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Metabolism of the Raffinose Family Oligosaccharides in Leaves of *Ajuga reptans* L.[1]

Inter- and Intracellular Compartmentation

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We recently suggested that leaves of the frost-hardy species *Ajuga reptans* L. (Lamiaceae) contain two pools of raffinose family oligosaccharides (RFO): a large long-term storage pool in the mesophyll, possibly also involved in frost resistance, and a transport pool in the phloem (M. Bachmann, P. Matile, F. Keller [1994] Plant Physiol 105: 1335–1345). In the present study, the inter- and intracellular compartmentation of anabolic RFO metabolism was investigated by comparing whole-leaf tissue with mesophyll protoplasts and vacuoles. The studies showed the mesophyll to be the primary site of RFO synthesis in *A. reptans*. Mesophyll protoplasts were capable of RFO formation upon in vitro $^{14}CO_2$ photosynthesis. Sucrose-phosphate synthase, galactinol synthase, and the galactinol-independent galactosyltransferase, which is responsible for RFO chain elongation, were located predominantly in the mesophyll protoplasts. The percentage of stachyose synthase in the mesophyll changed greatly during the cold-acclimation period (from 26% at the beginning to 88% after 20 d). The remainder was most probably in the intermediary cells of the phloem. Compartmentation studies in which mesophyll protoplasts were compared with vacuoles isolated from them showed that, of the components of the RFO storage pool, galactinol synthase, stachyose synthase, *myo*-inositol, galactinol, and sucrose were extravacuolar (most probably cytosolic), whereas galactinol-independent galactosyltransferase and higher RFO oligomers (with degree of polymerization 4) were vacuolar. Raffinose was found in both locations and might serve as a cryoprotectant.

In a recent paper (Bachmann et al., 1994), we showed that the frost-hardy, perennial labiate *Ajuga reptans* stores large amounts of RFO (up to 200 mg/g fresh weight) in the leaves and translocates mainly Sta (the RFO tetramer) in the phloem. We suggested the presence of two separate RFO pools, a storage pool and a transport pool. To fulfill their storage role, RFO are expected to be synthesized and accumulated in the mesophyll, the largest tissue of an *A. reptans* leaf. There is now increasing evidence that Sta is loaded symplastically through plasmodesmata (for a recent review, see Grusak et al., 1996). The symplastic phloem-loading model developed by Turgeon (Turgeon and Gowan, 1990; Turgeon, 1991) states that Suc is synthesized in the mesophyll and diffuses into specialized companion cells of the phloem (IC) via narrow plasmodesmata, where Sta is finally synthesized. Sta is thought to be physically trapped, because it is larger than the functional pore size of the plasmodesmata and, therefore, cannot diffuse back into the mesophyll. As a result, a concentration gradient builds up in the IC and Sta is forced to enter the sieve elements. If this model is true for *A. reptans*, the dual role of Sta as main phloem translocate and as precursor for storage RFO suggests a distinctive compartmentation of Sta synthesis. The RFO storage pool would imply that the mesophyll is the site of Sta synthesis, and symplastic phloem loading would imply that the IC is a separate site of synthesis of transported Sta.

In this paper, we show that the two RFO pools are located in different tissues of *A. reptans* leaves, the mesophyll (storage RFO) and the phloem (transport RFO). Furthermore, we show that the storage pool is located mainly in the vacuoles of mesophyll cells.

MATERIALS AND METHODS

Materials

Biochemicals were obtained from Fluka unless stated otherwise in the text. Gly betaine (Betafin BP) was from Finnsugar Ltd. (Helsinki, Finland) and $NaH^{14}CO_3$ (2.17 GBq/mmol) was from Centre d'Etudes Atomique (Gif-sur-Yvette, France). Cellulase Y-C and pectolyase Y-23 were purchased from Seishin Pharmaceuticals Co. (Tokyo, Japan).

Plant Material and Growth

A. reptans was grown in a mixture of soil and sand (10:1) in a glass greenhouse as previously described (Bachmann et al., 1994). After reaching a stage at which they had 10 to 12 leaves, the plants were transferred to an environmental

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Abbreviations: DP, degree of polymerization; GGT, galactan: galactan galactosyltransferase; Gol, galactinol; GS, galactinol synthase; IC, intermediary cells; Raf, raffinose; RFO, raffinose family oligosaccharide(s); RS, raffinose synthase; SPS, Suc-phosphate synthase; Sta, stachyose; STS, stachyose synthase.

chamber and were subjected to a 12-h photoperiod (200 μmol m^{-2} s^{-1}) at 10/3°C and 58/72% RH (day/night). Cold-acclimated *A. reptans* plants (10–15 d at the conditions described above) were used for all compartmentation experiments, because (a) they contained higher concentrations of RFO and (b) protoplasts isolated from them resulted in a higher yield, purity, and viability than protoplasts isolated from control plants (grown at 25°C).

