

Raffinose in Chloroplasts is Synthesized in the Cytosol and Transported across the Chloroplast Envelope

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In chloroplasts, several water-soluble carbohydrates have been suggested to act as stress protectants. The trisaccharide raffinose (α -1,6-galactosyl sucrose) is such a carbohydrate but has received little attention. We here demonstrate by compartmentation analysis of leaf mesophyll protoplasts that raffinose is clearly (to about 20%) present in chloroplasts of cold-treated common bugle (*Ajuga reptans* L.), spinach (*Spinacia oleracea* L.) and *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.] plants. The two dedicated enzymes needed for raffinose synthesis, galactinol synthase and raffinose synthase, were found to be extra-chloroplastic (probably cytosolic) in location, suggesting that the chloroplast envelope contains a raffinose transporter. Uptake experiments with isolated *Ajuga* and *Arabidopsis* chloroplasts clearly demonstrated that raffinose is indeed transported across the chloroplast envelope by a raffinose transporter, probably actively. Raffinose uptake into *Ajuga* chloroplasts was a saturable process with apparent K_m and v_{max} values of 27.8 mM and $3.3 \mu\text{mol mg}^{-1} \text{Chl min}^{-1}$, respectively.

Keywords: Abiotic stress • Carbohydrate compartmentation • Chloroplast • Raffinose transport.

Abbreviations: ARB, *Arabidopsis* resuspension buffer; Chl, chlorophyll; CPB, chloroplast purification buffer; DB, digestion buffer; DIC, differential interference contrast; GalS, galactinol synthase; HPR, 2-hydroxypyruvate reductase; MDH, malate dehydrogenase; MS, Murashige and Skoog; PIBB, protoplast isolation buffer B; PPM, protoplast purification medium; RafS, raffinose synthase; RFO, raffinose family oligosaccharide; RH, relative humidity; SRB, spinach resuspension buffer.

Introduction

Because land plants are sessile organisms, their survival depends on the ability to cope with adverse environmental stresses.

The development of stress tolerance strategies is complex and involves a plethora of processes starting with stress perception and signaling, finally leading to molecular, biochemical, cellular, physiological and even morphological changes. The accumulation of non-reducing water-soluble carbohydrates is one of the most commonly observed responses of plants to abiotic stresses. Interestingly, such a general observation does not pay tribute to the fact that plants are highly compartmentalized both on the cellular and subcellular levels; i.e. the local concentration of a potential stress protectant in a particular location is important (Lunn 2007).

In this study, we focus on one compartment, the chloroplast, and ask which of the potentially stress-protecting water-soluble carbohydrates it contains and how they may get there to fulfill their protective roles. Several such chloroplast-bound carbohydrates have been described, e.g. the polyols, mannitol and *myo*-inositol (Bohnert et al. 1995) and the di- and trisaccharides, sucrose and raffinose, respectively, sucrose more often (Heber 1959, Santarius and Milde 1977, Leidreiter et al. 1995, Moore et al. 1997a, Moore et al. 1997b, Gerrits et al. 2001, Voitsekhovskaja et al. 2006, Benkeblia et al. 2007) than raffinose, which has only been reported in chloroplasts of cold-treated cabbage and wheat plants (Heber 1959, Santarius and Milde 1977). The protective effect of sucrose and raffinose on proteins and membranes was shown with both isolated thylakoid membranes (Lineberger and Steponkus 1980) and liposomes (Hincha et al. 2003). An increase in sucrose and raffinose concentrations during cold acclimation has been reported for many plant species including *Ajuga reptans* (Bachmann et al. 1994) and *Arabidopsis* (Zuther et al. 2004). The proposed protection mechanisms include interaction with protein and lipid bilayer surfaces (Hoekstra et al. 2001). Additionally, raffinose has the ability to delay sucrose crystallization (Caffrey et al. 1988) preventing membrane damage. Even if raffinose alone might not be responsible for stress protection (Zuther et al. 2004),

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it most probably still plays some role *in planta* which is yet to be more fully understood.

With the exception of *myo*-inositol, which is synthesized directly in the chloroplasts (Adhikari et al. 1987, RayChaudhuri and Majumder 1996, Lackey et al. 2003), all of the above-mentioned carbohydrates, including raffinose, are proposed to be synthesized extra-chloroplastically (Bird et al. 1974, Bachmann and Keller 1995), most probably in the cytosol and, therefore, chloroplastic membrane transport mechanisms need to be postulated to allow the import of the protective carbohydrates synthesized in the cytosol. However, no such transporters have been identified. In this paper, we provide first evidence that raffinose is present, but not synthesized, in the chloroplast; rather it is taken up from the cytosol, its site of synthesis, by a most probably active raffinose transporter located at the chloroplast envelope.

