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RESEARCH PAPER

Methyl-β-D-glucopyranoside in higher plants: accumulation and intracellular localization in *Geum montanum* L. leaves and in model systems studied by ^{13}C nuclear magnetic resonance

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Abstract

Using ^{13}C-NMR, methyl-β-D-glucopyranoside (MeG) was characterized as a major compound in the leaves of the alpine herb *Geum montanum* L. MeG continuously accumulated during the life span of *G. montanum* leaves, and accounted for up to 20% of the soluble carbohydrates in aged overwintering leaves, without being reallocated during senescence. Incubating intact plant tissues, culture cells, and purified organelles with ^{13}C-labelled substrates showed that MeG was synthesized in the cytosol of cells, directly from glucose and methanol molecules. There was no contribution of the C-1 pathway. MeG was subsequently stored in the vacuole without being re-exported to the cytoplasm. All the dicots tested contained the enzymatic machinery permitting MeG synthesis from methanol and glucose, but the plants accumulating this compound at concentrations higher than 1 μmol g^{-1} wet wt were mainly members of the Rosaceae family belonging to the Rosoideae subfamily. It is suggested that the synthesis of MeG may contribute to reduce the accumulation in the cytoplasm of methanol and its derived compounds.

Key words: *Geum montanum*, leaf life span, methyl-β-D-glucopyranoside, NMR spectroscopy, pathway compartmentation, taxonomic distribution.

Introduction

In this paper, ^{13}C-NMR was used to analyse the metabolic pattern of the herb *G. montanum*, which grows in the subalpine and alpine belts of the Alps. This plant has been chosen as a model to study plant adaptations to extreme environmental conditions and, in particular, the resistance to photoinhibition (Manuel *et al*., 1999). The present study revealed that methyl-β-D-glucopyranoside (MeG) is a major soluble compound in *G. montanum* leaves and other plants of the Rosaceae family. Using HPLC, MeG has been identified as a soluble compound in rose petals (Ichimura *et al*., 1997) and it has also been reported in suspension-cultured sycamore cells after incubation in the presence of methanol (Gout *et al*., 2000). However, little was known about the location of this compound in plants (except rose petals), its synthesis pathway, intracellular compartmentation, and cellular function.

These questions are addressed in this paper using *G. montanum* leaves and suspension-cultured sycamore

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Abbreviations: $H_4PteGlu_n$, 5,6,7,8 tetrahydropteroylpolyglutamate (tetrahydrofolate); MeG, methyl-β-D-glucopyranoside; SAM, *S*-adenosylmethionine; PCA, perchloric acid; asl, above sea level.

cells, a material more convenient for cell fractionation. The results show that MeG was synthesized in the cytosol of cells, from glucose and methanol, and that it was stored in the vacuole and not metabolized further. An overview of the taxonomic distribution of MeG in various plants is also given and its possible role is discussed.

Materials and methods

Leaves from plants belonging to various Rosaceae and other families were collected between 10.00 h and 11.00 h during the summer season, on the dates indicated in the figure legends, in the following places: (i) in the Alpine Botanical Garden of the Col du Lautaret (French Alps, 2100 m asl) and in the surrounding fields; (ii) in the arboretum of Grenoble University Joseph Fourier (220 m asl) and in the fields near Grenoble. Leaves were kept at melting ice temperature during transport to the laboratory. For PCA extraction, they were frozen in liquid nitrogen. Suspension-cultured cells from *Daucus carota, Silene vulgaris*, and *Zea mays* strains were also used.

For labelling experiments, leaves were cut into small pieces (*c*. 10 mm^2) with a razor blade, floated on Murashige and Skoog (MS) medium containing labelled substrates, as indicated in figure legends, and gently infiltrated (Dorne and Bligny, 1993). The infiltrated leaf pieces were then placed in a flask containing 200 ml of the infiltration medium and agitated under the light (300 μmol photons m^{-2} s^{-1}) for 1 d. Suspension-cultured sycamore cells were cultivated and incubated as described in Gout *et al*. (2000). Leaf pieces and suspension-cultured cells were rapidly rinsed at the end of incubation and frozen in liquid nitrogen.

PCA extracts were prepared from 10 g (fresh weight) frozen samples. Samples were ground to a fine powder with a mortar and pestle with 1 ml of 70% (v/v) perchloric acid, at liquid nitrogen temperature. The frozen powder was then placed at −10 °C and thawed. The thick suspension obtained was centrifuged at 15 000 *g* for 10 min to remove particulate matter, and the supernatant was neutralized with 2 M $KHCO_3$ to about pH 5.0. The supernatant was then centrifuged at 10 000 *g* for 10 min to remove $KClO_4$ and the resulting supernatant was lyophilized. This freeze-dried material was dissolved in 2.5 ml water containing 10% D_2O and stored frozen at −20 °C.

Vacuoles, chloroplasts, and mitochondria were isolated from protoplasts. Protoplasts were prepared from 12 g fresh material using a cocktail of enzymes as stated by Pugin *et al*. (1986). The purification of vacuoles from protoplast contamination was carried out using a discontinuous gradient of Ficoll 400. The gradient was prepared in a buffer of 0.7 M mannitol, 25 mM MES/TRIS, 2 mM EDTA (pH 7.0) (buffer A). After centrifugation at 150 000 *g* for 1 h (Beckman 70 Ti rotor), liberated vacuoles were located at two different interfaces; 'heavy vacuoles' and 'light vacuoles' corresponding to bands at the 6/4 and 4/0 interfaces (numbers refer to the percentage of Ficoll), respectively. The two vacuole populations were combined and washed with buffer A, and, subsequent to a centrifugation step at 300 *g* for 10 min, the vacuolar pellet was resuspended in 2.5 ml of buffer A containing 10% D_2O for NMR analyses. The purifications of chloroplasts and mitochondria were done from crude tissues or from protoplasts according to Douce and Joyard (1982), and Neuburger *et al*. (1982), respectively.

Spectra of neutralized PCA extracts and vacuole suspensions were recorded on a NMR spectrometer (AMX 400, Bruker Billerica MA) equipped with a 10 mm multinuclear probe tuned at 161.9 MHz for ^{13}C-NMR studies. The deuterium resonance of ^2H_2O (100 μl added ml^{-1} of extract) was used as a lock signal. ^{13}C-NMR acquisition conditions: 90° pulses (19 μs) at 6 s intervals; spectral width, 20 kHz; Waltz-16 ^{1}H decoupling sequence with 2.5 W decoupling during acquisition and 0.5 W during the delay; free induction decays collected as 32 000 data points, zero-filled to 64 000, and processed with a 0.2 Hz exponential line broadening. Spectra were referenced to hexamethyldisiloxane at 2.7 ppm. Mn^{2+} ions were chelated by the addition of 1 mM CDTA.