Isolation of Protoplasts

Leaves were detached from cold-acclimated plants 3 h into the light period. The lower leaf surface was gently abraded with a mixture of Carborundum and protoplast purification buffer (25 mM Mes-Tris buffer [pH 5.5], 0.7 M Gly betaine, 10 mM $CaCl_2$, 1 mM DTT, 1 mM sodium ascorbate, 0.1% [w/v] BSA, and 0.1% [w/v] PVP 40,000) using a piece of firm foam rubber. About three to four leaves, with their abraded surface in contact with the medium, were floated on 10 mL of digestion medium (2% [w/v] cellulase Y-C and 0.1% [w/v] pectolyase Y-23 in protoplast purification buffer) in a Petri dish (5.5 cm in diameter). Complete digestion of mesophyll tissue was achieved after 2 to 3 h of incubation at room temperature on a rotary shaker (50 rpm). Leaf tissue was filtered through a 25-μm nylon net to remove epidermal protoplasts (>50 μm in diameter) and any cell-wall fragments and washed with 20 mL of protoplast purification buffer. The filtrate was centrifuged at 10*g* for 5 min to pellet the mesophyll protoplasts. The protoplast pellet was resuspended in 5 mL of protoplast purification buffer and centrifuged again at 10*g* for 5 min.

Light Microscopy and Probes of Protoplast Integrity

Leaf tissue was fixed in a mixture of 1.5% (v/v) acrolein and 1.25% (v/v) glutaraldehyde in phosphate buffer, pH 7.2, at room temperature, dehydrated, and embedded in Historesin according to the method of Honegger (1987). Protoplasts were routinely examined by light microscopy, and protoplast integrity was assessed by the uptake of fluorescein diacetate (Wetter and Constabel, 1982) and the exclusion of a 2.5% (w/v) solution of Evans blue (Kanai and Edwards, 1973). The integrity of the tonoplast was confirmed by the protoplasts' ability to accumulate 1% (w/v) neutral red (in protoplast purification buffer) in their vacuoles. Protoplast numbers were determined using a hemocytometer.

Intercellular Compartmentation

One-half of an *A. reptans* leaf was processed for protoplast isolation (total of about 2 g), and the other half was treated the same way except that the digestion enzymes were omitted in the incubation medium. Purified mesophyll protoplasts (1.5 mg of Chl) were extracted by lysing them in 1 mL of enzyme extraction buffer (20 mM Hepes-KOH [pH 7.5], 5 mM DTT, 5 mM $MgCl_2$, 2% [w/v] PEG-20,000, and 2% [w/v] PVP 40,000). Undigested control leaves were extracted in 2 mL of extraction buffer in a chilled glass homogenizer. Protoplast and leaf extracts were centrifuged at 14,000*g* for 15 min in a microcentrifuge. An aliquot (200 μL) of the supernatant was deionized using a small-scale procedure (Bachmann et al., 1994) and analyzed for carbohydrates by HPLC with pulsed amperometric detection using a Sugar-Pak I (300 × 6.5 mm; Waters) or an SS-100 Ca^{2+} carbohydrate (300 × 7.8 mm; Benson Polymeric, Reno, NV) column (Bachmann et al., 1994). The remaining supernatant was desalted by the centrifuge technique of Helmerhorst and Stokes (1980) and assayed for enzyme activities as described below.

Photosynthesis Experiments

For in vitro photosynthesis, protoplasts were resuspended in protoplast purification buffer (400 μL) to give a final concentration of 80 μg/mL Chl and placed in a 1-mL tightly capped glass vial. Photosynthesis was allowed to occur at 200 μE m^{-2} s^{-1} (incandescent lighting) and room temperature and was initiated by the addition of 185 kBq of $NaH^{14}CO_3$. The samples were gently agitated every 2 min.

Photosynthesis with leaf strips or discs was performed under the same conditions as described above except that 370 kBq of $NaH^{14}CO_3$ were used. Leaf strips (2 × 3–5 mm) or leaf discs (19 mm in diameter) were incubated in 800 μL of protoplast purification medium lacking Gly betaine (80 μg Chl/mL) in a 2-mL crimped-top glass vial. Plasmolysis of leaf strips was obtained by abrading the lower leaf surface with Carborundum and floating them for 3 h on protoplast purification medium containing 0.7 M Gly betaine.