Results

Intact chloroplasts were isolated from *Ajuga*, spinach and *Arabidopsis* leaves

Chloroplasts were isolated from *Ajuga*, spinach and *Arabidopsis* leaf protoplasts and their intactness was determined

either by measurement of ferricyanide-dependent oxygen evolution using an oxygen electrode or by determination of the intactness with differential interference contrast (DIC) microscopy. Independent of the species and the chosen growth and isolation methods, the chloroplast fractions showed an intactness of 75–88% and a combined mitochondrial, peroxisomal and cytosolic contamination of 6.8–9.0% [NADH-malate dehydrogenase (NADH-MDH); **Table 1**]. Activities of the vacuolar marker enzymes α -mannosidase and α -galactosidase were not detectable in any of the chloroplast fractions (**Table 1**). In spinach, the chloroplast fractions showed a peroxisomal contamination of $7.8 \pm 0.5\%$ as determined by the peroxisomal marker enzyme, 2-hydroxypyruvate reductase (HPR). In *Arabidopsis*, no HPR activity was detectable in the chloroplast fractions. In *Ajuga*, HPR was not investigated.

Raffinose, but no GolS and RafS activities, were found in *Ajuga*, spinach and *Arabidopsis* chloroplasts

Compared with the contamination marker enzyme distribution, a significantly higher chloroplastic distribution of raffinose was shown ($19.2 \pm 3.9\%$ for *Ajuga*, $22.1 \pm 1.4\%$

Table 1 Comparison of *Ajuga*, spinach and *Arabidopsis* leaf mesophyll protoplasts with chloroplasts isolated from them; protoplasts and chloroplasts were isolated from soil-grown cold-treated plants

Substance/enzyme	Species					
	<i>A. reptans</i>		<i>S. oleracea</i>		<i>A. thaliana</i>	
	Protoplasts (μg or nkat mg^{-1} Chl)	In chloroplasts (%)	Protoplasts (μg or nkat mg^{-1} Chl)	In chloroplasts (%)	Protoplasts (μg or nkat mg^{-1} Chl)	In chloroplasts (%)
α -Galactosidase	2.43 ± 0.15	n.d.	n.i.	n.i.	0.616 ± 0.002	n.d.
α -Mannosidase	1.49 ± 0.06	n.d.	1.43 ± 0.08	n.d.	0.658 ± 0.001	n.d.
NADH-MDH	357 ± 47	9.0 ± 1.4	331 ± 28	6.8 ± 2.1	61 ± 8	6.9 ± 4.2
HPR	n.i.	n.i.	247 ± 24	7.8 ± 0.5	27 ± 8	n.d.
GolS	0.471 ± 0.021	n.d.	0.019 ± 0.002	9.5 ± 2.4	n.d.	n.d.
RafS	0.023 ± 0.002	n.d.	0.066 ± 0.005	5.0 ± 1.3	0.043 ± 0.009	6.2 ± 4.6
<i>myo</i> -Inositol	196 ± 50	$35.6 \pm 5.5^*$	99 ± 29	$29.8 \pm 2.9^*$	n.p.	n.p.
Galactinol	615 ± 119	12.0 ± 1.5	181 ± 63	15.0 ± 2.0	n.d.	n.d.
Glucose	861 ± 464	3.6 ± 1.5	691 ± 78	4.1 ± 0.2	445 ± 261	8.4 ± 2.8
Fructose	187 ± 69	8.3 ± 1.6	579 ± 115	3.7 ± 0.4	561 ± 399	5.2 ± 1.8
Sucrose	1477 ± 363	$31.1 \pm 6.3^*$	4241 ± 1379	9.3 ± 0.9	257 ± 197	$28.4 \pm 5.6^*$
Raffinose	1015 ± 188	$19.2 \pm 3.9^*$	549 ± 156	$22.1 \pm 1.4^*$	142 ± 39	$19.7 \pm 6.6^*$
Stachyose	1047 ± 190	8.1 ± 3.0	n.d.	n.d.	n.d.	n.d.
Verbascose	1012 ± 236	6.1 ± 2.4	n.d.	n.d.	n.d.	n.d.
RFOs DP>5	2751 ± 398	4.1 ± 1.4	n.d.	n.d.	n.d.	n.d.

Carbohydrates were analyzed by HPLC-PAD on a BC100 column. To confirm *myo*-inositol and galactinol concentrations, samples were analyzed additionally by HPLC-PAD on a CarboPac MA1 column. Data are mean \pm SE of at least three independent experiments. n.d., not detected; n.i., not investigated; n.p., analysis not possible.

*Significantly different from marker enzyme contaminations at the $P \leq 0.05$ level. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparison test.

for spinach and $19.7 \pm 6.6\%$ for Arabidopsis; **Table 1**), which clearly indicates the presence of an intrachloroplastic raffinose pool in soil-grown cold-treated plants. Conversely, activities of the two dedicated raffinose biosynthetic pathway enzymes, GolS and RafS, were undetectable in *Ajuga* chloroplasts or, in spinach and Arabidopsis, they were distributed like the combined mitochondrial, peroxisomal and cytosolic marker enzyme, NADH-MDH, and the peroxisomal marker enzyme, HPR (**Table 1**). These findings indicate an extra-chloroplastic location of both raffinose biosynthetic enzymes. Furthermore, galactinol (α -1,1-galactosyl *myo*-inositol), a GolS product and RafS substrate, was exclusively extra-chloroplastic. Therefore, intrachloroplastic de novo synthesis of galactinol and raffinose via GolS and RafS can be excluded.