The identification of the peaks of resonance was done by comparing the spectra of standard solutions of known compounds at pH 7.5 with that of the PCA extracts. The definitive assignments were made after running a series of spectra of the extracts added with authentic compounds, at different pHs, according to previous publications (Aubert *et al*., 1996*a*). To quantify the compounds identified on the spectra accurately, the surfaces of their different resonance peaks were referred to that of the C-2+C-3 peak of maleate (100 μmol) added to samples before grinding. 20 s recycling time was used to obtain fully relaxed spectra. Biochemical measurements were carried out according to Bergmeyer (1974).

In order to evaluate the rate of contamination of the purified vacuoles by cytoplasm, the following marker enzymes were used: cytosol, alcohol dehydrogenase; mitochondria, fumarase; plastids, and ADP-glucose pyrophosphorylase. Assays were performed according to previous publications (Journet and Douce, 1985; Lunn *et al*., 1990).

Labelled substrates, 99% [^{13}C]methanol, 99.9% [^{12}C]methanol (^{13}C-depleted), 99% [1-^{13}C]glucose, 99.9% [U-^{12}C]glucose (^{13}C-depleted), 99% [2-^{13}C]glycine, and 99% [3-^{13}C]serine were purchased from Leman (Archamps, France). Other chemicals were purchased from Sigma-Aldrich (St Louis).

Results

Identification of MeG in the leaves of G. montanum

A typical ^{13}C-NMR spectrum from *G. montanum* leaves collected at the Col du Lautaret is shown in Fig. 1. Among the various solutes accumulated in this alpine plant, sucrose, glucose, and fructose were the major carbohydrates, with cell contents estimated as 65–70, 23–25, and 20–22 μmol g^{-1} wet wt, respectively. Malate (20–22 μmol g^{-1} wet wt) was the most abundant organic acid. Of particular interest were seven resonance peaks centred at 58.00, 61.58, 70.35, 73.99, 76.69, 76.71, and 104.00 ppm not yet reported in leaf extracts (Shachar-Hill and Pfeffer, 1996). These peaks were attributed to methyl-β-D-glucopyranoside (MeG), based on the following observations. First, adding authentic MeG to the extract enhanced the seven resonance peaks. The two peaks centred at 58.00 and 104.00 ppm (Fig. 1, insets) correspond to the methyl group and to C-1, respectively. They are specific to MeG and clearly separated from the resonance peaks of other sugars and other related compounds such as methyl-α-D-glucopyranoside, methyl-α-D-galactopyranoside, and 3-*O*-methyl-glucose. Second, using gas chromatography and mass spectroscopy according to Dejongh *et al*. (1974), it was observed that the mass spectra of the trimethyl derivatives of the putative *G. montanum* MeG and of the authentic MeG were identical (results not shown; Gout *et al*., 2000). Third, when *G. montanum* leaves were incubated with [^{13}C]methanol, the resonance corresponding to the methyl group of MeG (at 58.00 ppm) was specifically enhanced (see below; Figs $3A_2$, 6). Finally, when *G. montanum* leaves were incubated in the presence of

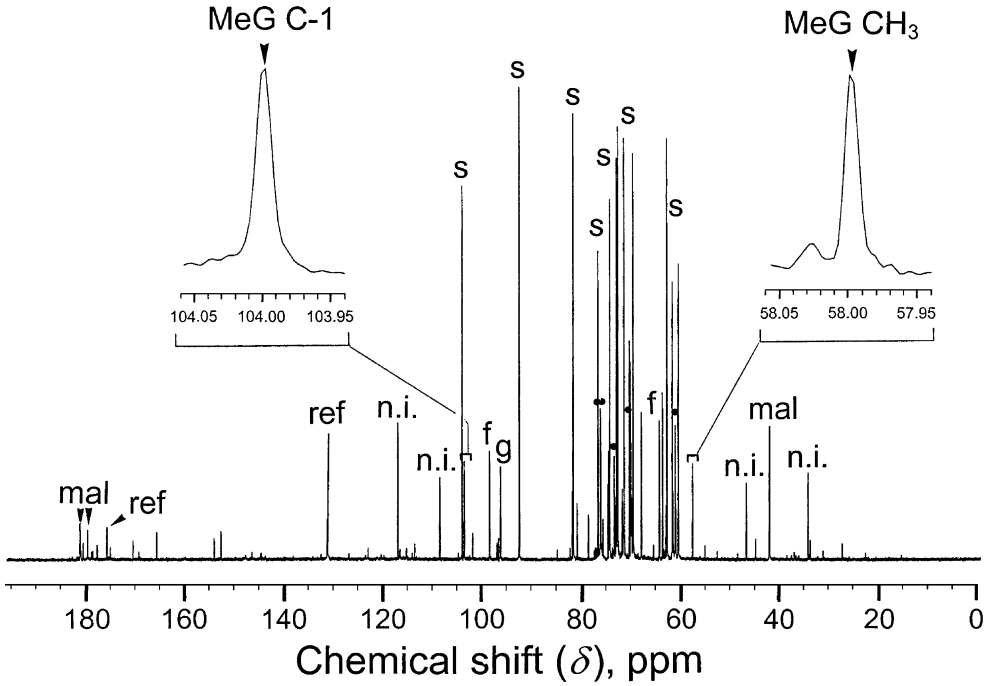

Fig. 1. Representative proton-decoupled *in vitro* ^{13}C-NMR spectrum of *G. montanum* leaves (PCA extract). This spectrum, recorded at 20 °C, is the result of 3600 transients with a 6 s repetition time (6 h). The PCA extract was prepared from adult (1-month-old) *G. montanum* leaves harvested during the first week of July at the Lautaret pass (2100 m asl) at 10.00 h, according to the procedure described in the Materials and methods. Insets, expanded scales (magnification, ×2) showing two of the seven resonance peaks of MeG, corresponding to the methyl group (58.00 ppm), and to the C-1 (104.00 ppm). The positions of the other MeG resonance peaks are indicated by solid circles. Peak assignments are as follows: MeG, methyl-β-D-glucopyranoside; s, sucrose; g, glucose; f, fructose; mal, malate; n.i., not identified; ref, reference (100 μmol maleate).

[1-^{13}C]glucose and [^{13}C]methanol, the existence of spin-spin interaction peaks at 104.00 and 58.00 ppm (Fig. 3A_4) demonstrated that the newly formed compound was glucose methylated on C-1. Measurements done from the spectrum shown in Fig. 1 indicate that the concentration of MeG in 1-month-old leaves harvested at the beginning of July was 17–19 μmol g^{-1} wet wt.

MeG is probably the unidentified abundant glucoside formerly observed by Bonnier and Douin (1911–1935) in ethanolic extracts of different plant species belonging to the genus *Geum*.

