened wheat leaves and potato tubers (Butt and Beevers, 1961; Malone et al., 2006; Stitt and ap Rees, 1978). In the present study on *Arabidopsis*, release of $^{14}CO_2$ from [1-^{14}C]gluconate was more rapid than that from [^{14}C]glucose and was approximately linear after a shorter initial lag of 2–4 h, but declined after the first 12 h due to depletion of the

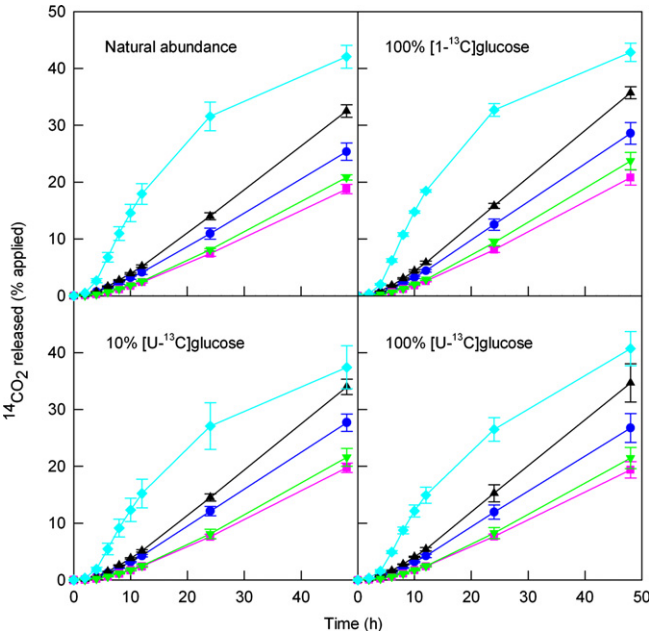

Fig. 2. Oxidation of specifically-labelled ^{14}C-substrates by cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose. Samples from 5-day-old cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose were incubated with 3.7 kBq [1-^{14}C]glucose (●), [2-^{14}C]glucose (■), [3,4-^{14}C]glucose (▲), [6-^{14}C]glucose (▼), or [1-^{14}C]gluconate (♦) for 48 h. Released $^{14}CO_2$ was collected at intervals as indicated. Cumulative $^{14}CO_2$ release was expressed as a proportion of applied label. Each value is the mean ± SE of three independent cultures except for measurements from cells grown in natural abundance glucose for which three cultures were used.

labelled substrate. Changes in the ratios of $^{14}CO_2$ release from different carbon positions within [^{14}C]glucose (Fig. 3) are likely to result from differences in the time taken to isotopically label the pools of metabolites that are the immediate precursors for decarboxylation of C-groups derived from specific positions within the original labelled substrate. In this context, the more rapid release of $^{14}CO_2$ from gluconate probably reflects a combination of the faster turnover rate of the 6-phosphogluconate pool relative to that for glucose 6-phosphate, and the fewer metabolic steps required for the direct decarboxylation of carbon in the labelled position in [1-^{14}C]gluconate (Garlick et al., 2002).

Three points emerge from the data presented in Figs. 2 and 3. First, the dominant yield of CO_2 from C3,4 indicates that an appreciable proportion of the supplied substrate is oxidised by conversion to pyruvate and subsequent decarboxylation via the pyruvate dehydrogenase complex (ap Rees, 1980). Secondly, the greater yield of CO_2 from C1 than from C6 (C1/C6 ratio greater than unity, Fig. 3e) suggests that an appreciable proportion of the added glucose is metabolised through the oxidative pentose phosphate pathway in these cells (ap Rees, 1980). This interpretation is supported by the values obtained for the difference between the yield of CO_2 from C1 and C6 expressed relative to the yield from C3,4 (Fig. 3h). The extent to which release from C1 exceeds that from C6 is indicative of oxidative pentose phosphate pathway activity, whereas release from C3 and C4 is likely to reflect the total amount of hexose phosphates metabolised by both pathways (see above). Thus (C1–C6)/C3,4 will be a measure of the proportion of pyruvate formed from hexose phosphates via the oxidative pentose phosphate pathway (Davies et al., 1964). Further indication that the oxidative pentose phosphate pathway makes a significant contribution to carbohydrate oxidation in the *Arabidopsis* cells is provided by the appreciable release of CO_2 from C1 of gluconate relative to that from glucose (Fig. 2; also compare Fig. 3e and f) since in plants exogenous gluconate is metabolised almost exclusively via this pathway (Garlick et al., 2002). Thirdly, the higher yields of CO_2 from C6 than from C2 at every sampling time in each culture cannot be explained by the conventional operation of glycolysis or the pentose phosphate pathway (Fig. 2). However, release of C6 of glucose as CO_2 may occur through the decarboxylation of UDPglucose during pentan synthesis, or following recycling of triose phosphates to hexose phosphates, which results in the transfer of carbon from C6 of glucose to the C1 position in hexose phosphates, thereby facilitating its release as CO_2 via the oxidative pentose phosphate pathway (Kruger et al., 2003). Pentan synthesis can make a significant contribution to release of CO_2 from C6 in some rapidly growing plant tissues (Averill et al., 1998; Stitt and ap Rees, 1978), while recycling from triose phosphates to hexose phosphates is a widespread, and possibly ubiquitous, feature of carbohydrate metabolism in plant cells (Fernie et al., 2001; Hatzfeld and Stitt, 1990; Roscher et al., 2000).