Similar to raffinose, *myo*-inositol was also significantly located in *Ajuga* and spinach chloroplasts ($35.6 \pm 5.5\%$ and $29.8 \pm 2.9\%$, respectively; **Table 1**). In Arabidopsis, *myo*-inositol determination was not possible because of low

myo-inositol concentration. Sucrose was found in chloroplasts of *Ajuga* ($31.1 \pm 6.3\%$) and Arabidopsis ($28.4 \pm 5.6\%$), but not spinach. All other carbohydrates were distributed like the combined mitochondrial, peroxisomal and cytosolic marker enzyme, NADH-MDH, and the peroxisomal marker enzyme, HPR (**Table 1**). Thus, an extra-chloroplastic location of these carbohydrates is most likely.

Raffinose and glucose were taken up by isolated *Ajuga* and Arabidopsis chloroplasts

In *Ajuga*, very rapid uptake of [3 H]raffinose into isolated chloroplasts was observed (**Fig. 1A**). Compared with the uptake of the intermembrane space control substance [14 C]sorbitol, [3 H]raffinose uptake was 3.8-fold higher after 0.85 s (net raffinose uptake $15.3 \text{ nmol mg}^{-1} \text{ Chl}$) and 10-fold higher after 10–60 s (net raffinose uptake $317 \text{ nmol mg}^{-1} \text{ Chl}$). The uptake was time saturated after 10 s at a net maximum concentration of $330 \text{ nmol mg}^{-1} \text{ Chl}$. Longer uptake times of up to 5 min did not result in a further increase in net raffinose

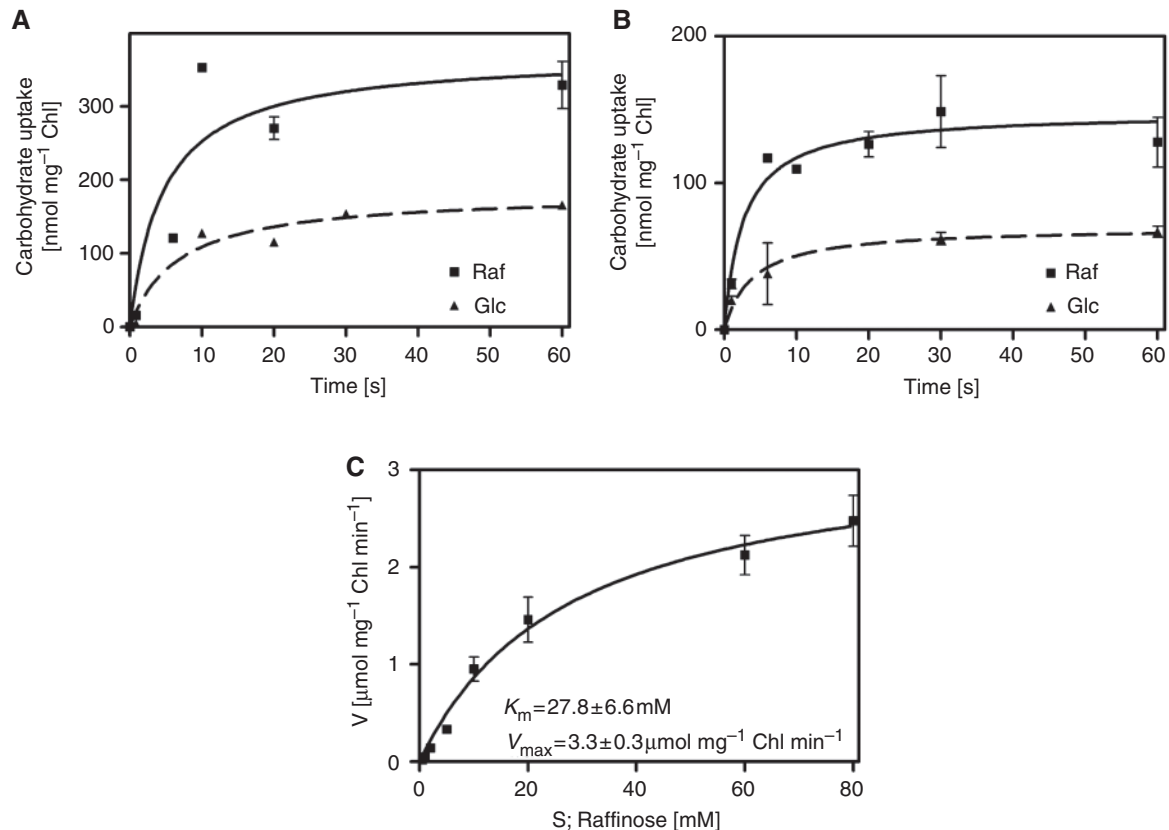


Fig. 1 Carbohydrate uptake into chloroplasts isolated from soil-grown cold-treated *Ajuga* and sterile-grown Arabidopsis plants. (A) Time dependence of 1 mM [3 H]raffinose and 4 mM [14 C]glucose net uptake into *Ajuga* chloroplasts. Data are mean \pm SE from two experiments run in triplicate. (B) Time dependence of 1 mM [3 H]raffinose and 4 mM [14 C]glucose net uptake into Arabidopsis chloroplasts. Data are mean \pm SE from one experiment run in triplicate. A second replicate with chloroplasts from Arabidopsis showed the same trend but differed in the absolute values. (C) Substrate dependence of [3 H]raffinose net uptake into *Ajuga* chloroplasts. Data are mean \pm SE from three experiments run in triplicates. All uptake experiments were performed at room temperature (22°C) and in the light. V, uptake rate (velocity); S, substrate concentration.