Distribution of MeG in G. montanum *during the growth cycle*

MeG was detected in *G. montanum* leaves, rhizome, and roots, but it was abundant only in leaves (Fig. 2). It increased in *G. montanum* leaves during ageing, accounting for up to 20% of soluble carbohydrates before senescence in overwintering leaves. MeG was not likely to be reallocated to roots and rhizome, since these storage organs contained only low levels of MeG throughout the vegetation season (<3 μmol g^{-1} wet wt in rhizomes and barely detectable in roots). By contrast, sucrose and glucose declined in old leaves, whereas they increased in rhizomes and roots, reflecting a probable reallocation of these sugars. Similarly, the highest levels of MeG were observed in overwintering leaves of the alpine Rosaceae plant *Dryas octopetala*, whereas it remained very low in the subterranean organs of this plant (not shown).

These results suggested that, after synthesis, MeG was not metabolized further. In agreement with this hypothesis, it was observed that MeG did not decline in leaves kept in the dark for several days, in contrast to sucrose and glucose which were consumed to sustain respiration (data not shown).

MeG synthesis pathway in G. montanum *and in sycamore cells*

It has been shown that the synthesis of MeG by suspension-cultured sycamore cells requires the addition of methanol to the incubation medium (Gout *et al*., 2000). By contrast, *G. montanum* leaves naturally accumulate large amounts of MeG (Fig. 1). This raises the question of the origin of the methyl group linked by a glucosidic bond to the C-1 of glucose in *G. montanum*: is it the C-1 pathway or methanol produced by the plant? According to Fall and Benson (1996) methanol is produced by pectin demethylation in expanding leaves. If this is the case in *G montanum*, methanol can be glycosylated directly or used as a source of methyl groups through folate-mediated single-carbon metabolism involving glycine and serine (Cossins, 1964; Gout *et al*., 2000). In order to know whether glucose was directly methylated via a glycosylation reaction or indirectly via the

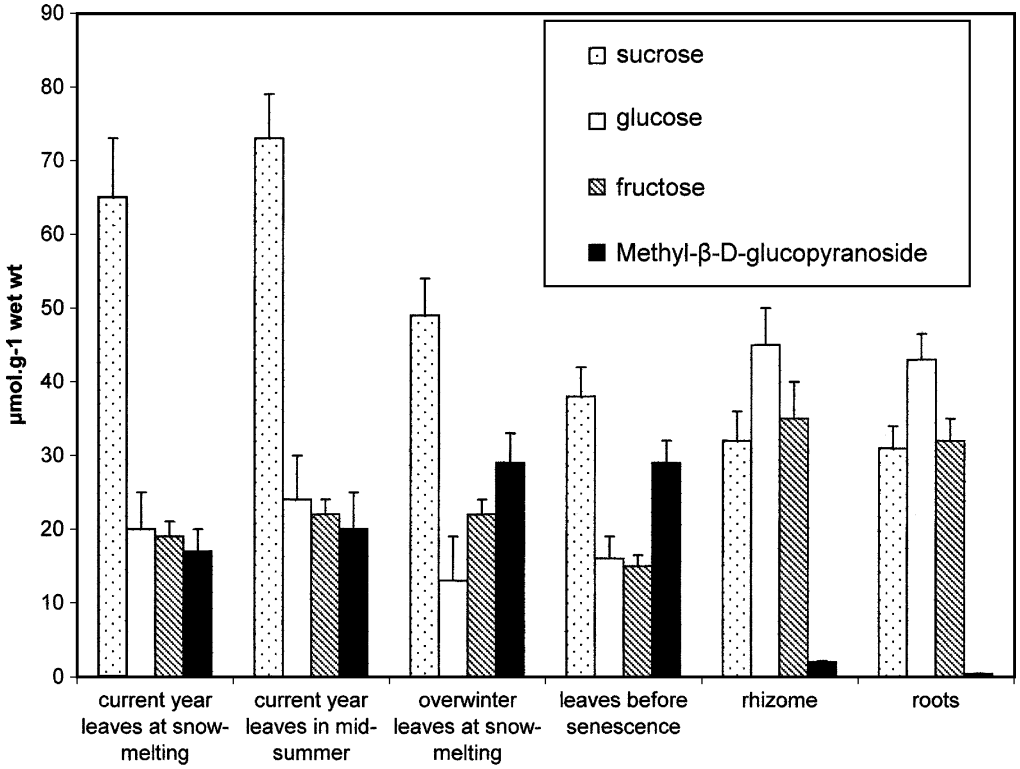

Fig. 2. Seasonal variation and organ-specific distribution of MeG, sucrose, fructose, and glucose in *Geum montanum* leaves. Quantification was done from metabolite profiles obtained using ^{13}C-NMR as described in the Materials and methods. Values are given as means ±SE (n=3).

C-1 pathway, or both, *G. montanum* leaf pieces were incubated in the presence of either [^{13}C]methanol or [2-^{13}C]glycine (or [3-^{13}C]serine).

Figure 3 shows the synthesis of MeG in *G. montanum* leaf tissues and suspension-cultured cells incubated in the presence of 5 mM [^{13}C]methanol (A_2, B_2) and 10 mM unlabelled glucose; 5 mM [^{12}C]methanol (unlabelled) and 10 mM [1-^{13}C]glucose (A_3, B_3); 5 mM [^{13}C]methanol and 10 mM [1-^{13}C]glucose (A_4, B_4). The portions of spectra shown are centred on C-1 and the methyl group of MeG at 104.00 and 58.00 ppm, respectively. The rate of methanol incorporation in *G. montanum* leaves estimated from the MeG CH_3 peak (A_2) was 0.23–0.27 µmol h^{-1} g^{-1} wet wt. The value measured from the MeG C-1 peak (A_3) was lower by *c*. 50%. This discrepancy may have two origins: (i) the presence of significant stores of unlabelled endogenous glucose (Fig. 1); (ii) the partial redistribution of glucose labelling from C-1 to C-6. Indeed, the redistribution of the C-1 and C-6 carbons of glucose is due to the cytosolic pathway cycle involving the conversion of hexose phosphates to triose phosphate, interconversion of triose phosphate by triose phosphate isomerase, and resynthesis of hexose phosphate in the cytosol (Keeling *et al.*, 1988). This cycling of hexose phosphate via triose phosphate is sustained by pyrophosphate: fructose-6-phosphate 1-phosphotranferase itself activated by fructose 2,6-bisphosphate (Fernie *et al.*, 2001). The authors observed that the redistribution of label from C-1 to C-6 could reach 25% in tobacco callus. When tissues were incubated in the presence of [^{13}C]methanol and [1-^{13}C]glucose, MeG C-1 split into a doublet, reflecting nuclear spin-spin interactions between the ^{13}C-1 and the next ^{13}C of the methyl group of MeG (A_4). Since the peak corresponding to the ^{13}C-1 of MeG was entirely split into two equivalent peaks, this demonstrates that MeG synthesized from newly incorporated glucose is methylated only by added methanol (99% ^{13}C-labelled), and proves that no significant internal pool of methanol, or donor of methyl groups was involved. By contrast, the fact that only 25–30% of MeG CH_3 split in a doublet (arrows) suggests that an important portion of newly synthesized MeG originated from unlabelled intracellular stores of glucose. In order to confirm this point, sycamore cells, which contain very little free hexoses (Gout *et al.*, 2000) and no NMR-detectable MeG (B_1), were utilized in parallel experiments. In this case, MeG was synthesized at a rate of 0.050–0.058 µmol h^{-1} g^{-1} wet wt when 5 mM methanol was added to the incubation medium (B_2, B_3, B_4). The spectrum B_4 shows that glucose was methylated only by added methanol (99% ^{13}C-labelled) since all C-1 split in a doublet, as in *G. montanum* leaves (A_4). In sycamore cells, however, *c*. 50% of MeG CH_3 split in a doublet (arrows), showing that a higher proportion of MeG molecules originated from the methylation of added [1-^{13}C]glucose. In this study's cell culture conditions, the above-mentioned redistribution of ^{13}C between carbon