Overall, the yields of CO_2 from different positions within glucose and C1 of gluconate indicate that carbohydrate oxidation in the *Arabidopsis* cell culture occurs principally through a combination of glycolysis and the oxidative pentose phosphate pathway to provide pyruvate for subsequent decarboxylation through the tricarboxylic acid cycle, with a degree of recycling of triose phosphates to hexose phosphates and/or withdrawal of a fraction of the hexose phosphates for pentan synthesis.

To assess the influence of ^{13}C-enrichment on the relative activities of these processes, we compared the pattern of release of $^{14}CO_2$ from the various labelled substrates in two ways. First, the time-course of cumulative $^{14}CO_2$ release for all substrates was compared between the cultures grown in all four ^{13}C-enrichments using repeated measures analysis of variance (ANOVA), an approach which recognises that successive measurements in the time-course are taken from the same culture and therefore do not represent independent samples (Field, 2005). This analysis confirmed the obvious differences in the extent of $^{14}CO_2$ release from the different ^{14}C-labelled substrates [$F(4,50) = 157.1$, $P < 0.001$], but revealed no significant effect of the different ^{13}C-enriched growth media on the pattern of $^{14}CO_2$ release between cultures [$F(3,50) = 1.968$, $P = 0.131$]. Secondly, since the results of specifically-labelled ^{14}C feeding studies are often inter-