uptake (data not shown). In addition to chloroplastic [^3H] raffinose uptake, time-dependent [^{14}C]glucose uptake was observed (Fig. 1A), similar to that described for spinach chloroplasts (Weber et al. 2000). The concentration dependence of the *Ajuga* chloroplastic raffinose uptake was determined using 1 s uptake assays. It was substrate saturable and showed Michaelis–Menten type kinetics with apparent K_m and v_{max} values of $27.8 \pm 6.6 \text{ mM}$ and $3.3 \pm 0.3 \mu\text{mol mg}^{-1} \text{ Chl min}^{-1}$, respectively (Fig. 1C).

Similar to *Ajuga*, [^3H]raffinose uptake was also clearly detectable in chloroplasts isolated from sterile-grown *Arabidopsis* plants (Fig. 1B). Sterile-grown plants were chosen because it is known that chloroplasts isolated from them are more stable than those isolated from soil-grown plants (Fitzpatrick and Keegstra 2001). The raffinose uptake into *Arabidopsis* chloroplasts was again very rapid, saturating after 10 s. The [^3H]raffinose uptake was 1.5-fold higher than that of the [^{14}C]sorbitol control (Fig. 1B). After 0.85 s, the net raffinose uptake was $31.2 \text{ nmol mg}^{-1} \text{ Chl}$ and was saturated after 10 s at a concentration of $125 \text{ nmol mg}^{-1} \text{ Chl}$. [^{14}C]Glucose uptake into *Arabidopsis* chloroplasts was also rapid, leveling off after 10 s (Fig. 1B). Longer incubation times of up to 5 min did not lead to higher uptake rates (data not shown). This rapid glucose uptake corresponds to that published for spinach chloroplasts (Weber et al. 2000).

Contrary to the passive chloroplastic glucose uptake, raffinose uptake seems to be active

Glucose and raffinose concentrations in the chloroplast stroma were calculated according to Heldt (1980). In both uptake systems used, a chloroplastic glucose concentration was determined that corresponded to the external concentration (Table 2), suggesting that glucose enters the chloroplast by facilitated diffusion. For raffinose, the situation was clearly different. Raffinose accumulated in the chloroplasts of *Ajuga* and *Arabidopsis* by a factor of 8.3 and 8.9, respectively (Table 2), suggesting an active process.

Discussion

The chloroplastic water-soluble carbohydrate location found in *Ajuga*, spinach and *Arabidopsis* is in line with results from other plant species

To determine the water-soluble carbohydrate distribution between protoplasts and chloroplasts, the aqueous fractionation technique was used. Compared with the non-aqueous fractionation technique, it has the advantage of using almost exclusively one cell type, the mesophyll, as the starting material; it has the disadvantage that water-soluble, low-molecular weight substances, such as the carbohydrates considered in this study, might putatively leak out of the protoplasts and chloroplasts or may be redistributed or metabolized. Therefore, the aqueous isolation technique was performed as quickly as possible. Protoplast isolation (about 2–4 h) was quickly followed by fast release and purification of chloroplasts. Typically, the chloroplast isolation and purification time was <1 min. Because of very low amounts of raffinose present in the protoplasts of warm-grown plants (below the detection limit of our HPLC-PAD system), we focused on the determination of raffinose compartmentation using cold-grown plants.

Our chloroplastic carbohydrate location results were quite similar to those obtained by the non-aqueous fractionation technique used for other plants (Moore et al. 1997a, Voitsekhovskaja et al. 2006, Benkeblia et al. 2007, Nadwodnik and Lohaus 2008), except for the hexoses, which we found to be to 88% cytosolic in cold-treated *Ajuga* leaves, contrary to all other instances (Table 3). Chloroplasts from *Ajuga* and spinach leaves contained a considerable proportion of the total cell *myo*-inositol (36 and 30%, respectively). Chloroplastic *myo*-inositol location seems to be widespread and independent of abiotic stress, because plants growing under non-stress conditions also contained *myo*-inositol in their chloroplasts (Table 3).

In contrast to *myo*-inositol, a chloroplastic sucrose location seems to be less widespread. Relatively high proportions

Table 2 Carbohydrate concentrations in chloroplasts of cold-treated *Ajuga* and sterile-grown *Arabidopsis* plants used for transport studies compared with the uptake medium

Species	Substrate	Concentration in uptake medium (mM)	Concentration in chloroplasts (mM)	Transport type
<i>A. reptans</i>	Glucose	4	3.6	Facilitated diffusion
	Raffinose	1	8.3	Active accumulation
<i>A. thaliana</i>	Glucose	4	4.4	Facilitated diffusion
	Raffinose	1	8.9	Active accumulation

The concentrations were calculated after saturation of the time-dependent uptake of 4 mM external [^{14}C]glucose and 1 mM external [^3H]raffinose had occurred. Comparison of the chloroplastic concentration with the external concentration indicates the transport type involved. Stromal carbohydrate concentrations were calculated according to Heldt (1980).