In situ labeled protoplasts were produced by labeling leaf strips for 10 min as described above and subsequently isolating protoplasts from them.

Photosynthesis was terminated after 10 min by the addition of 600 μL of ice-cold methanol:chloroform:formic acid:water, 12:5:1:2 (Bieleski and Redgwell, 1977). After the tissue was homogenized in a chilled glass homogenizer, the aqueous phase was separated into acidic, basic, and neutral fractions by centrifugation through Serdolit ion-exchange resins (Serva, Heidelberg, Germany). The neutral fraction was further analyzed by HPLC using a Sugar-Pak I Ca column (Bachmann et al., 1994) and a Radiomatic Flo-One radiodetector (Canberra Packard, Zurich, Switzerland).

Isolation of Vacuoles

Vacuoles were obtained from isolated mesophyll protoplasts by selective rupture of the plasma membrane. An aliquot (50 μL) of purified protoplasts was added to 1 mL of the lysing medium containing 25 mM Tricine-KOH (pH 8.0), 0.6 M Gly betaine, 12.5 mM EDTA, and 2 mM DTT. After 1 min of gentle swirling, the protoplasts were completely lysed, releasing their large central vacuoles. The vacuoles were mixed with an equal volume of vacuole medium (25 mM Tricine-KOH [pH 7.6], 0.7 M Gly betaine, 2 mM DTT, 1 mM EDTA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.1% [w/v] BSA). After sedimentation, the supernatant was carefully withdrawn, and the vacuoles were washed twice with vacuole medium and sedimented (1*g* for 5 min).

Enzyme Assays and Additional Analytical Methods

GS activity was determined by an isotopic assay, and RS, STS, and GGT activities were determined by HPLC as previously detailed (Bachmann et al., 1994). SPS activity was measured with saturating substrate concentrations in the absence of Pi ("V_{max} assay") using the method described by Hubbard et al. (1989). Activities of α-mannosidase and β-*N*-acetylglucosaminidase were assayed using the *p*-nitrophenyl substrates, and NADH-malate dehydrogenase activity was determined photometrically (Frehner et al., 1984). Chl was extracted in 80% (v/v) acetone and determined according to the method of Lichtenthaler and Wellburn (1983).

RESULTS AND DISCUSSION

Minor Vein Anatomy

In a wide variety of plants, Sta has the principal function of a long-distance transporter of carbon skeletons and is thought to be loaded symplastically into the phloem (Grusak et al., 1996). These plants seem to have an ultrastructural difference in the configuration of the minor vein as compared to plants that load apoplastically. Their companion cells are specialized to facilitate symplastic loading: they are especially large, densely filled with cytoplasm, mitochondria, and small vacuoles, and display a particularly high abundance of plasmodesmata at the interface toward the surrounding bundle-sheath cells (Turgeon et al., 1975). Figure 1 shows the minor vein anatomy of a mature *A. reptans* leaf in cross-section. The cells of the minor veins are arranged in a highly regular pattern: the sieve elements are always partly surrounded by three to four cytoplasm-rich companion cells. We assume that these companion cells are identical with the IC described for the cucurbits *Cucurbita pepo* (Turgeon et al., 1975) and *Cucumis melo* (Schmitz et al., 1987), as well as for the labiates *Coleus blumei* (Fisher, 1988) and *Lamium album* (Gamalei et al., 1994). To prove this unequivocally, ultrastructural studies would be necessary to establish the presence of numerous and highly branched plasmodesmata typical of IC (Turgeon et al., 1975; Fisher, 1988). However, there is a strong indication that only plant species that translocate a considerable amount of Sta have true IC (Turgeon et al., 1993), and phloem sap analyses with *A. reptans* demonstrated that Sta was, indeed, the predominant translocated carbohydrate (Bachmann et al., 1994). Furthermore, all members of the Lamiaceae that have been studied so far showed a minor vein anatomy with IC (Gamalei, 1989). Summarizing, *A. reptans* leaves seem to have the necessary minor vein ultrastructure to allow a symplastic phloem-loading pathway.

Intercellular Compartmentation of RFO Metabolism

In *A. reptans*, RFO function as both the main transport and storage carbohydrates. According to the symplastic phloem-loading model (Turgeon, 1991), it is imperative that the synthesis site of Sta used for transport is strongly separated from that of Sta used for storage. To address this question, we isolated viable mesophyll protoplasts to assay them for the presence of RFO and their capacity for direct RFO synthesis.