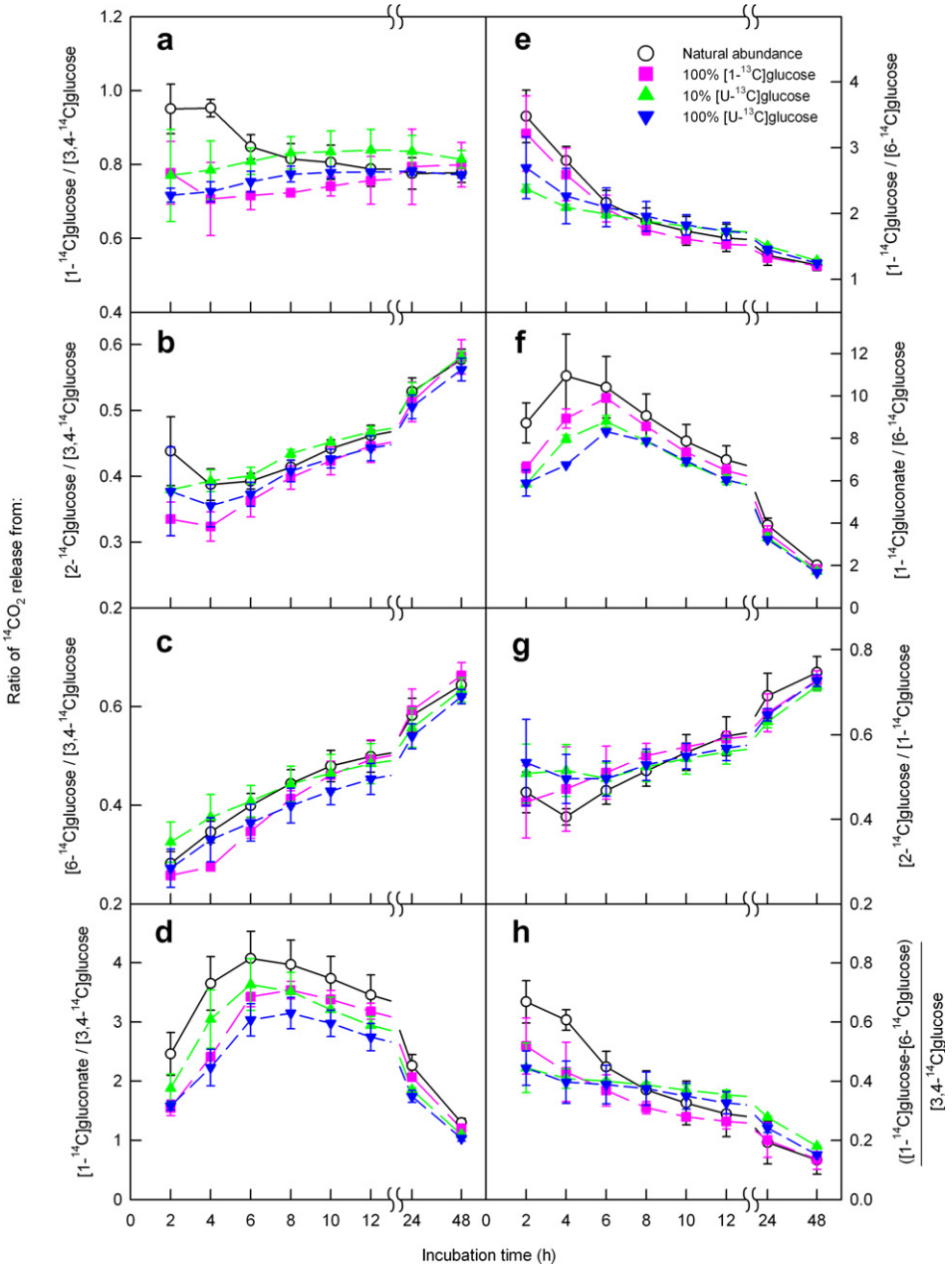

Fig. 3. Comparison of relative rates of oxidation of specific positions within labelled substrates by cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose. Ratios of $^{14}CO_2$ release from different combinations of positionally labelled substrates were calculated for cumulative $^{14}CO_2$ released during defined time intervals following metabolism of ^{14}C-labelled substrate by cell suspension cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose using data presented in Fig. 2. Shown are the ratios of $^{14}CO_2$ release from: a, [1-^{14}C]glucose:[3,4-^{14}C]glucose; b, [2-^{14}C]glucose:[3,4-^{14}C]glucose; c, [6-^{14}C]glucose:[3,4-^{14}C]glucose; d, [1-^{14}C]gluconate:[3,4-^{14}C]glucose; e, [1-^{14}C]glucose:[6-^{14}C]glucose; f, [1-^{14}C]gluconate:[6-^{14}C]glucose; g, [2-^{14}C]glucose:[1-^{14}C]glucose; h, ([1-^{14}C]glucose–[6-^{14}C]glucose):[3,4-^{14}C]glucose. Each value is the mean ± SE for ratios determined from three independent cultures except for those for cells grown in natural abundance glucose which are derived from five cultures.

preted as ratios of specific yields from particular C positions rather than absolute values (ap Rees, 1980), we examined the time-course of ratios of release of $^{14}CO_2$ from particular combinations of ^{14}C-substrates (Fig. 3). Repeated measures ANOVA revealed no significant differences between the cultures grown in the different ^{13}C-enriched media, irrespective of whether the ratios were analysed separately or in various combinations (Table 3). These comparisons establish that ^{13}C-enrichment has no effect on the relative activities of the principal routes of carbohydrate oxidation in the *Arabidopsis* cell suspension culture.

2.3. Influence of ^{13}C isotopic enrichment on the ^{1}H NMR metabolic fingerprint of Arabidopsis cells

For a broader assessment of the extent to which isotopic composition might influence plant cell metabolism, we used

Table 3
Repeated measures ANOVA comparison of time course of $^{14}CO_2$ release from specifically-labelled substrates by cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose

Comparison of ratio of $^{14}CO_2$ release from:	df	*F*-ratio	*P*-value
[1-^{14}C]glucose:[3,4-^{14}C]glucose	3, 10	3.20	0.071
[2-^{14}C]glucose:[3,4-^{14}C]glucose	3, 10	0.70	0.571
[6-^{14}C]glucose:[3,4-^{14}C]glucose	3, 10	0.43	0.738
[1-^{14}C]gluconate:[3,4-^{14}C]glucose	3, 10	1.74	0.223
([1-^{14}C]glucose–[6-^{14}C]glucose):[3,4-^{14}C]glucose	3, 10	0.66	0.593
[1-^{14}C]glucose:[6-^{14}C]glucose	3, 10	0.44	0.730
[1-^{14}C]gluconate:[6-^{14}C]glucose	3, 10	1.18	0.366
[2-^{14}C]glucose:[1-^{14}C]glucose	3, 10	0.03	0.993
Each ^{14}C substrate:[3,4-^{14}C]glucose	3, 50	2.63	0.061
Combination of all above ratios	3, 80	2.61	0.057

Analysis was conducted on cumulative $^{14}CO_2$ released during defined time intervals following metabolism of [1-^{14}C]glucose, [2-^{14}C]glucose, [3,4-^{14}C]glucose and [6-^{14}C]glucose and [1-^{14}C]gluconate by cell suspension cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose using data summarised in Fig. 2. Comparisons between the four ^{13}C-growth media were conducted on a logarithm transformation of the ratio of $^{14}CO_2$ release from different combinations of ^{14}C-positionally-labelled substrates.

^{13}C-decoupled ^{1}H NMR to provide metabolite fingerprints of extracts from the same *Arabidopsis* cells (Fig. 4). The extent of ^{13}C-coupling in the ^{1}H spectra was strongly dependent on the degree and nature of the ^{13}C-enrichment in the growth medium, and ^{13}C-decoupling was essential to facilitate a comparison between the fingerprints obtained from different samples. The resulting spectra showed the usual range of overlapping signals expected for a plant cell extract (Charlton et al., 2004; Ward et al., 2003), with intensities varying over several orders of magnitude. Carbohydrate signals dominated the spectra, but there were numerous signals from amino acids and other metabolites.