Table 3 Distribution of carbohydrates between the chloroplastic, cytoplasmic and vacuolar compartments of leaf mesophyll cells in different plant species

Plant species (growth condition)	Hexoses (%)			Sucrose (%)			<i>myo</i> -Inositol (%)			Raffinose (%)			Reference
	Chl	Cyt	Vac	Chl	Cyt	Vac	Chl	Cyt	Vac	Chl	Cyt	Vac	
<i>A. reptans</i> (cold treated) ^a	6	88	6 ^b	31	59	10 ^b	36	58	6 ^b	19	21	60 ^b	This study
<i>A. thaliana</i> (cold treated) ^a	7	–	–	28	–	–	n.p.	–	–	20	–	–	This study
<i>S. oleracea</i> (cold treated) ^a	4	–	–	9	–	–	30	–	–	22	–	–	This study
<i>Apium graveolens</i> (warm grown) ^c	1	1	98	1	20	79	44	9	48	–	–	–	Nadwodnik and Lohaus (2008)
<i>A. barclaiana</i> (warm grown) ^c	1	1	98	14	35	51	53	24	23	n.d.	n.d.	n.d.	Voitsekhovskaja et al. (2006)
<i>A. meridionalis</i> (warm grown) ^c	1	1	98	21	44	35	81	13	6	0 ^d	6 ^d	94 ^d	Voitsekhovskaja et al. (2006)
<i>Anthrimum majus</i> (warm grown) ^c	0	0	100	1	84	15	55	27	18	–	–	–	Moore et al. (1997a)
<i>Brassica oleracea</i> (frost hardy) ^c	23	–	–	20	–	–	–	–	–	n.p. ^e	–	–	Santarius and Milde (1977)
<i>B. oleracea</i> (dehardened, mid-summer) ^c	9	–	–	8	–	–	–	–	–	n.d.	–	–	Santarius and Milde (1977)
<i>Glycine max</i> (field grown) ^c	21	29	51	25	55	21	32	38	31	–	–	–	Benkeblia et al. (2007)
<i>Hedera helix</i> (field grown, mid-summer) ^c	0	0	100	16	26	58	–	–	–	–	–	–	Moore et al. (1997b)
<i>Petroselinum hortense</i> (warm grown) ^c	3	0	97	5	57	38	43	23	34	–	–	–	Moore et al. (1997a)
<i>Nicotiana sylvestris</i> (warm grown) ^c	1	0	99	0	92	8	–	–	–	–	–	–	Moore et al. (1997a)
<i>Plantago major</i> (warm grown) ^c	3	1	96	1	44	55	60	35	6	–	–	–	Nadwodnik and Lohaus (2008)
<i>Plantago maritima</i> (warm grown) ^c	1	1	98	1	56	44	82	1	17	–	–	–	Nadwodnik and Lohaus (2008)
<i>Prunus persica</i> (warm grown) ^c	1	1	98	7	16	77	40	15	45	–	–	–	Nadwodnik and Lohaus (2008)
<i>Solanum tuberosum</i> (warm grown) ^c	1	1	98	27	40	33	–	–	–	–	–	–	Leidreiter et al. (1995)

Plants were grown as indicated. Chl, chloroplast; Cyt, cytoplasm; n.d., not detectable; n.p., analysis not possible; Vac, vacuole; –, not investigated. ^aData obtained by aqueous fractionation; ^bdata are from Bachmann and Keller (1995); ^cdata obtained by non-aqueous fractionation; ^ddata represent the sum of raffinose and stachyose; ^eclearly present, but not quantified.

of sucrose were detected in chloroplasts of *Ajuga* (31%) and *Arabidopsis* (28%), cabbage (20%) (Santarius and Milde 1977), *Alonsoa meridionalis* (21%) and *Asarina barclaina* (14%) (Voitsekhovskaja et al. 2006), ivy (16%) (Moore et al. 1997b), potato (27%) (Leidreiter et al. 1995) and soybean (25%) (Benkeblia et al. 2007) (Table 3). In all other investigated plant species, including spinach in this study, only low sucrose proportions that are not significantly different from cytoplasmic contamination were located in chloroplasts (Table 3).

The presence of raffinose in chloroplasts as reported earlier for frost-hardy cabbage plants (Santarius and Milde 1977) was clearly confirmed in this study for cold-treated *Ajuga* (19%), spinach (22%) and soil-grown *Arabidopsis* (20%) plants.