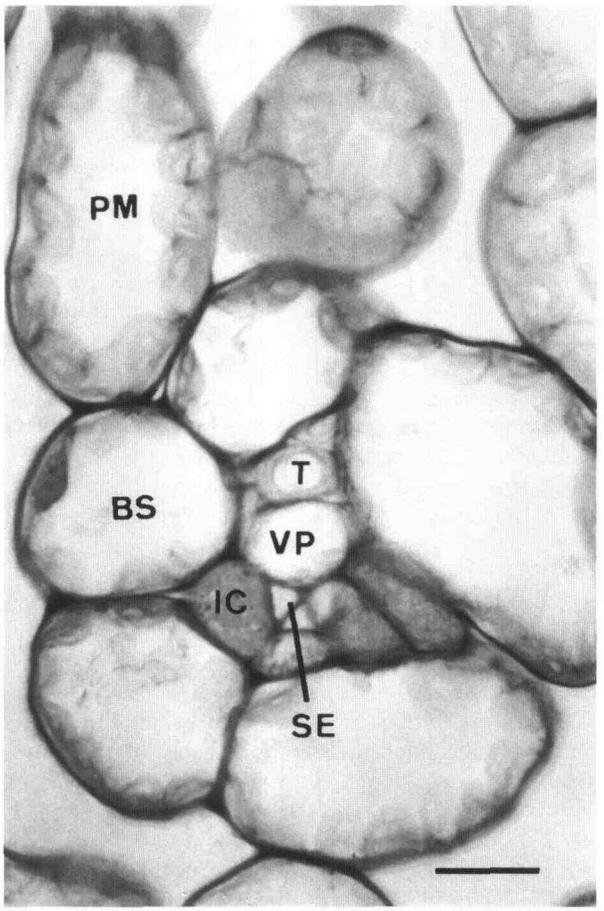

Figure 1. Light micrograph of an *A. reptans* leaf cross-section stained with a mixture of methylene blue and basic fuchsin. Sieve elements (SE) are partly surrounded by IC, which are in contact with the bundle-sheath cells (BS). PM, Palisade mesophyll; VP, vascular parenchyma cell; T, tracheary element. Bar = 10 μm (×920).

The lower surface of the leaves was gently abraded to allow the cell-wall-degrading enzymes to gain proper access to the mesophyll cells. This abrasion technique caused less tissue damage when compared to preparations made with sliced leaf strips and, thus, provided higher yield, purity, and stability of the protoplasts. Complete enzymatic digestion with cellulase and pectolyase was achieved after 2 to 4 h, depending on the plant material used. Examination of the pellet by light microscopy revealed a pure preparation of mesophyll protoplasts with no observable contamination by either vascular or epidermal protoplasts. The isolated protoplasts were virtually free of undigested cells, cell debris, or remnants of vascular tissue but contained a few vacuoles (up to 2%). Evans blue was excluded by more than 90% of the protoplasts, indicating their high viability. A rapid accumulation of fluorescein from fluorescein diacetate into the cytoplasm was also observed, confirming the intactness of the plasmalemma and retention of esterase activity. Neutral red accumulated in the

vacuoles, showing the acidic pH of the vacuole and integrity of the tonoplast membrane. The mesophyll protoplasts showed only a slight variation in size within a given preparation, with the average diameter being about 37 μm in a medium of 0.7 M osmolarity (Bachmann, 1993).

For the intercellular compartmentation studies, isolated mesophyll protoplasts were compared with extracts of whole leaves, which, as a control, had been floating on protoplast purification medium for the same duration as was needed for protoplast isolation. As a mesophyll marker, we used Chl, since IC do not contain chloroplasts (Table I). Marker enzymes such as NADH-malate dehydrogenase, α-mannosidase, and β-*N*-acetylglucosaminidase were located in the mesophyll protoplasts to levels of about 85%. Because of inherent limitations in the method, we are unable to quantitatively determine the proportions of solutes or enzymes in the mesophyll and phloem. However, it is clear that enzymes of the anabolic Suc (SPS) and RFO (GS, RS, STS, GGT) pathway were located primarily in the mesophyll. These results demonstrated that in *A. reptans* the mesophyll seems to function as an RFO production factory as well as an RFO store. The mesophyll as a pronounced long-term RFO-storing tissue might be typical mainly of frost-hardy RFO plants such as *A. reptans*.