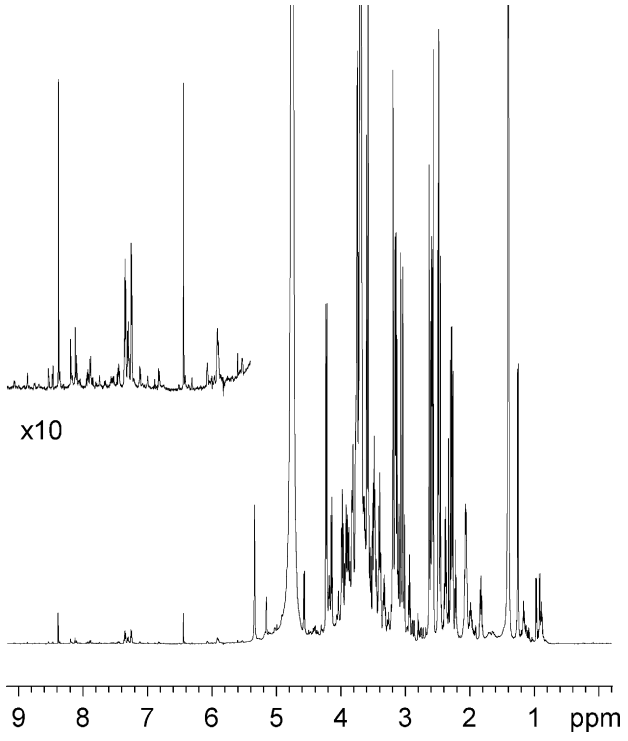

Fig. 4. ^{13}C-decoupled ^{1}H NMR spectrum of a perchloric acid extract of an *Arabidopsis* cell suspension culture grown in [U-^{13}C]glucose. Signals from carbohydrates dominate the spectrum, but there are also many signals from amino acids, organic acids and aromatic compounds. The inset is a 10-fold scale expansion of the aromatic region of the spectrum.

The spectra were compared by reducing to integrated regions of equal width, and applying principal component analysis (PCA) to the resulting data bins. This procedure generates a set of principal components in which each successive component is a linear combination of the original binned data that explains a maximal proportion of the remaining variance between samples. As a result, each spectrum is reduced to a single factor score for each component axis. Prior to this analysis the spectral data were refined by removing bins for which the relative spectral intensity was less than 10^{-7} in the majority of samples and less than 10^{-6} in the remaining spectra. This procedure eliminated 196 bins in which the intensities are considered to represent spectral noise. The intensities in each of the remaining 640 bins were re-normalised relative to their summed value. The principal components were extracted from these data using either the covariance matrix, which preserves the absolute values of the signal intensities and their variance, or the correlation matrix, which standardises the values and normalises the variance of signal intensities between bins. The first five principal components derived using these two approaches described 94% and 78%, respectively, of total variance between spectra (Table 4). Subjectively, there was no obvious correlation between the isotopic composition of the growth medium and the factor scores for the five principal components obtained from the covariance matrix either individually or in any pair-wise combination (Fig. 5, see also graphical abstract for comparison of the first three components). Equivalent results were obtained when comparing the principal com-

Table 4
Multivariate analysis of metabolite fingerprints of cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose

Number of principal components	Cumulative percentage variance explained based on analysis of:	
	Covariance matrix	Correlation matrix
1	37.3	24.9
2	64.9	47.5
3	79.4	62.2
4	90.7	71.0
5	94.0	78.4

Principal components analysis was conducted on ^{1}H NMR spectra of perchloric acid extracts of cell suspension cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose. Principal components were obtained from both the covariance matrix and correlation matrix of 640 binned variables from 14 samples.