Raffinose is synthesized in the cytosol and transported into chloroplasts

Exact information on the subcellular location of the raffinose biosynthetic pathway is minimal. Several indirect lines of

evidence suggest it to be cytosolic. (i) In *Ajuga* leaf mesophyll cells, GolS activity was localized exclusively to the extra-vacuolar space of the cytoplasm (Bachmann and Keller 1995). (ii) In *Cucurbita pepo* leaves, GolS was suggested to reside in the cytosol of intermediary cells (Beebe and Turgeon 1992). (iii) GolS and RafS activities showed neutral pH optima, corresponding to the pH of the cytosol (Bachmann et al. 1994). (iv) The substrates, *myo*-inositol, galactinol and sucrose co-localized with the corresponding enzymes, GolS and RafS, in *Ajuga* leaf mesophyll cells (Bachmann and Keller 1995). (v) In this study, a chloroplastic GolS and RafS location was excluded by the lack of enzyme activities in *Ajuga*, spinach and *Arabidopsis* chloroplasts (Table 1). (vi) An in silico study using different sequence-based predictors (Emanuelsson et al. 2007) revealed no chloroplast transit peptides in all known GolS and RafS protein sequences (data not shown). The obvious consequence of the dichotomy of cytosolic raffinose synthesis and a chloroplastic raffinose location is that raffinose has to be transported from the cytosol across the envelope into the

chloroplast by a transporter. This was clearly demonstrated here for *Ajuga* and *Arabidopsis* (Fig. 1). Raffinose uptake was both rapid and active as indicated by time saturation after 10 s (Fig. 1A, B) and an 8- to 9-fold accumulation (Table 2). The transport kinetics of *Ajuga* chloroplasts (Fig. 1C) were similar to those of the only known plant RFO transporter, the stachyose/H⁺ antiporter in *Stachys* tuber vacuoles (Keller 1992). Like the vacuolar stachyose transporter ($K_m = 53$ mM), the chloroplastic raffinose transporter showed Michaelis–Menten kinetics ($K_m = 28$ mM). Is this relatively high K_m value physiologically relevant? Because the cytosolic raffinose concentration is not known from direct measurements, we have to draw on indirect calculations. Assuming (i) a raffinose concentration in cold-acclimated *Ajuga* leaves of 5–10 mg g⁻¹ FW (Bachmann et al. 1994, Peters and Keller 2009, 80% leaf water content), (ii) a 21% cytosolic raffinose location (Table 3) and (iii) a 9% cytosolic cell volume contribution (average value for different dicotyledonous leaves; Nadwodnik and Lohaus 2008), a cytosolic raffinose concentration of 29–58 mM is calculated which is indeed in the range of the K_m value for the raffinose transporter described here. Whether chloroplastic raffinose transport is proton dependent like vacuolar stachyose transport was not investigated. A proton gradient across the chloroplast inner envelope is formed by an ATPase (Neuhaus and Wagner 2000), leading to a slightly alkaline pH in the chloroplasts (pH 8). A raffinose/H⁺ symporter may be postulated. Other possible co-substrates include cations, such as K⁺ or Ca²⁺, both of which can enter the chloroplasts via channels (Neuhaus and Wagner 2000). P_i as co-substrate is unlikely, because coupled transport of an unphosphorylated substance like raffinose would lead to an imbalance in the well-regulated intrachloroplastic P_i concentration (Flügge 1999). For *Arabidopsis*, a special problem arose in that chloroplasts isolated from soil-grown cold-treated plants were not stable enough to obtain reliable uptake data. Therefore, we isolated chloroplasts from sterile-grown *Arabidopsis* plants, which is well known for the production of high quality chloroplasts (Fitzpatrick and Keegstra 2001). Importantly, the carbohydrate spectra of sterile-grown plants were similar to those of soil-grown cold-treated plants (viz. increased Suc and Raf concentrations; data not shown).

Our results clearly validated proceeding with the next step, to systematically characterize the chloroplastic raffinose transporter at the biochemical level and to search for putative raffinose transporter genes in *Arabidopsis* using recent chloroplast envelope proteomics (Ferro et al. 2003, Froehlich et al. 2003) as well as general genomics data. Once candidate genes are identified, a reverse genetic approach will facilitate the determination of the importance of chloroplastic raffinose in low-temperature tolerance. Furthermore, recombinant expression strategies will allow the raffinose transport system to be fully characterized.

Materials and Methods

Plant material and growth

Ajuga reptans plants were collected from the University of Zürich Botanical Garden, treated with a 0.1% (w/v) Benlate (DuPont, Switzerland) solution for 10 min, washed with water and potted in Perlite or soil (Einheitserde Werksverband, Sinntal Jossa, Germany). Plants were grown at 22°C (100 μmol s⁻¹ m⁻² for 12 h) and 18°C (dark) at 70% RH. After 6–8 weeks, plants were transferred to cold-treatment conditions at 8°C (70 μmol s⁻¹ m⁻² for 12 h) and 3°C (dark) at 70% RH for a minimum of 6 weeks.

Spinach plants (*Spinacia oleracea* cv. Winterriese) were grown in soil under daylight conditions and 22°C for 5 weeks. They were then transferred to cold-treatment conditions at 8°C (90–100 μmol s⁻¹ m⁻² for 12 h) and 3°C (dark) at 70% RH for a minimum of 1 week.

Arabidopsis thaliana (Col-0) plants were grown in soil at 22°C (150–200 μmol s⁻¹ m⁻² for 16 h) and 20°C (dark) at 60% RH. After 4–5 weeks, plants were transferred to cold-treatment conditions at 1°C (150 μmol s⁻¹ m⁻² for 16 h) at 60% RH for a minimum of 1 week.

Sterile *Arabidopsis* plants were grown from sterilized seeds sown on plates containing 0.5×MS salt and vitamin mixture, 0.8% (w/v) phyto agar and 1% (w/v) sucrose. After 3 d at 4°C, the plants were grown at 22°C (100 μmol m⁻² s⁻¹ for 16 h) for a minimum of 3 weeks.