We have preliminary evidence that the amount of STS in the mesophyll changes greatly during the cold-acclimation period. In nonacclimated leaves, STS activity was only partly recovered in the mesophyll (26%), whereas it was predominantly recovered there (88%) after 20 d. The high preference for Sta as phloem translocate in *A. reptans* would favor the IC of the phloem as the second possible site of Sta synthesis. Immunolocalization studies with *C. melo* leaves showed that the STS was, indeed, located in the IC (Holthaus and Schmitz, 1991). Generally, the size of the observed mesophyll storage pool may easily vary with the physiological state of the plant (Madore, 1992), whereas that of the phloem site is more constant.

Photosynthesis with Mesophyll Protoplasts and Leaf Tissues

In another approach to detect the distinct RFO synthetic pathway in the mesophyll, we monitored $^{14}CO_2$ photosynthesis with different leaf tissues (Table II). Biosynthesis of [^{14}C]Sta was indeed found to occur upon in vitro $^{14}CO_2$ photosynthesis with isolated mesophyll protoplasts. About 1% of the label of the neutral fraction was present in Sta. Its formation, however, was markedly lower compared to that in intact leaf discs (9.5%) or leaf strips (10.8%). In a similar approach, Madore and Webb (1982) detected only a very small amount of Sta (0.4%) synthesized upon in vitro ^{14}C photosynthesis with isolated mesophyll cells of *C. pepo*. Also, when *A. reptans* leaf strips were plasmolyzed, Sta formation was greatly impaired (2.3%; Table II). This decreased capacity of RFO synthesis is most likely the result of a general inhibition of photosynthesis by the decreased osmotic potential (Sharkey and Badger, 1982; Kaiser, 1984; Saradadevi and Raghavendra, 1994). The possibility that the freshly synthesized Raf and Sta had been hydrolyzed by invertase and/or α-galactosidase activities seems rather unlikely, because no hydrolytic products were found. When mesophyll protoplasts were isolated from $^{14}CO_2$-labeled whole leaves, 7.8% of the neutral fraction of fixed radioactivity was recovered in Sta (Table II). This increased incorporation of ^{14}C into Sta of in situ labeled protoplasts (7.8%) compared to in vitro labeled protoplasts (1.0%) could be explained by the 3-h chase period applied for protoplast isolation. Similarly, when some of the pulse-labeled, plasmolyzed leaf strips were subjected to a 3-h

Table I. *Comparison of mesophyll protoplasts with whole-leaf extracts of cold-acclimated A. reptans leaves*

Values are means ± SD of at least three replicates. ppl, Protoplasts.

Substance	Whole-Leaf Extract	Mesophyll Protoplast Fraction	Percentage ppl[a]
	mg or milliunits/g fresh wt	*mg or milliunits (2×10^7 ppl)*	
Chl	1.77 ± 0.04	1.32 ± 0.01	100 ± 2
NADH-malate dehydrogenase	2180 ± 70	1390 ± 150	86 ± 11
α-Mannosidase	299 ± 14	190 ± 24	85 ± 14
β-*N*-acetylglucosaminidase	257 ± 35	163 ± 11	85 ± 15
SPS	419 ± 73	262 ± 21	84 ± 19
GS	597 ± 81	345 ± 42	77 ± 18
RS	37.0 ± 6.8	18.8 ± 2.1	68 ± 21
STS	63.0 ± 14.9	28.8 ± 6.0	61 ± 31
GGT	84.0 ± 2.9	53.5 ± 2.0	85 ± 5
RFO DP > 6	108.4 ± 7.1	77.8 ± 5.2	96 ± 7
Ajugose	34.1 ± 6.2	30.8 ± 5.8	121 ± 26
Verbascose	41.5 ± 5.5	30.6 ± 4.0	99 ± 19
Sta	39.4 ± 7.0	21.0 ± 0.6	71 ± 18
Raf	13.5 ± 2.4	10.7 ± 2.8	106 ± 32
Suc	12.4 ± 4.6	8.9 ± 1.5	96 ± 41
Gol	8.7 ± 1.2	4.7 ± 0.7	72 ± 20

[a] Calculated from mean values assuming Chl to be 100% located in mesophyll protoplasts.