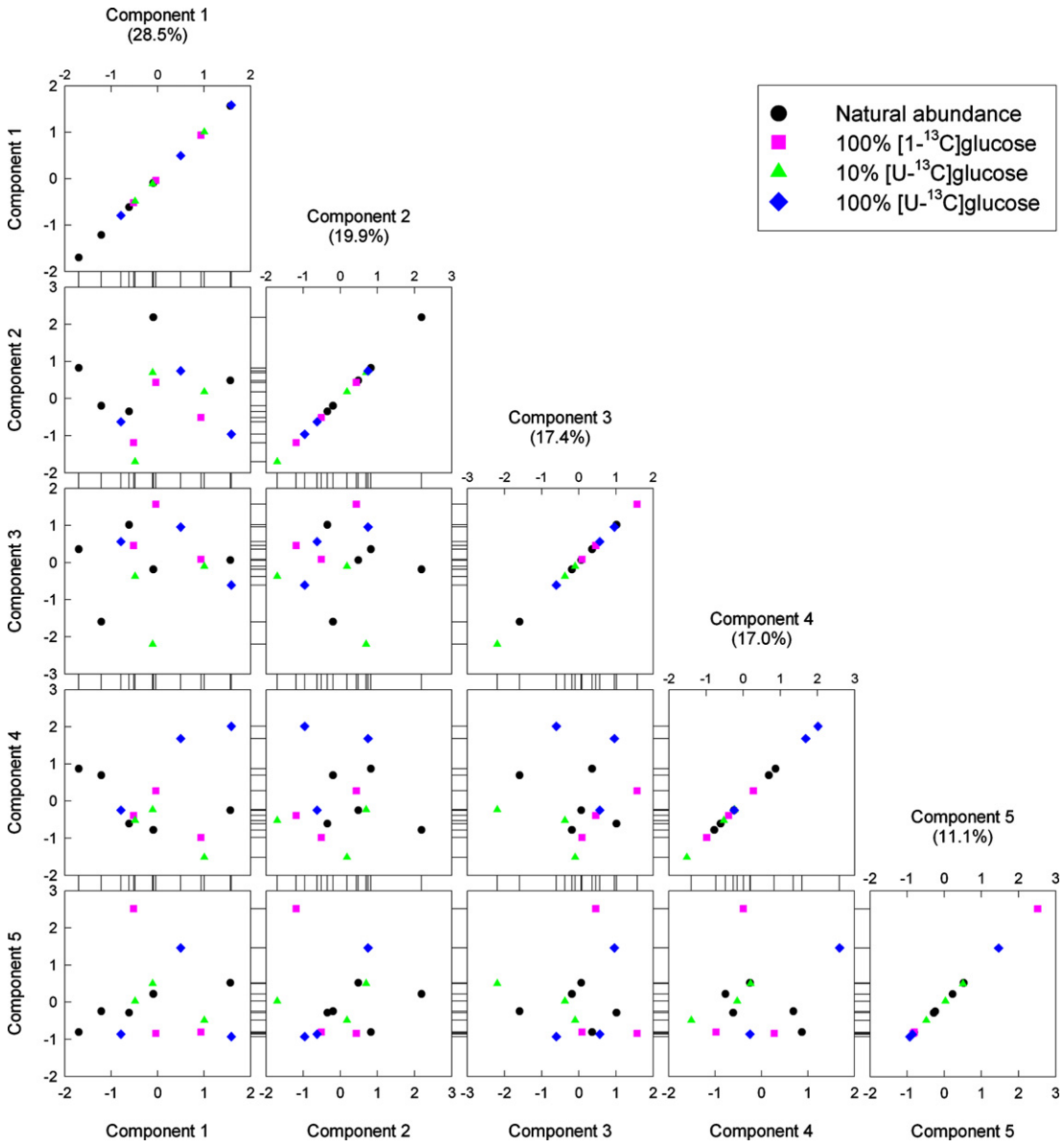

Fig. 5. Scatter plots of PCA factor scores for ^{1}H NMR metabolite fingerprints of cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose. Uncorrelated mean-normalised factor scores scaled to unit variance were obtained from the components extracted from the covariance matrix and subjected to orthogonal axis rotation. Data are presented as a rug plot (Tufte, 1983). Points represent the factor scores of individual extracts obtained from cells cultured in un-enriched glucose (●), 100% [1-^{13}C]glucose (■), 10% [U-^{13}C]glucose (▲) or 100% [U-^{13}C]glucose (♦). Values in parentheses indicate the percentage of variance explained by each principal component.

ponents derived from the correlation matrix (data not shown). Similar analysis of factor scores obtained from extracting 2–4 principal components or of factor scores derived without rotation of the component axes failed to identify any obvious clustering of samples from cells fed with identically-labelled substrate (data not shown).

We also assessed the extent of clustering between samples grown in media of the same isotopic composition by analysis of similarity (ANOSIM). This is a non-parametric, permutation-based procedure. Pair-wise measures of dissimilarity between samples are calculated and the mean dissimilarity between and within groups of samples are compared. The ANOSIM test statistic, R, reaches a maximum value of 1.0 when all samples within groups are more similar to one another than they are to any samples from other groups (Clarke and Warwick, 2001). Statistical significance is determined by comparing the sample R with those produced by randomly assigning samples to groups. The proportion of random arrangements with R-values higher than the sample value is the significance level of the test (Clarke, 1993). For this analysis we used the Euclidean distance between eigenvectors obtained during PCA as a measure of the dissimilarities between samples. Irrespective of the number of principal components considered or the source matrix from which they were extracted, there was no evidence for significant clustering of samples based on the ^{13}C composition of the growth medium (Table 5).

Table 5
ANOSIM comparison of metabolite fingerprints of cell suspension cultures of *Arabidopsis* grown at different specific abundances of [^{13}C]glucose

Number of principal components	ANOSIM based on:			
	Covariance matrix		Correlation matrix	
	R	*P*-value	*R*	*P*-value
3	0.047	0.329	−0.124	0.798
4	0.022	0.430	−0.117	0.776
5	0.018	0.442	−0.130	0.831

Eigenvectors obtained during PCA of ^{1}H NMR spectra of perchloric acid extracts from 14 samples were used to assess the dissimilarity between the metabolite composition of cell suspension cultures grown in glucose at natural abundance ^{13}C or enriched in [1-^{13}C]glucose or [U-^{13}C]glucose. *R*, the ANOSIM test statistic, approaches unity when the dissimilarities between samples within groups are all smaller than those between groups.

3. Discussion

The data obtained in this study suggest that there is no major effect of ^{13}C-stable isotope composition on the principal pathways of metabolism in *Arabidopsis* cells. We were unable to detect any statistically significant effects of the degree of ^{13}C-enrichment of respiratory substrates on either the pattern of metabolism of [^{14}C]glucose or the metabolite profile of cell extracts as assessed by ^{1}H NMR. The significance of the failure to detect any differences between cells grown in different media by these approaches is strengthened by three considerations.

First, both the redistribution of label following metabolism of [U-^{14}C]glucose and the time-course of release of $^{14}CO_2$ from specifically labelled ^{14}C-substrates provide direct assessments of metabolic activity and are markedly affected by metabolic perturbations (Malone et al., 2006). Although the fate of [^{14}C]-labelled sugars is likely to reflect principally the major pathways of carbohydrate oxidation, these are the sections of the metabolic network which are most commonly the focus of metabolic flux analysis and in which distortions of metabolism due to differences in isotopic composition of the respiratory substrate would have the greatest implications for quantification of fluxes.