Protoplast isolation

Ajuga leaf protoplasts were isolated according to Bachmann and Keller (1995), with the following changes. Protoplast purification medium [PPM, 0.8–1.2 M glycine–betaine, 25 mM MES–Tris pH 5.5, 5 mM CaCl₂, 1 mM DTT, 1 mM Na-ascorbate, 0.1% (w/v) BSA, and 0.1% (w/v) PVP-K-30] was supplemented with 2% (w/v) cellulase Y-C and 0.1% (w/v) pectolyase Y-23. Digested leaf tissue was filtered through a double layer of 40 μm nylon mesh. The filtrate was washed by centrifugation at 50×g for 5 min through a Percoll density step gradient of PPM. A top layer of 30% (v/v) Percoll allowed the removal of most of the unwanted cell debris. To remove the remaining carborundum, the protoplast fraction was underlayered with 100% (v/v) Percoll PPM phase. The supernatant was removed and the purified protoplasts on the top of the 100% Percoll were resuspended in PPM and pelleted by centrifugation at 10×g for 5 min. Protoplasts were centrifuge washed (10×g, 5 min) in PPM and finally resuspended in PPM.

For spinach leaf protoplast isolation, the lower leaf epidermis was removed using fine sandpaper and leaves were cut into squares (1×1 cm) and transferred to buffer A [1.5 M glycine–betaine, 20 mM MES–KOH pH 5.5, 0.5 mM CaCl₂, 3% (w/v) cellulase Y-C, and 0.75% (w/v) pectolyase Y-23].

The tissue was digested for 2–3 h at 30°C in the dark. Protoplasts were filtered through a 200 µm nylon mesh, collected by centrifugation at 50×g for 5 min and centrifuge washed twice with spinach resuspension buffer (SRB; 1.5 M glycine–betaine, 20 mM MES–KOH pH 6, 0.5 mM CaCl₂).

For leaf protoplast isolation of cold-treated *Arabidopsis* plants, leaves were chopped in protoplast isolation buffer B [PIBB; 1.6 M glycine–betaine, 20 mM MES–KOH pH 5.6, 1 mM CaCl₂, 0.1% (w/v) BSA, 0.1% (w/v) PVP K-30 and 2 mM Na-ascorbate] and washed with the same buffer. The tissue was digested with 1.5% (w/v) cellulase Y-C and 0.125% (w/v) pectolyase Y-23 in PIBB for 2 h at 30°C. Protoplasts were filtered through a 200 µm nylon mesh, collected by centrifugation at 50×g for 5 min and centrifuge washed twice with *Arabidopsis* resuspension buffer (ARB; 1.6 M glycine–betaine, 20 mM MES–KOH pH 6, 0.5 mM CaCl₂).

Protoplasts from sterile-grown *Arabidopsis* plants were isolated as reported by Fitzpatrick and Keegstra (2001). Entire plants were chopped in digestion buffer (DB; 400 mM sorbitol, 20 mM MES–KOH pH 5.2, 0.5 mM CaCl₂) and washed with DB. The tissue was digested with 1.5% (w/v) cellulase Onozuka R-10 and 0.375% (w/v) Macerozyme R-10 in DB for 4 h at 20°C and 80 µmol m⁻² s⁻¹ light. Protoplasts were filtered through a 200 µm nylon mesh, collected by centrifugation at 50×g for 5 min and centrifuge washed twice with resuspension buffer (400 mM sorbitol, 20 mM MES–KOH pH 6, 0.5 mM CaCl₂).

Chloroplast isolation

Chloroplast isolation from protoplasts was performed similarly for all plant sources used. After an additional centrifugation (50×g, 5 min), the protoplast pellet was resuspended in chloroplast purification buffer (CPB; 330 mM sorbitol, 50 mM HEPES–KOH pH 7.6, 2 mM EDTA, 1 mM MgCl₂ and 1 mM MnCl₂). The obtained suspension was squeezed through a 5 ml syringe closed with a 20 and 11 µm nylon mesh. Released chloroplasts were resuspended in CPB, centrifuge washed twice (425×g, 4°C and 5 min) and resuspended in CPB to a Chl concentration of 0.2 mg ml⁻¹.

Protoplast and chloroplast integrity and purity

Protoplast integrity was determined by staining with fluorescein diacetate and inspecting visually by bright field and DIC microscopy. Random samples were investigated using ferricyanide as a marker for chloroplast integrity (Lilley et al. 1975).

For chloroplast purity, NADH-MDH activity was used as a marker enzyme for extra-chloroplastic contamination with mitochondria, peroxisomes and cytosol. It was analyzed photometrically in an assay mixture containing, in a total volume of 1 ml, 100 mM Tris–HCl pH 7.5, 2 mM MgCl₂, 0.24 mM NADH, 3 mM oxaloacetate and 10–30 µl of sample. Peroxisomal contamination was determined by the

measurement of HPR activity according to Titus et al. (1983). Vacuolar contamination was determined by the soluble vacuolar marker enzymes, α-mannosidase and α-galactosidase (Keller and Matile 1985). Chl concentration was determined according to Lichtenthaler and Wellburn (1983).

