Galactan:galactan galactosyltransferase (GGT) is a unique enzyme of the raffinose family oligosaccharide (RFO) biosynthetic pathway. It catalyzes the chain elongation of RFOs without using galactinol (α-galactosyl-myoinositol) by simply transferring a terminal α-galactosyl residue from one RFO molecule to another one. Here, we report the cloning and functional expression of a cDNA encoding GGT from leaves of the common bugle (Ajuga reptans), a winter-hardy long-chain RFO-storing Lamiaceae. The cDNA comprises an open reading frame of 1215 bp. Expression in tobacco (Nicotiana plumbaginifolia) protoplasts resulted in a functional recombinant protein, which showed GGT activity like the previously described purified, native GGT enzyme. At the amino acid level, GGT shows high homologies (>60%) to acid plant α-galactosidases of the family 27 of glycosylhydrolases. Physiological studies on the role of GGT confirmed that GGT plays a key role in RFO chain elongation and carbon storage. When excised leaves were exposed to chilling temperatures, levels of GGT transcripts, enzyme activities, and long-chain RFO concentrations increased concomitantly. On a whole-plant level, chilling temperatures induced GGT expression mainly in the roots and fully developed leaves, both known RFO storage organs of the common bugle, indicating an adaptation of the metabolism from active growth to transient storage in the cold.

Raffinose family oligosaccharides (RFOs) are sucro-syl galactosides, in which the Gal residues are linked in an α-1,6 fashion to the C-6 of the Glc moiety of Suc. These water-soluble, nonreducing carbohydrates are widespread in the plant kingdom. In many cases, they are direct photosynthetic products and are used for storage, translocation, and utilization of carbon as well as for protection against different abiotic stresses such as those caused by frost, drought, and salt (for review, see Kandler and Höpf, 1984; Keller and Pharr, 1996; Avigad and Dey, 1997; Peterbauer and Richter, 2001). The best-studied and most widespread RFOs are the two short-chain RFOs, the trisaccharide raffinose (Gal1-Suc) and the tetrasaccharide stachyose (Gal2-Suc). Much less is known about the long-chain RFOs, which may occur with degrees of polymerization (DPs) of up to 15 (Bachmann et al., 1994). The focus of our recent research efforts has, therefore, been the study of the physiology and metabolism of the whole range of RFOs. One plant species that allows such studies is the common bugle (Ajuga reptans). This frost-hardy, perennial labiate runs on both the short-chain as well as the long-chain RFOs. It produces, translocates, and stores RFOs and might even use them as antifreeze or antisalt stress agents (Bachmann et al., 1994; Bachmann and Keller, 1995; Gilbert et al., 1997; Sprenger and Keller, 2000; Inan Haab and Keller, 2002).

While the biosynthetic pathway of raffinose and stachyose is well established and known to be galactinol (α-galactosyl-myoinositol) dependent, a novel galactinol-independent pathway responsible for the synthesis of long-chain RFOs was recently described (Bachmann et al., 1994; Inan Haab and Keller, 2002). RFO chains are elongated from raffinose and stachyose by the activity of the enzyme galactan:galactan galactosyltransferase (GGT). GGT catalyzes the direct transfer of a terminal Gal residue from one RFO molecule to another, resulting in the next higher and lower RFO oligomers, respectively. This soluble galactosyltransferase is a glycoprotein, has an acid pH optimum, and resides in the vacuole. Its activity correlates positively with the accumulation of long-chain RFOs (Braun and Keller, 2000; Inan Haab and Keller, 2002). Such a galactinol-independent mode of RFO chain elongation has only been described in two other instances so far. Coleus blumei leaves clearly showed increased GGT activities when plants were salt stressed in their root zones (Gilbert et al., 1997). Secondly, a GGT-like activity was observed in maturing pea (Pisum sativum) seeds producing verbascose (Gal3-Suc) from stachyose alone, but it showed an atypical neutral pH optimum (Peterbauer et al., 2001). Subsequent detailed studies revealed that a multifunctional stachyose synthase, not a bona fide GGT, was responsible for RFO chain elongation in pea seeds (Peterbauer et al., 2002, 2003).