Secondly, since the vast majority of cellular intermediates contain hydrogen, ^{1}H NMR fingerprints report on metabolite levels across wide sections of the metabolic network (Krishnan et al., 2005). In practice the range of metabolites amenable to this approach is restricted to those that are soluble in the solvents used for extraction and whose concentrations are high enough to be detected by NMR. Nevertheless, intermediates of the central pathways of carbon metabolism are likely to be represented in the ^{1}H NMR spectra since most of these metabolites are soluble in aqueous solution, stable during the extraction procedure used here, and present at average cell concentrations much greater than the estimated detection threshold of 5 μM in the present study (data not shown). Thus, any appreciable perturbation of the principal pathways of carbohydrate metabolism should be reflected in these spectra, and should be revealed by the multivariate analysis in the same way that ^{1}H NMR profiles have been used to differentiate between *Arabidopsis* ecotypes (Ward et al., 2003).

Thirdly, although it was not feasible to examine directly the potential impact of all possible [^{13}C]glucose species that might be exploited in plant metabolic flux analysis, the ^{13}C-substrates were chosen to cover some of the most important labelling strategies. Both [1-^{13}C]glucose and 10% [U-$^{13}C_6$]glucose (or a similar low proportion) are commonly used to introduce label into plant metabolic networks (Ratcliffe and Shachar-Hill, 2006) and these treatments are indicative of the conditions that may be encountered in a typical analysis. In contrast, 100% [U-$^{13}C_6$]glucose is unsuitable for steady state flux analysis, since it leads to uniform labelling of the whole system. However, this would be expected to provoke the maximum possible isotopic effect, and so the metabolic response of cells grown in 100% [U-$^{13}C_6$]glucose is likely to encompass that of cells supplied with respiratory substrates that are ^{13}C-enriched to a lower degree. It is striking that even when grown at this high level of ^{13}C-isotopic enrichment, metabolism in the *Arabidopsis* cell culture was not measurably affected.

At present there is no simple explanation for the marked difference between *Paracoccus*, which showed considerable sensitivity to the ^{13}C-composition if its growth medium (Dunstan et al., 1990a,b), and the results obtained here for *Arabidopsis*. Metabolically, *Paracoccus* differs considerably from *Arabidopsis* in possessing active Entner–Doudoroff and G6 pathways, and lacking a functional glycolytic sequence. However, it seems unlikely that differences in kinetic isotope effects on the component steps of these pathways would be sufficient to account for the marked differences in sensitivity to the isotopic composition of respiratory substrate. The earlier researchers suggested that the unexpected sensitivity of *Paracoccus* to the positional isotopic labelling of glucose might arise as a consequence of differential expression of the bacterial genome resulting in differences in the levels of enzyme induction in response to different isotope distributions (Dunstan et al., 1990b). If correct, it is plausible to envisage such an effect being more significant in an organism with a faster growth rate. However, such ideas remain speculative.

Nevertheless, irrespective of the impact of ^{13}C-enriched glucose on metabolism in *Paracoccus*, or its origin, this response is not an inevitable or universal phenomenon. The data demonstrate that there is no detectable effect of ^{13}C abundance on the principal pathways of carbon metabolism in *Arabidopsis*, and that there is no reason to believe that growth of plants in ^{13}C-labelled respiratory substrates will necessarily alter metabolism. This finding is also relevant to the current interest in using plants that have been uniformly labelled with either ^{13}C or ^{15}N for metabolomic analysis (Harada et al., 2006; Ippel et al., 2004; Kikuchi et al., 2004) since it provides evidence in support of the assumption that the isotopic effects of the labelling strategy will be negligible. In conclusion metabolic flux analysis

based on steady-state ^{13}C labelling does not in itself inevitably distort the fluxes through the metabolic network that it seeks to measure, thus removing a potential obstacle for its increased application in studies of plant metabolism.

4. Experimental

4.1. Materials

Cell suspension cultures of *A. thaliana* (L.) Heynh ecotype Landsberg *erecta*, derived from an original stock established by May and Leaver (1993), were maintained in Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 160 mM glucose, 0.5 μg ml^{-1} naphtheleneacetic acid and 0.05 μg ml^{-1} kinetin at 22 °C in the light in a controlled environment aerated on an orbital shaker at 100 rpm. The suspension was subcultured every 7 d by adding 10 ml of culture to 90 ml of fresh MS medium in a 250 ml conical flask. To examine the influence of ^{13}C-enrichment on metabolism, 5 ml cell suspension was sub-cultured into 50 ml growth medium containing unlabelled glucose (with ^{13}C at natural abundance), 100% [1-^{13}C]glucose, 100% [U-$^{13}C_6$]glucose or 10% [U-$^{13}C_6$] glucose + 90% unlabelled glucose and incubated for 5 d in darkness prior to analysis. During this period the fresh weight of the cells approximately tripled. All analyses were conducted on cell cultures grown simultaneously and initiated with inocula from the same stock culture.