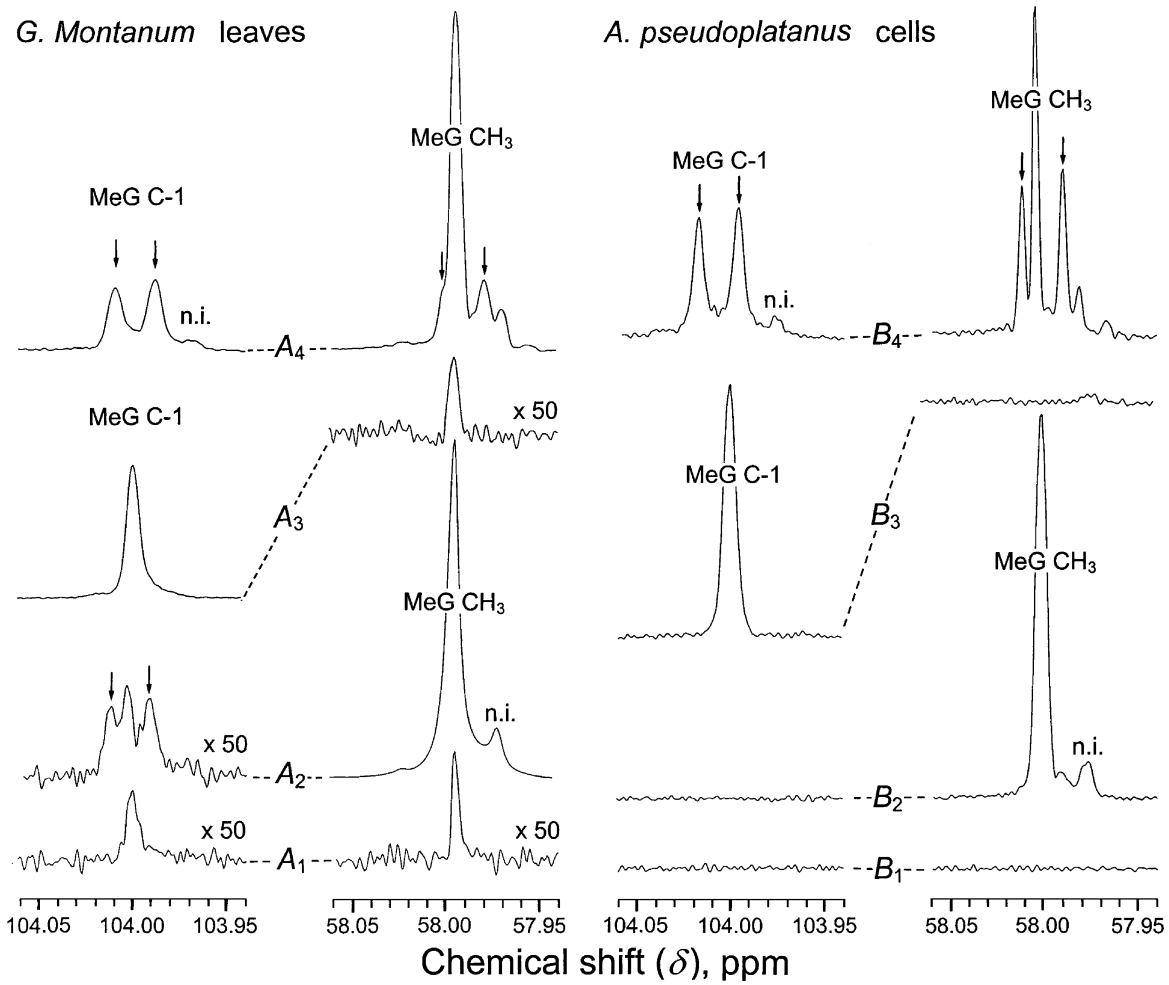

Fig. 3. Representative proton-decoupled *in vitro* ^{13}C-NMR spectra (expanded scales centred around 58 ppm and 104 ppm) showing the synthesis of MeG in *G. montanum* leaf fragments (*A*), and suspension-cultured sycamore cells (*B*) after labelling with 99% [^{13}C]methanol or 99% [1-^{13}C]glucose, or both. PCA extract preparation and NMR acquisition parameters were as described in the Material and methods; number of transients, 225 (22.5 min). Tissue fragments and cells were incubated for 1 d in the presence of: 5 mM [^{13}C]methanol and 10 mM [U-^{12}C]glucose (A_2, B_2); 5 mM [^{12}C]methanol and 10 mM [1-^{13}C]glucose (A_3, B_3); 5 mM [^{13}C]methanol and 10 mM [1-^{13}C]glucose (A_4, B_4). A_1 and B_1, reference spectra at time zero (no added substrate). Peak assignments are as in Fig. 1.

atoms 1 and 6 of glucose was *c*. 25% (not shown). The remaining 25% of MeG not labelled on C-1 probably corresponded to the methylation of glucose released from sucrose or starch stores. These results together suggest that a glycosylation process in the presence of glucose and methanol took place. However, the possibility that methyl groups originating from added methanol were also transferred to glucose via folate-mediated metabolism was not excluded.