Chloroplast uptake assays

Radioactive uptake assays were performed at room temperature (22°C) in the light using two different silicone oil centrifugation methods. Uptake times from 6 to 60 s were achieved by single layer silicone oil centrifugation (Heldt 1980). After the corresponding uptake time, uptake was stopped by centrifugation at 13,000×g for 20 s using a vertical rotor in a Beckman Microfuge E. For uptake times <6 s, the double layer silicone oil centrifugation method was used (Gross et al. 1990). Microcentrifuge tubes (400 µl) contained the five layers (from bottom to top), 20 µl 10% (v/v) perchloric acid, 75 µl silicone oil, 100 µl of CPB containing the radiolabeled substrates, 75 µl silicone oil and 100 µl of chloroplast suspension. Centrifugation under those conditions resulted in an uptake time of 0.8–1 s (Gross et al. 1990). *Ajuga* chloroplasts were centrifuged through a 1:3 (v/v) mixture of the silicone oils, AR20 and AR200 (Wacker, Burghausen, Germany). For *Arabidopsis* chloroplasts, only silicone oil AR200 was used. The chloroplast pellets were transferred to 300 µl of water and centrifuged at 13,000×g to separate the oil from the water phase. Radioactivity was determined in an aliquot of 150 µl of the water phase by liquid scintillation counting. In addition to the corresponding uptake rates, stromal carbohydrate concentrations were calculated according to Heldt (1980) from values obtained after time saturation of the uptake (6–60 s).

Carbohydrate compartmentation

Carbohydrate distribution between protoplasts and chloroplasts was determined by aqueous compartmentation assays. Protoplasts and chloroplasts were isolated as mentioned with the exception that sorbitol was replaced by glycine–betaine in all media. After chloroplast isolation, 100 µl of chloroplast suspension were directly loaded into 400 µl microcentrifuge tubes containing 20 µl of 1.5 M glycine–betaine and 100 µl of silicone oil [a 1:3 (v/v) mixture of AR20 and AR200] for *Ajuga* and AR200 for spinach and *Arabidopsis*) and centrifuged at 13,000×g for 20 s using a vertical rotor. The tube tips were cut and the pellets within the 20 µl of glycine–betaine of 10 tubes were resuspended and pooled. Aliquots of the purified protoplasts and chloroplasts were used for Chl, carbohydrate and marker enzyme analysis.

Water-soluble carbohydrate extraction

Carbohydrates from protoplast and chloroplast fractions were extracted, desalted and freed from phenolic compounds as

described (Bachmann et al. 1994), with the following modifications. A 1 ml MoBiCol column prepared with 150 µl of Amberlite CG400 II (HCO₂⁻-form), 100 µl of PVPP and 50 µl of Serdolit CS-2C (H⁺-form) was washed with 600 µl of water by centrifugation (3,000×g, 4°C, 4 min). Sample aliquots (50 µl) were added and centrifuged as mentioned. Carbohydrates were eluted by rinsing the columns twice with 100 µl of water and analyzed by HPLC-PAD.

Raffinose biosynthetic pathway enzyme activities

Protoplast and chloroplast extracts were prepared by resuspending pellets in 50 mM HEPES–KOH pH 7.5, 5 mM MgCl₂, 1 mM CaNaEDTA, 2% (w/v) PVP K-30, 2% (w/v) PEG 6000, 0.1% (v/v) Triton X-100, 20 mM DTT, 2% (w/v) PVPP, 50 mM Na-ascorbate and 0.1% (w/v) BSA to a final Chl concentration of 0.3 mg ml⁻¹. The homogenate was centrifuged (13,000×g, 10 min, 4°C) and desalted by centrifuge desalting. GolS activity was determined in a 40 µl assay containing desalted crude enzyme extract, 50 mM *myo*-inositol, 2.5 mM UDP-galactose in 25 mM HEPES–KOH pH 7.5, 5 mM DTT and 1 mM MnCl₂. The reaction was incubated at 30°C for 15 min and stopped by boiling. Control samples were boiled immediately. Samples were centrifuged (13,000×g, 4°C, 5 min) and desalted as described. Galactinol formation was determined in 20 µl aliquots by HPLC-PAD. RafS activity was determined in a 100 µl assay containing desalted crude enzyme extract, 25 mM raffinose and 25 mM *myo*-inositol in 25 mM MES–KOH pH 6.5. The reaction was incubated at 30°C for 30 min and stopped by boiling. Control samples were boiled immediately. Samples were centrifuged and desalted as described above. Raffinose formation was determined in 20 µl aliquots by HPLC-PAD.

Carbohydrate analysis

Two different HPLC-PAD systems were used to allow optimum separation for each particular experiment. Desalted protoplast and chloroplast fractions and enzyme assays were analyzed with a Ca²⁺-column (BC100, 7.8×300 mm; Benson Polymeric, Reno, NV, USA; 90°C, 50 mg l⁻¹ Ca/Na₂-EDTA in H₂O). Polyols were analyzed with a CarboPac MA1 column (4×250 mm, 600 mM NaOH; Dionex, Sunnyvale, CA, USA). Carbohydrates were detected by pulsed amperometric detection with a gold working electrode.

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