Table II. *Distribution of ^{14}C in the neutral fraction of different parts of A. reptans leaves following photosynthesis with $^{14}CO_2$*

All samples were labeled for 10 min at 200 $\mu E\ m^{-2}\ s^{-1}$ by incubation in 25 mM Mes-Tris buffer (pH 5.5), 10 mM $CaCl_2$, 1 mM DTT, 1 mM sodium ascorbate, 0.1% (w/v) BSA, and 0.1% (w/v) PVP 40,000 containing 185 kBq of $NaH^{14}CO_3$. Plasmolysis was achieved by inclusion of 0.7 M Gly betaine. For in situ labeling, protoplasts were isolated from $^{14}CO_2$-labeled leaf discs. In vitro photosynthesis with protoplasts was performed by incubating isolated protoplasts in the labeling solution as described above. All samples were extracted as described in "Materials and Methods," and neutral fractions were analyzed by HPLC on a Sugar-Pak I Ca column. Data are means ± SD of two to six replicates. Suc is the sum of Suc and maltose; Gol is the sum of Gol and Glc. Suc/maltose and Gol/Glc were not separated by the HPLC column used in these experiments. Control runs of representative samples using an amino column indicated that maltose and Glc were only minor components (<25%) of the major components Suc and Gol, respectively.

Sample	^{14}C Distribution in Neutral Fraction				
	Sta	Raf	Suc	Gol	Other
			%		
Leaf discs	9.5 ± 2.3	11.9 ± 9.5	42.5 ± 10.1	31.3 ± 26.4	4.9 ± 4.5
Leaf strips	10.8 ± 6.2	9.8 ± 6.0	50.5 ± 14.4	23.2 ± 7.6	5.8 ± 3.5
Leaf strips, plasmolyzed	2.3 ± 0.5	3.0 ± 0.9	66.2 ± 15.1	20.4 ± 8.8	4.3 ± 2.7
Leaf strips, plasmolyzed, 3-h chase	19.2 ± 10.0	6.3 ± 1.7	51.5 ± 14.6	17.0 ± 2.0	6.2 ± 1.0
Protoplasts, in vitro	1.0 ± 0.6	1.8 ± 0.4	70.8 ± 8.4	19.7 ± 9.5	6.1 ± 4.7
Protoplasts, in situ	7.8 ± 5.1	2.9 ± 0.2	76.1 ± 4.4	12.5 ± 1.7	0.6 ± 0.9

chase period, ^{14}C incorporation into Sta was markedly increased (19.2 versus 2.3%).

Intracellular Compartmentation of RFO Metabolism

The vacuolar/extravacuolar compartmentation of RFO and their anabolic enzymes were studied by comparison of mesophyll protoplasts and vacuoles isolated from them. Intact vacuoles were released from purified protoplasts by selective lysis of the plasma membrane. This was successfully achieved by treating the protoplasts with 5 mM EDTA under hypotonic conditions at high pH (8.0) and allowed the simple purification procedure of sedimenting the vacuoles with subsequent withdrawal of the supernatant. The quantitative estimation of the yield and purity of the isolated vacuoles was based on α-mannosidase as a vacuolar marker and Chl and NADH-malate dehydrogenase as extravacuolar markers. A yield of 15 to 20% of vacuoles was obtained from protoplasts used for lysis (Table III). The isolated vacuoles were contaminated to levels of about 20%; this was caused mainly by chloroplasts and some intact protoplasts co-sedimenting with the vacuoles. Nevertheless, yield and purity of *A. reptans* mesophyll vacuoles were sufficiently high to allow compartmentation analysis.

The results are summarized in Table III and may be simplified by distinguishing three different patterns of subcellular distributions: (a) GGT, Sta, and higher RFO oligomers, as well as Gal, are vacuolar; (b) GS, STS, *myo*-inositol, Gol, Suc, Glc, and Fru are extravacuolar; whereas (c) Raf is more or less evenly distributed between the two compartments. The presence of Gal in the vacuole might be the result of some α-galactosidase action. However, Gal concentration in leaves is extremely low (approximately 2 mM in the cryosap; M. Bachmann and F. Keller, unpublished results), a finding we have always observed.

Table III. *Comparison of A. reptans mesophyll protoplasts with vacuoles isolated from them*

Data are of one representative experiment.

Substance	Protoplast	Vacuoles	Percentage in Vacuole[a]
	μg or millunits		
α-Mannosidase	6.57	1.25	100
Chl	63.3	2.46	20
NADH-malate dehydrogenase	133.8	6.13	24
GS	18.3	0.11	3
STS	1.93	0.03	8
GGT	4.31	0.87	106
RFO DP > 6	3342	693	109
Ajugose	1687	343	107
Verbascose	1594	321	106
Sta	937	173	97
Raf	617	71	60
Suc	1959	36	10
Gol	195	5	13
Glc	723	9	7
Gal	47	12	134
Fru	231	2	5
myo-Inositol	83	1	6

[a] Calculated assuming α-mannosidase to be 100% vacuolar.