To determine if the synthesis of MeG also involved the C-1 pathway via $CH_3H_4PteGlu_n$, *G. montanum* leaves were incubated in the presence of 5 mM [2-^{13}C]glycine over 40 h. Figure 4 shows that, besides the accumulation of [2-^{13}C]glycine (C-2 at 42.40 ppm), various molecules were labelled, notably serine at positions C-2 (triplet at 61.28, 61.10, and 60.92 ppm) and C-3 (triplet at 57.48, 57.30, and 57.12 ppm). Note that the triplet signals correspond to the doublet due to nuclear spin-spin interactions between C-2 and C-3 carbon atoms of serine labelled on the same molecule and the single labelled isotopomer (central peak). Similarly, β-hydroxypyruvate, originating from serine transamination, appeared as a triplet on C-2 (67.13, 66.94, and 66.75 ppm) and C-3 (not shown in the figure), as well as alanine (transamination product of pyruvate) with a C-2 triplet at 51.63, 51.45, and 51.27 ppm). These results confirm the observation in *Arabidopsis* leaves (Prabhu *et al*., 1996) and in sycamore cells (Mouillon *et al*., 1999), where the incorporation of [2-^{13}C]glycine resulted in serine labelling at both positions C-2 and C-3, reflecting the involvement of $^{13}CH_3H_4PteGlu_n$ through glycine decarboxylase and serine hydroxymethyltransferase activities. By contrast, no increase of the resonance peak at 58.00 ppm compared with the peak at 104.00 ppm was noticed. This indicates that the methyl group of MeG was not labelled following the addition of [2-^{13}C]glycine. Similarly, it was verified that no labelled MeG accumulated after incubating leaves with

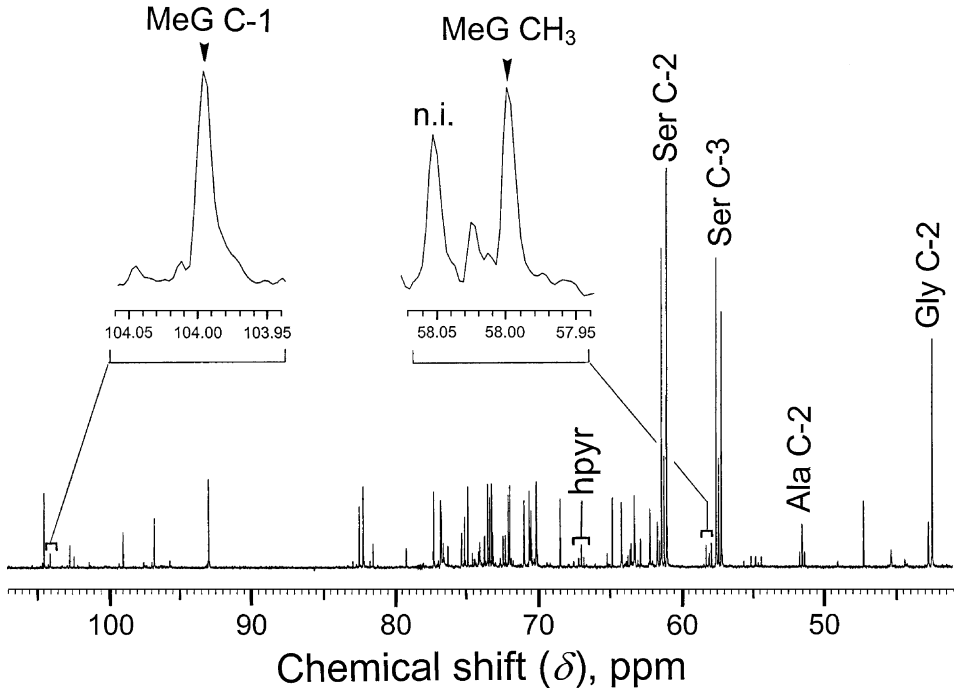

Fig. 4. Representative proton-decoupled *in vitro* ^{13}C-NMR spectrum (scale from 41 ppm to 107 ppm) of *G. montanum* leaf fragments incubated for 40 h in the presence of 5 mM [2-^{13}C]glycine. PCA extract preparation and NMR acquisition parameters were as described in the Materials and methods; number of transients, 3600 (6 h). Insets, expanded scales (magnification identical to that of Fig. 1) showing two of the seven resonance peaks of MeG, corresponding to the methyl group (58.00 ppm), and to the C-1 (104.00 ppm). Peak assignments are as follows: MeG, methyl-β-D-glucopyranoside; Ala, alanine; Gly, glycine; hpyr, β-hydroxypyruvate; Ser, serine; n.i., not identified.

[3-^{13}C]serine (not shown). Consequently, these results demonstrate that, as in sycamore cells (Gout *et al.*, 2000), the folate-mediated single-carbon metabolism was not involved in the synthesis of MeG in *G. montanum* leaves. They also confirm the requirement of methanol for MeG synthesis.

Finally, incubating *G. montanum* leaf tissues or sycamore cells in the presence of [^{13}C]methanol and under anoxic conditions showed little difference concerning MeG synthesis (not shown result). Since the pools of glycolysis intermediates are very low in plant cells in the absence of oxygen (Gout *et al.*, 2001), it seems unlikely that MeG synthesis started at the level of hexoses (or trioses) phosphate. It seems also unlikely that this synthesis required an energy supply.

Subcellular compartmentation of MeG and its synthesis

In order to determine the subcellular location of MeG between cytoplasmic and vacuolar compartments, the purification of cell organelles was tried. However, since it was difficult to purify intact organelles from *G. montanum*, experiments were done with suspension-cultured cells which are more convenient for this kind of fractionation (Pugin *et al.*, 1986). Vacuoles were purified and MeG was compared with sucrose and malate, assuming that these compounds are almost exclusively stored in vacuoles (Gout *et al.*, 1993). Sycamore cells chosen for this experiment were first incubated with [^{13}C]methanol over 2 d, resulting in a significant accumulation of ^{13}C-MeG (Fig. 5*A*). The ^{13}C-NMR spectrum obtained from purified vacuoles (*B*) indicates that the ratio of MeG versus sucrose or malate was almost identical in cells and vacuoles. This demonstrates that MeG was mainly (>95%) stored in the vacuoles and points to the question of the localization of MeG synthesis in plant cells.

There are three possible hypotheses concerning the site of MeG synthesis: (i) the vacuole where it is stored; (ii) the apoplasm; and (iii) the cytoplasm. In order to determine whether the methylation of glucose occurred inside or outside the vacuole, sycamore cells were first incubated in the presence of [1-^{13}C]glucose in order to fill the vacuole with this substrate, then rinsed, and subsequently incubated with [^{13}C]methanol either in the absence of glucose (Fig. 6*A*), or in the presence of [U-^{12}C]glucose (Fig. 6*B*). In the first case, carbohydrates are released from vacuoles to sustain cell metabolism (Roby *et al.*, 1987), whereas in the second case glucose is continuously incorporated into cells, fuelling metabolism and preventing carbohydrates being released from vacuoles. Figure 6 shows a split peak of MeG C-1 on spectrum *A* and no MeG C-1 peak on spectrum *B*, whereas MeG CH_3 peaks were equivalent in both spectra. Spin-spin interaction peaks (arrows) between carbons C-1 and methyl-C of MeG are present on spectrum *A* only. They are weaker than in Fig. 3B_4 probably due to the dilution of initially incorporated [1-^{13}C]glucose by unlabelled carbohydrates present in vacuole. Taken together, these results