[1-^{13}C]glucose (specific abundance 99 atom%) and [U-$^{13}C_6$]glucose (specific abundance 99 atom%) were from Isotec (http://www.sigmaaldrich.com/). [1-^{14}C]glucose (specific activity 2.00 GBq $mmol^{-1}$), [6-^{14}C]glucose (specific activity 2.07 GBq $mmol^{-1}$), and [U-^{14}C]glucose (specific activity 0.14 GBq $mmol^{-1}$) were purchased from Amersham Biosciences (http://www1.gelifesciences.com/). [1-^{14}C]gluconate (specific activity 2.04 GBq $mmol^{-1}$) and [3,4-^{14}C]glucose (specific activity 1.11 GBq $mmol^{-1}$) were obtained from American Radiolabeled Chemicals Inc. (http://www.arc-inc.com/). [2-^{14}C]glucose (specific activity 1.67 GBq $mmol^{-1}$) was from PerkinElmer NEN (http://las.perkinelmer.com/). All enzymes were from Roche Diagnostics Ltd. (http://www.roche.com/). General chemicals and chromatography resins were purchased from Sigma–Aldrich (http://www.sigmaaldrich.com/) or Merck (http://www.merckbiosciences.co.uk/).

4.2. Incubation of Arabidopsis cell culture with ^{14}C-labelled substrates

A 4 ml aliquot of an *Arabidopsis* cell suspension grown for 5 d in a medium containing glucose of defined ^{13}C-enrichment was incubated in the dark at 22 °C in a 100 ml conical flask. The incubation was started by addition of 0.1 ml MS medium containing 3.7 kBq specifically labelled [^{14}C]glucose, 3.7 kBq [1-^{14}C]gluconate or 37 kBq [U-^{14}C]glucose. Each flask was then sealed with a rubber bung and aerated on an orbital shaker at 100 rpm. For incubations involving [1-^{14}C]gluconate, the concentration of gluconate in the incubation medium was adjusted to 0.3 mM.

Released $^{14}CO_2$ was collected in 0.5 ml of 10% ($^{w}/_{v}$) KOH in a vial suspended in the flask. For incubations involving specifically labelled substrate, the KOH solution was replaced every two hours for 12 h and again 24 h and 48 h after the beginning of the incubation. Incubations with [U-^{14}C]glucose were conducted in the absence of an alkaline trap in 100 ml conical flasks each fitted with a suba-seal bung from which was suspended an empty 1.5 ml vial. After 4 h the incubation was terminated by injecting 1 ml 25 M HCO_2H into the incubation medium through the bung, and 0.5 ml 10% KOH was injected into the empty vial suspended in the flask. The flask was incubated for a further 1 h with shaking at 22 °C and then at 4 °C for 8 h prior to extraction of the incubation mixture.

4.3. Extraction of Arabidopsis cell culture using ethanol

The acidified incubation mixture was quantitatively transferred to a 15 ml tube using a total of 6 ml EtOH. The tube was sealed and incubated at 80 °C for 60 min. After cooling, the extract was centrifuged at 3500*g* for 5 min and the supernatant was removed. The pellet was washed with 5 ml EtOH three times and the washings added to the supernatant. The combined soluble fraction derived from each sample was reduced to dryness by rotary evaporation at 40 °C and redissolved in 5 ml 50 mM Na–OAc buffer (pH 5.5). The insoluble residue following ethanol extraction was resuspended in water to a final volume of 2 ml. All fractions were stored at −80 °C prior to fractionation.

4.4. Fractionation of ^{14}C-labelled extracts

The soluble fraction obtained following extraction in EtOH was separated into acidic, basic and neutral components by ion-exchange chromatography through Dowex 50 W × 8-200 (H^+ form) and Dowex 1 × 8-200 (OAc^- form – generated from the Cl^- form by treating 50 g of resin with 100 ml 1 M Na–OAc followed by 150 ml 0.1 M HOAc) using a Visiprep solid-phase extraction vacuum manifold (Supelco, http://www.sigmaaldrich.com/). Up to 1 ml soluble fraction (at pH 5–6) followed by 15 ml of H_2O were passed through 0.5 ml columns of Dowex 50 and Dowex 1 linked in series. The effluent passing through both columns constituted the neutral fraction (consisting mainly of soluble sugars). Basic components (principally amino acids) were eluted from the Dowex 50 resin using 5 ml 1 M NH_4OH, and acidic compounds (largely organic acids and phosphate esters) were eluted from the Dowex 1 resin using 5 ml 4 M HCO_2H (Canvin and Beevers, 1961). An aliquot of the basic fraction recovered from the Dowex 50 column was lyophilised, redissolved in H_2O, and

chromatographed through the anion exchange resin (Dowex 1), as described above, to separate acidic amino acids (mainly glutamate and aspartate, which were retained by the resin) from the neutral and basic amino acids (which did not bind to the column) as described by Cossins and Beevers (1963). The neutral fraction was evaporated to dryness, resuspended in water, and further fractionated by TLC as described by Scott and Kruger (1995).

The EtOH-insoluble fraction was autoclaved for 3 h at 121 °C (104 kPa). A 1 ml aliquot of the suspension was digested by incubation with 400 μl 200 mM Na–OAc (pH 5.8) containing 10 U amyloglucosidase and 2 U amylase for 16 h at 37 °C, followed by addition of 400 μl 400 mM Tris–Cl (pH 7.8) containing 10 U pronase (protease XIV – *Streptomyces griseus*) and incubation for a further 16 h at 37 °C. The digest was centrifuged at 13,000*g* for 5 min. Ion-exchange chromatography, as described above, was used to separate neutral and basic fractions of the resulting supernatant, arising from starch and protein, respectively. The acidic fraction of the supernatant and the undigested residue were assumed to derive from cell wall components.