The completely vacuolar location of the higher RFO oligomers is consistent with their proposed role as storage carbohydrates. With an estimated total concentration of about 350 mM, their deposition in any other compartment than the mesophyll vacuole would have been surprising. The vacuolar accumulation of higher RFO oligomers and the acidic pH optimum found for the RFO chain-elongation reaction by GGT (Bachmann et al., 1994) both suggested a vacuolar location of GGT, which was indeed confirmed by the intracellular compartmentation study. It is interesting that the vacuolar location of the anabolic metabolism of higher RFO oligomers in *A. reptans* leaves is in complete analogy to fructan metabolism in barley leaves and Jerusalem artichoke tubers in which both the fructans and the chain-elongation enzyme fructan:fructan fructosyltransferase are also confined to the vacuoles (Wagner et al., 1983; Frehner et al., 1984).

According to their neutral pH optima (7.5 and 7.0, respectively; Bachmann et al., 1994), GS and STS activities were expected to be extravacuolar in location, and our results confirm that that is the case. The fact that some of the key substrates and products of the Sta synthetic pathway (i.e. *myo*-inositol, Gol, Suc, and to a certain extent Raf) are extravacuolar (most probably cytosolic) is also in agreement with the extravacuolar location of GS and STS. Because Sta synthesis obviously occurs outside the vacuole (most probably in the cytosol) and Sta is located mainly in the vacuole, Sta transport across the tonoplast is necessary. Such transport, possibly against a concentration gradient from cytosol to vacuole (assuming the vacuole to occupy 70–80% and the cytosol 5–10% of the protoplast volume; Winter et al., 1994), might be similar to that recently found in *Stachys* tubers, where uptake of Sta was stimulated by Mg·ATP and PPi and operated by an H^+-sugar antiport system (Keller, 1992; Greutert and Keller, 1993).

The results of the inter- and intracellular compartmentation of RFO metabolism in mature *A. reptans* leaves are summarized in the tentative scheme shown in Figure 2. According to this model, Sta is synthesized in different tissues, depending on its future physiological fate. Storage Sta is synthesized in the mesophyll; transport Sta is synthesized in the IC. Sta synthesis in the mesophyll takes place in the cytosol by the classical pathway and is subsequently taken up by the vacuole, the site of RFO chain elongation and storage. Sta synthesis in the IC proceeds by STS using Gol and Raf formed either totally or partly from Suc arriving symplastically from the mesophyll.

RFO and Cold Hardiness

The intracellular compartmentation of RFO raises some interesting questions about the function of higher RFO in terms of cold hardiness. In contrast to Suc and starch, RFO are stored in high concentrations and are not subject to rapid depletion (Bachmann et al., 1994). Even in periods of minimum accumulation, RFO concentrations found in *A. reptans* greatly exceeded that of starch or Suc combined, as reported for other species (Lewis, 1984; Brocklebank and Hendry, 1989). This indicates that RFO may have functions in addition to serving as a carbon reserve or energy source.