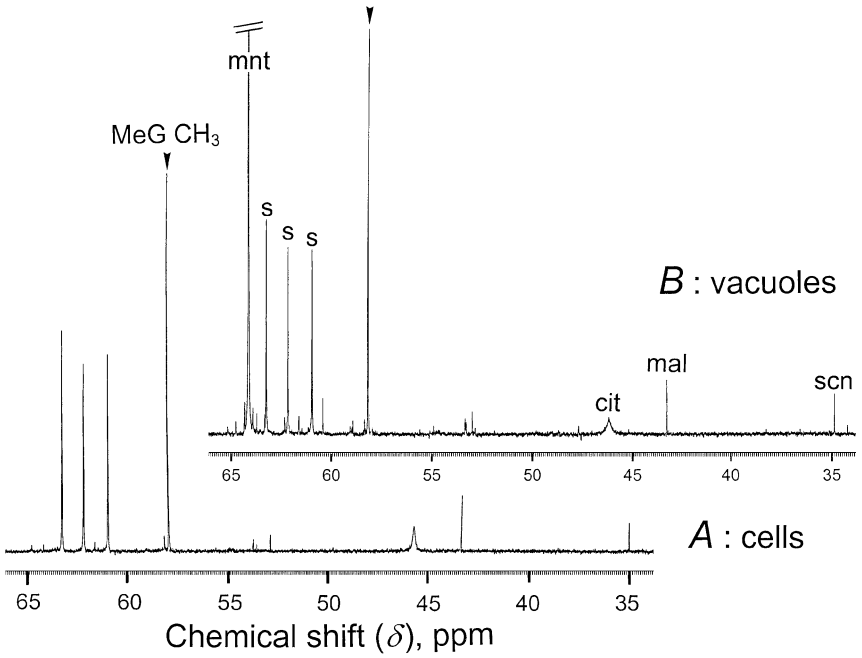

Fig. 5. Representative proton-decoupled *in vitro* ^{13}C-NMR spectra (scale from 34 ppm to 66 ppm) of sycamore (*Acer pseudoplatanus* L.) cells and their purified vacuoles showing that MeG accumulated in vacuoles. Sycamore cells were first incubated over 2 d in the presence of 5 mM [^{13}C]methanol. 10 g of cells were utilized directly for PCA extraction (sample *A*), and 12 g for the preparation of vacuoles as described in the Materials and methods. According to the measurement of malate or citrate taken as a vacuolar marker (Gout *et al*., 1993), the rate of vacuole extraction was about 80% after purification. The cytoplasmic contaminants were estimated to less than 5% according to the measurement of the remnant alcohol dehydrogenase, fumarase, and ADP-glucose pyrophosphorylase activities. Cold (0 °C) perchloric acid (300 µl) was added to the suspension of vacuoles (3 ml in buffer A containing 0.7 M mannitol) for PCA extraction (sample *B*). NMR acquisition parameters were as described in the Materials and methods; number of transients, 900 (90 min). Peak assignments are as follows: MeG, methyl-β-D-glucopyranoside; mnt, mannitol; s, sucrose; cit, citrate; mal, malate; scn, succinate.

indicate that MeG was not synthesized in the vacuole, but in the cytoplasm or in the apoplasm.

The possibility that MeG was synthesized in the cell wall, where methanol can be produced according to pectin demethylation processes (Nemecek-Marshall *et al*., 1995; Fall and Benson, 1996), and then incorporated to cells, could not be discarded *a priori*. However, this hypothesis was unlikely since: (i) MeG synthesis occurred after the C-1 and C-6 redistribution (see above and Fig. 3B_1) which is known to take place in the cytoplasm; (ii) excretion of carbohydrates has never been observed with these cells. Consequently, MeG was very likely synthesized in the cytoplasm. Furthermore, the absence of MeG synthesis in purified cell mitochondria or plastids incubated with labelled methanol and glucose (not shown) suggested that MeG was synthesized in the cytosol, prior to transfer to the vacuole.

To verify that MeG can be transported into the vacuole, sycamore cells and *G. montanum* leaf tissues were incubated over 24 h in the presence of 1 mM MeG. In both cases MeG was incorporated at a rate of 2.5 and 1.2 µmol h^{-1} g^{-1} wet wt, respectively. In addition, MeG uptake was competitively inhibited by glucose ($K_i \approx 30$ µM). In sycamore cells, using purified vacuoles, it was then verified that this exogenous MeG was stored in the vacuoles (not shown). Considering the total amount of MeG accumulated in *G. montanum* after 24 h (*c*. 25 µmol g^{-1} wet leaf), and the very limited volume of cytoplasm versus vacuole, it was hypothesized that most of MeG was translocated to vacuole in this plant as it was in culture cells.

Finally, when cells were incubated in a carbohydrate-free medium subsequent to MeG accumulation, intracellular MeG, in contrast to carbohydrates, remained constant, indicating that it was not used as a carbon source to sustain respiration and to prevent autophagy (not shown). These data show that MeG was not re-exported from the vacuole and was not further metabolized. They are consistent with the observation that *G. montanum* leaves kept in prolonged darkness are not using MeG as a source of carbon to sustain respiration (not shown), and that MeG remained nearly constant in senescent leaves whereas sucrose, fructose, and glucose decreased (Fig. 2).

Taxonomic distribution of MeG in plants

The level of MeG accumulation and the ability to synthesize MeG from [^{13}C]methanol was investigated in leaves of various plants including different Rosaceae (Table 1). Note that the absence of MeG detection may mean that: (i) MeG is absent in the species; (ii) MeG is present but at a level lower than the detection threshold in PCA extracts using natural abundance ^{13}C-NMR (10 µmol); or (iii) MeG levels vary during the year and leaves were collected at a period when it was not accumulated. These points taken into account, it

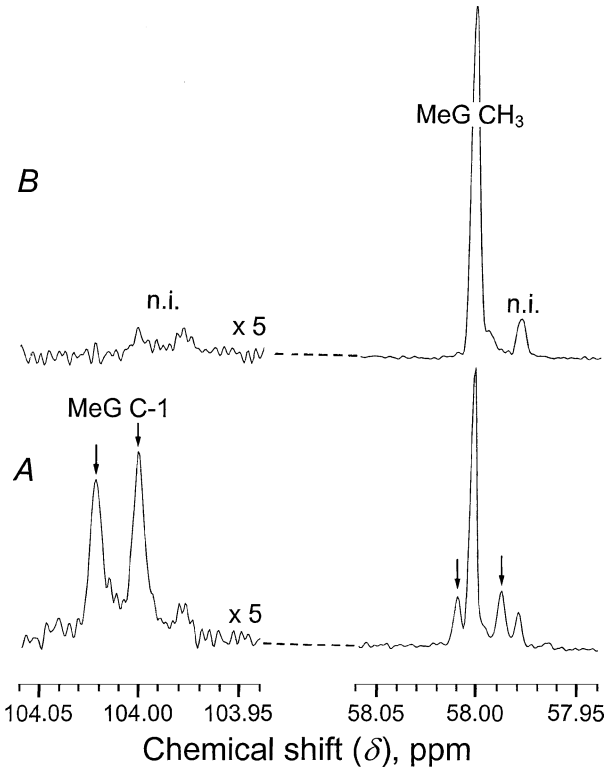

Fig. 6. Representative proton-decoupled *in vitro* ^{13}C-NMR spectra (expanded scales centred around 58 ppm and 104 ppm) of suspension-cultured sycamore cells showing that MeG was not synthesized in the vacuole. Cells were first incubated for 1 d in the presence of 10 mM [1-^{13}C]glucose, then rinsed three times with water, and subsequently incubated in the presence of [^{13}C]methanol either in a carbohydrate-free nutrient medium (*A*) or in a nutrient medium containing 10 mM [U-^{12}C]glucose (*B*). PCA extract preparation and NMR acquisition parameters were as described in the Materials and methods; number of transients, 225 (22.5 min). Peak assignments are as in Fig. 1.