4.5. Determination of radioactivity

The amount of ^{14}C in aqueous samples was determined by liquid scintillation counting after addition of four volumes of Optiphase 'HiSafe' 3 (Wallac, http://las.perkinelmer.co.uk/). The efficiency of counting was typically greater than 90%.

4.6. Perchloric acid extraction of Arabidopsis cell culture for NMR analysis

Cells from 25 ml of an *Arabidopsis* suspension culture grown for 5 d in a medium containing glucose of defined ^{13}C-enrichment were recovered by vacuum filtration through glass fibre paper on a Buchner funnel and washed with about 150 ml of MS medium (from which glucose and plant growth substances were omitted). The washed cells were frozen in liquid nitrogen and stored at −80 °C prior to extraction.

Frozen cells (about 2.5 g) were ground to a fine powder in liquid nitrogen in a mortar and pestle and combined with 4 ml 3 M $HClO_4$. The frozen mixture was transferred to a 50 ml centrifuge tube and allowed to thaw at 0 °C. The homogenate was supplemented with 10 ml 1 M $HClO_4$ and then centrifuged at 48,000*g* for 15 min at 4 °C. The resulting supernatant was retained, and the pellet was extracted using a further 15 ml 1 M $HClO_4$ and centrifuged, as before. The supernatant was retained, and the pellet was re-extracted using 15 ml H_2O and centrifuged. The resulting supernatant and pellet were separated. The supernatants obtained from an individual sample were combined, and adjusted to pH 5.0 using 2 M KOH. Insoluble $KClO_4$ was removed by centrifugation, and the resulting supernatant was lyophilised and redissolved in 1 ml H_2O. The sample was centrifuged at 13,500*g* for 5 min, and the resulting supernatant lyophilised before being redissolved in H_2O and diluted to a final volume of 2.5 ml containing 10 mM EDTA and 10 mM Na-Pi and adjusted to pH 7.5. The sample was finally lyophilised and redissolved in 2.5 ml D_2O containing 25 mM 1,4-dioxane.

4.7. NMR spectroscopy

^{13}C-decoupled ^{1}H NMR spectra of the acid-soluble fractions from *Arabidopsis* cell extracts were recorded at 20 °C on a Varian Unity Inova 600 spectrometer (http://www.varian.com/) using a 5-mm diameter $^{1}H\{^{13}C/^{15}N\}$ triple resonance probe, a 90° pulse angle, a 6000 Hz spectral width, a 60 s relaxation delay to minimise heating effects, a 2 s acquisition time with GARP ^{13}C decoupling, a decoupler modulation frequency of 30000 Hz, and 96 transients per sample. Free induction decays were processed with a 1 Hz line broadening using Nuts for Windows (Acorn NMR Inc., http://www.acornnmr.com/). ^{1}H NMR chemical shifts in the spectra were referenced to the dioxane signal at 3.692 ppm.

NMR spectra were converted to numerical values and stored as ASCII files using the histogram routine in Nuts. The −0.1 to 9 ppm region of the spectrum was reduced to a set of bins at 0.01 ppm resolution, each bin corresponding to 16 data points. The bins for the dioxane (3.63–3.69 ppm) and water (4.61–4.96 ppm) signals were removed, and the values for the remaining 836 bins were normalised relative to their summed value.

4.8. Statistical analysis

Multivariate analysis of variance (MANOVA) and repeated measures analysis of variance (ANOVA) based on Type III sums-of-squares were performed using SPSS 12.0 for Windows (SPSS, http://www.spss.com/). Prior to analysis, percentage values and ratios were subjected to arcsine $[\arcsin(y/100)^{0.5}]$ and log transformation, respectively, to reduce potential departures from normality and homoscedasticity (Wardlaw, 1985). Homogeneity of variance of the dependent variable(s) in MANOVA and repeated measures ANOVA was confirmed using Levine's test prior to assessing the significance of between-subject effects (isotopic composition of growth medium).

Principal component analysis (PCA) of metabolite fingerprints obtained by ^{1}H NMR analysis was performed using SPSS. The principal components were extracted from the binned data using either the covariance matrix or the correlation matrix. The outputs were, additionally, subjected to orthogonal rotation to focus the contribution of each bin into a single principal component. This was achieved using the Varimax procedure which also maximises the dispersal of bins between component axes. Factor scores for individual samples were obtained using the Anderson–Rubin method that yields uncorrelated mean-normalised scores scaled to unit variance for each component (Field, 2005).

Analysis of similarity (ANOSIM) of ^{1}H NMR metabolite fingerprints was conducted using VEGAN (version 1.8-5, http://cc.oulu.fi/~jarioksa/softhelp/vegan.html) an add-on to the R statistics package (version 2.4, http://www.r-project.org/). Dissimilarity between samples was based on the Euclidean distance in n-component space between samples, obtained during PCA of the binned spectral data. Problems of autocorrelation and duplication among the original 640 bin scores were removed by using the orthogonal eigenvector scores.

Statistical comparisons for which $P < 0.05$ are considered significant.

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