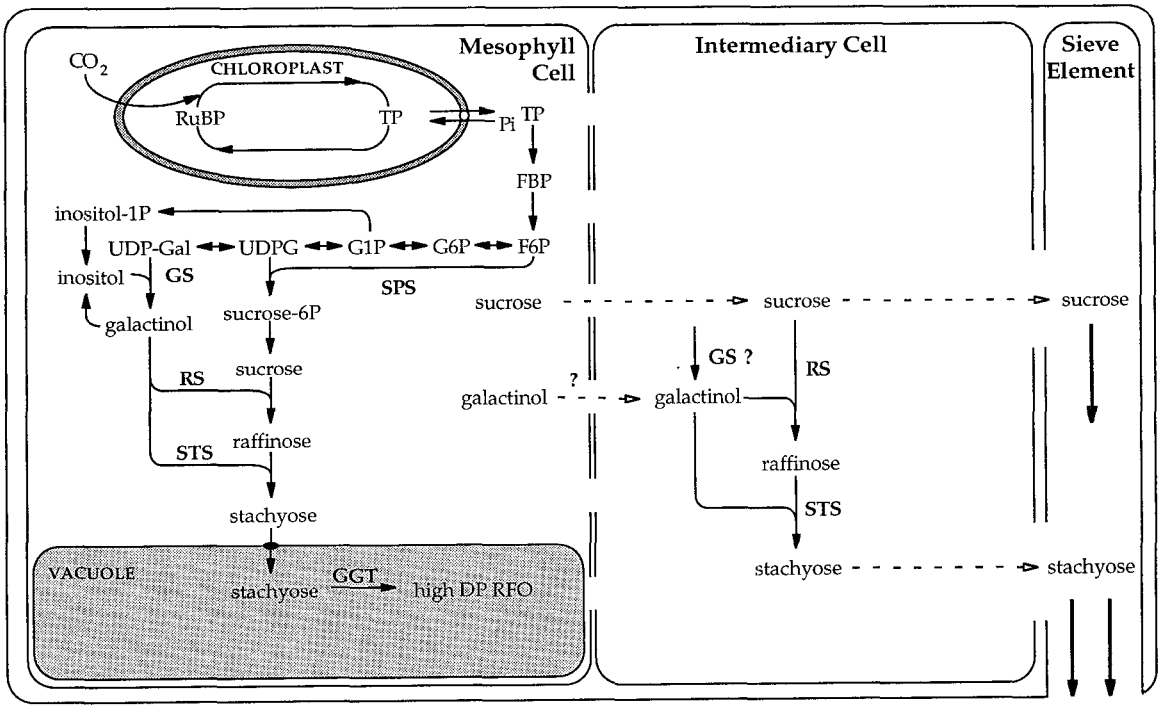

Figure 2. Tentative scheme depicting the inter- and intracellular compartmentation of the RFO and their metabolism in a mature *A. reptans* leaf. FBP, Fru-bisP; F6P, Fru-6-P; G1P; Glc-1-P; G6P, Glc-6-P; RuBP, ribulose-bisP; TP, triose-P; UDPG, UDP-Glc.

Because RFO accumulate during cold hardening, cryoprotective qualities have been attributed to them. Raf levels have been strongly correlated with increased cold hardiness (Hinesley et al., 1992; Wiemken and Ineichen, 1993). Studies with Puma rye showed that Raf accumulated during cold acclimation but was located primarily in the extravacuolar volume (Koster and Lynch, 1992). In *A. reptans*, where vacuolar RFO of higher DPs function as carbon-storage compounds, at least a portion of Raf was also found to be cytosolic and could serve as cryoprotectant. Raf is known to delay the crystallization of Suc and to show structure-conserving abilities upon binding to proteins and membranes (Caffrey et al., 1988).

Whether the vacuole-stored high-DP oligomers have a principal or direct role in low-temperature tolerance is questionable. Based on the correlation of fructan accumulation and the perennation of temperate plant species, a role for fructans as cryoprotectants has been proposed (Olien, 1984; Pontis, 1989). Studies with different wheat accessions, however, showed no correlation between DP > 4 fructan content and winter hardiness; in contrast, spring-habit accessions generally had more of the high-oligomer fructans than those of winter habit (Livingston et al., 1993). Polymerization and breakdown of RFO alter the vacuolar osmotic potential and hence may alter turgor pressure. It is conceivable that high-DP RFO may also serve as an osmotic regulator, as was proposed for fructans, for which fructan-to-Fru conversion was shown to be directly linked with petal expansion in the ephemeral daylily flower (Bieleski, 1993).

CONCLUDING REMARKS

A. reptans contains two pools of RFO, a storage pool in the mesophyll and a transport pool in the phloem. In the present study we report the inter- and intracellular compartmentation of the RFO metabolism. The mesophyll was shown to be the primary site of RFO synthesis. Isolated mesophyll protoplasts were capable of RFO synthesis upon in vitro $^{14}CO_2$ photosynthesis. The striking compartmentation of the RFO storage pool (i.e. mesophyll vacuoles) and the transport pool (i.e. IC) is in line with the current model for symplastic phloem loading. It seems that Sta synthesized in the IC is used directly for export, whereas Sta and higher oligomers synthesized in the mesophyll are used primarily for storage.

The subcellular compartmentation found in *A. reptans* mesophyll cells allows for the interpretation that Sta synthesis occurs in the cytosol and the subsequent RFO chain elongation occurs in the vacuole by GGT from Sta that had been taken up across the tonoplast. Research aimed at further substantiating this new model for RFO storage in leaves is underway. In particular, GGT is being thoroughly characterized, and the proposed Sta transporter on the tonoplast and the formation of higher RFO from Sta by isolated vacuoles are being investigated.

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