appeared that various members of the subfamily Rosoideae of Rosaceae accumulated more than 1 μmol MeG g^{-1} wet wt. A substantial accumulation was observed in nine out of the ten genera tested, but not in five species of the genus *Alchemilla*. Among plants belonging to a given genus, MeG accumulated much more in some species than in others. For example, the leaves of only seven species of *Geum* among ten tested accumulated MeG. The species of other subfamilies (Spiraeoideae, Maloideae, and Prunoideae) did not accumulate MeG above 1 μmol g^{-1} wet wt. By contrast, MeG was not detected in the leaf extracts of 250 other plants belonging to the main families of the French flora. Only two exceptions were noticed: *Oxytropis campestris* and *Pisum sativum* (Fabaceae). Table 1 also indicates that all the dicots tested, including non-photosynthetic suspension-cultured cells, showed an activity of methanol incorporation to glucose detected in the presence of [^{13}C]methanol. The Rosaceae in which MeG was detected in the absence of added methanol synthesized MeG at a higher rate (up to 6.5–7.5 μmol d^{-1} g^{-1} wet wt) compared with the non-accumulating species (usually less than 1 μmol d^{-1} g^{-1} wet wt in other tested Rosaceae and dicots). The rate of MeG accumulation in the tested monocots was not measurable by ^{13}C-NMR, or was very low. Gymnosperms, pteridophytes, and mosses did not accumulate MeG nor incorporate ^{13}C-methanol in MeG.

Discussion

Methylation is a classical technique for the study of glycosyl linkages in plant cell wall polysaccharides (Selvendran and Ryden, 1990). Methylated monosaccharides and methyl-glucosides (including MeG) resulting from these treatments are, therefore, well-known compounds. Surprisingly, as far as the authors are aware, MeG was reported to be present as a natural compound in a plant tissue (rose petals) only recently (Ichimura *et al*., 1997). A synthesis of MeG has also been described in sycamore cells incubated in the presence of methanol (Gout *et al*., 2000). However, since this glucoside was not detected in cells in the absence of methanol, the possibility that the synthesis of MeG could be artificially triggered by the addition of methanol was not excluded. The present results show that MeG is a major natural compound present in different plants, principally in the leaves of the Rosaceae family (Rosoideae subfamily) members.

Some pectic polysaccharides like rhamnogalacturonans contain methylated glucosides, for example 2-*O*-methyl fucose or 2-*O*-methyl xylose (for a review, see Bacic *et al*., 1988). *O*-methyl sugars are also known to occur in secondary metabolites. For instance, cardenolides contain 3-*O*-methyl-glucose, 2,3-di-*O*-methyl glucose, and various methylethers of deoxy-glucose or deoxy-galactose (Connolly and Hill, 1991). In these cases, the sugar polymers are formed by a glucosidic linkage involving carbon C-1, and methylation is therefore not observed on this latter position. The MeG observed in Rosaceae is probably not linked to an aglycone for the following reasons. First, the methylation takes place at position C-1, which is the position of most of the linkages involving sugars and aglycones. Second, ^{13}C-NMR spectra did not show resonance peaks, with intensities comparable to MeG, which could originate from a possible aglycone. In addition, a loss of aglycones during perchloric acid extraction is unlikely since metabolite profiles were not different when obtained from *in vivo* or *in vitro* (extracts) ^{13}C-NMR spectra (not shown).

Various *O*-methyl derivatives from myo-, chiro-, muco-, and scyllo-inositol have also been described (for a review, see Popp and Smirnoff, 1995), such as pinitol (3-*O*-methyl-chiro-inositol) or sequoyitol (5-*O*-methyl-myo-inositol). These monomethylethers of inositol can accumulate to high levels in the cells from different plant families, especially under water-stress conditions (Ford, 1982; Gorham *et al*., 1984). It has been shown that the expression of the gene *Imt1* encoding the *S*-adenosylmethionine-dependent

Table 1. *Accumulation of methyl-β-D-glucopyranoside in different plants*

The plants selected in this table were those naturally containing ^{13}C-NMR detectable MeG, and those incubated in the presence of [^{13}C]methanol (see Materials and methods) in which MeG was measured or not detected (nd). The names of 250 other analysed plants distributed in the main families of the French flora, in which MeG was not detected, are not mentioned. In particular, MeG was not detected in ten Rosaceae from the subfamilies Spiraeoideae and Maloideae.

				μmol g^{-1} wet wt	% Soluble sugars (mol mol^{-1})	^{13}C-methanol incorporation μmol d^{-1} g^{-1} wet wt
Dicots	Rosaceae	Rosoideae	*Acaena microphylla* Hook. fil	3.4–4.0	3.5–4.0	2.4–2.8
			Alchemilla pentaphyllea L.	nd	–	0.10–0.12
			Dryas octopetala L.	35–40	28–32	
			Fragaria vesca L.	1.3–2.2	2.7–3.3	
			Geum coccineum Sibth. & Sm.	14–17	5.6–6.0	
			Geum heterocarpum Boiss.	3.5–4.0	3.4–3.8	
			Geum montanum L.	16–20	12–14	5.5–6.5
			Geum pyrenaicum Miller	30–35	15–18	
			Geum reptans L.	25–29	12–15	
			Geum rivale L.	4.0–4.6	16–20	
			Geum triflorum Pursh	11–14	10–12	
			Potentilla grandiflora L.	15–20	11–13	6.5–7.5
			Rosa canina L.	3.5–4.0	7.8–9.0	
			Rubus fruticosus	6.9–8.0	6.5–7.4	
			Sanguisorba minor L.	15–18	10–12	
			Sibbaldia procumbens L.	18–22	11–14	
			Waldensteinia fragarioides Tratt.	16–21	36–40	
			Woronowia speciosa (Albov) Juz.	16–20	17–20	
		Prunoideae	*Prunus* sp.	nd	–	0.04–0.08
	Aceraceae		*Acer pseudoplatanus* L.[a]	nd	–	1.2–1.4
	Apiaceae		*Daucus carota* L.[a]	nd	–	0.05
	Caryophyllaceae		*Silene alba* (Miller) Krause[a]	nd	–	0.04
	Chenopodiaceae		*Spinacia oleracea* L.	nd	–	0.26–0.32
	Fabaceae		*Oxytropis campestris* (L.) DC.	9.5–12	34–40	
			Pisum sativum L.	2.2–3.0	–	0.56–0.68
	Ranunculaceae		*Ranunculus acris* L.	nd	–	0.16–0.19
	Solanaceae		*Solanum tuberosum* L.	nd	–	0.76–0.84
Monocots	Amaryllidaceae		*Narcissus pseudonarcissus* L.	nd	–	0.40–0.50
	Araceae		*Arum maculatum* L.	nd	–	nd
	Iridaceae		*Iris* sp.	nd	–	0.005–0.01
	Liliaceae		*Tulipa* sp.	nd	–	0.01–0.02
	Orchidaceae		*Himantoglosum hircinum* (L.) Sprengel	nd	–	0.003–0.005
	Poaceae		*Zea mays*	nd	–	nd
			Zea mays[a]	nd	–	nd
Gymnosperms			*Cedrus* sp.	nd	–	nd
Pteridophytes			*Athyrium*	nd	–	nd
Bryophytes			*Bryum* sp.	nd	–	nd

[a] Suspension-cultured cells.

myo-inositol *O*-methyl transferase, the enzyme involved in the first step of conversion of *myo*-inositol to pinitol, is controlled by salt and cold stress (Vernon and Bohnert, 1992; Vernon *et al*., 1993). Methyl group transfers catalysed by specific methyltransferases using *S*-adenosylmethionine (Ibrahim, 1997) are involved in several other pathways that lead to the synthesis and accumulation of compounds used in osmoprotection and/or osmotic adjustment such as quaternary ammonium (like glycine betaine) or tertiary sulphonium compounds (for a review, see Bohnert and Jensen, 1996). A role of transmethylation in stress tolerance is further supported by the observation that mRNA for SAM synthetase accumulates during osmotic stress in tomato (Espartero *et al*., 1994).

These experiments carried out with [^{13}C]methanol and [1-^{13}C]glucose show that MeG originates from a direct transfer of methanol on glucose C-1 via a glycosylation reaction, and suggest that this reaction requires no energy supply. The methylation of glucose through folate-mediated single-carbon metabolism is not likely to be involved since MeG was not detected after incubating plant material in the presence of [2-^{13}C]glycine or [3-^{13}C]serine (for a review on single-carbon metabolism and its regulation, see Rébeillé and Douce, 1999). On the other hand, MeG was not an artefact due to PCA extraction since it was also observed in fresh leaves (not shown) and in sycamore cells using *in vivo* ^{13}C-NMR (Gout *et al*., 2000).

Concerning the substrates of the glycosylation reaction, glucose was abundant in *G. montanum* leaves (23–25 μmol g^{-1} wet wt; Fig. 1) and methanol, when not added during experiments, can be produced by young expanding leaves (Fall and Benson, 1996). In an attempt to explain the huge differences of MeG accumulation observed between different plants of the Rosaceae family (and in some other

families), one may suppose either that the enzyme catalysing the synthesis of MeG may vary according to plants, or that some plants produce more methanol than others, assuming that all of them contain non-limiting amounts of glucose in their cytoplasm. Interestingly, after leaf incubation with [^{13}C]methanol, it was observed that the amount of labelled MeG was dependent on the level of MeG initially present in the sample. Plants accumulating high amounts of MeG showed much higher glucose methylation activities than non-accumulating species (Table 1). Consequently, it seems that the differences of accumulated MeG observed in the analysed plants originate, at least partly, from the level of enzyme activity. However, it cannot be excluded that, in some plants, the availability of methanol limits MeG synthesis under the threshold of ^{13}C-NMR detection, as observed in suspension-cultured sycamore cells. In this context, the accumulation of MeG in various Rosaceae species of the subfamily Rosoideae and its absence of detection in all the species of the other subfamilies (Spiroideae, Maloideae, Prunoideae) is a further argument for the chemotaxonomy of the family Rosaceae, mostly based on the patterns of phenolic compounds and sorbitol (Challice, 1981). However, the accumulation of MeG is not exclusive for the subfamily of Rosoideae since two Fabaceae also accumulate high amounts of the compound.

Incubation of *G. montanum* leaf tissues with ^{13}C-labelled methanol and/or glucose showed that MeG was not synthesized in the apoplasm where methanol is released (Fall and Benson, 1996), but inside cells. In addition, experiments carried out with sycamore cells demonstrated that MeG was synthesized in the cytosol and that it subsequently accumulated in the vacuole where it remained sequestrated. These observations raise several questions about the transport of MeG across the tonoplast. For example, does MeG utilize the same transporter as glucose, as suggested at the level of the plasmalemma by the competitive inhibition of MeG uptake by glucose? If so, is there an exchange across the tonoplast between newly synthesized MeG and glucose stored in the vacuole as suggested by Fig. 6? What kind of transporter(s) is (are) involved? Obviously, further experiments on isolated vacuoles are necessary to address these questions.

The physiological significance of MeG accumulation in Rosaceae remains unclear. Like many other methylated molecules (quaternary ammonium, tertiary sulphonium, methyl-inositols, etc), MeG could be involved in osmotic stress tolerance. Interestingly, using chlorophyll fluorescence and CO_2 measurements, it was observed that *G. montanum* is highly resistant to water deficit (Manuel *et al*., 1999). However, preliminary experiments suggested that MeG does not act as an osmoprotectant in the bacteria of the Rhizobiaceae family (X Pocard, personal communication). MeG could also be involved in hydroxyl radical scavenging, as reported by Popp and Smirnoff (1995) for methyl-inositols. In this context, MeG accumulation was observed mostly in mountain Rosaceae, which are exposed to high light intensities resulting in reactive oxygen species (ROS) production (Asada, 1994; Streb *et al*., 1998). However, the vacuolar location of MeG would not permit the protection of the cytoplasmic cell components.

Moreover, although MeG represents an important part of soluble sugars in some species, it is probably not a storage compound for carbon since: (i) it did not significantly accumulate in the rhizome of *G. montanum*; (ii) it did not show a reallocation pattern in the leaves over the vegetative season, by contrast with sucrose and glucose; (iii) leaves kept in prolonged darkness and senescent leaves hydrolysed sucrose and glucose, but not MeG; (iv) in sycamore cells, MeG resulting from ^{13}C-methanol incorporation was not metabolized further and did not prevent autophagy upon prolonged carbon starvation (Aubert *et al*., 1996*b*).

A last hypothesis is that the synthesis in the cytosol of MeG, which is subsequently stored in the vacuole, may help in limiting the accumulation of methanol in the cytoplasm. Indeed, methanol is a potential solvent of membrane lipids in plant tissues overproducing this alcohol. In addition, its oxidation produces formaldehyde which is a very reactive component considered as toxic to cell metabolism. Finally, the metabolization of methanol via the folate-mediated single-carbon metabolism (Gout *et al*., 2000) might also interfere with the regulation of this pathway. Experiments will be undertaken to identify a possible correlation between the level of MeG accumulation and the amount of methanol produced in the leaves.

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