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To further the understanding of the physiological importance of GGT, we have continued to study the common bugle. In this article, we report on the successful cloning of GGT, based on peptide sequence information derived from the major 48-kD band of the purified GGT preparation of the common bugle published previously (Inan Haab and Keller, 2002). We functionally expressed and characterized recombinant GGT in tobacco (Nicotiana plumbaginifolia) protoplasts and showed that GGT transcript levels and enzyme activities, as well as long-chain RFO concentrations, clearly increased in cold-treated plants. The results favor the conclusion that GGT plays a key role in long-chain RFO formation in the common bugle.

RESULTS
A cDNA Encoding GGT-I from Common Bugle Was Cloned and Analyzed

Tryptic digestion and peptide sequencing of the purified GGT protein had yielded fragments with the amino acid sequences LADYVHSK and DLWEHKVDP, respectively, which are highly homologous to plant α-galactosidasases (α-Gals; Inan Haab and Keller, 2002). Using degenerate forward and reverse oligonucleotide primers based on these two sequences, a 774-bp internal fragment of the GGT gene from source leaf cDNA was amplified by PCR. The fragment was cloned, sequenced, and used to screen a cDNA library from common bugle source leaves. After repeated screenings, two clones scored positive and covered the predicted 3' and 5' ends of GGT, respectively; they were identical with the 774-bp internal fragment initially obtained. The sequence information was further utilized to design specific forward and reverse oligonucleotide primers for subsequent RT-PCR using common bugle source leaf RNA as the template. A GGT clone identical with the λ-clones was obtained and the gene designated ArGGT-1. The ArGGT-1 cDNA sequence comprises 1,391 nucleotides (excluding a poly(A⁺) tail of 20 nucleotides) and contains an open reading frame from nucleotide 29 to 1,243 corresponding to a protein of 404 amino acids (Fig. 1).

Southern-blot analysis was performed at high stringency conditions with a specific 250-bp probe from the GGT 3' end, including the 3' untranslated region (UTR; Fig. 2). Restriction digestion with HindIII (Fig. 2, lane 1) and XhoI (Fig. 2, lane 2) resulted in two bands. Because none of these enzymes cut within the 3' UTR probe, the results suggest the presence of two highly homologous genes.

GGT Is Highly Homologous to Acid α-Gals of Family 27 of Glycosylhydrolases

We compared the deduced amino acid sequence of GGT with sequences in the GenBank databases and obtained high homologies to α-Gals of family 27 of glycosylhydrolases (according to the CAZY database, http://afmb.cnrs-mrs.fr/CAZY/; Fig. 1). The α-Gals from Carica papaya (AAP04002), rice (Oryza sativa; BAB12750), Phaseolus vulgaris (AAA73964), and Coffea arabica (AAA33022), were selected with respect to their catalytic characterization and homology to GGT. Like the hydrolases, GGT has a melibiase domain, including an α-Gal motif, and contains also a putative signal peptide, which indicates entering into the secretory pathway (Dey and Pridham, 1972; Zhu and Goldstein, 1994; Nielsen et al., 1996).

Based on the cDNA-deducted amino acid sequences, we constructed an unrooted radial phylogenetic tree from plant α-Gals, galactosyltransferases, and common bugle leaf GGT using ClustalX (Jeanmougin et al., 1998) and TreeView32 (Page, 1996; Fig. 3). Two main groups and four subgroups can be discerned. Family 36 of glycosylhydrolases harbors alkaline α-Gals, SIPs (seed imbibition proteins), raffinose synthases, and stachyose synthases, whereas family 27 of glycosylhydrolases harbors acid α-Gals and GGT. The deduced amino acid sequence of common bugle GGT clearly groups with the acid α-Gals of family 27 (Fig. 3, subgroup I; 64% to 78% identical). It does not group with the recently defined alkaline α-Gals/SIPs (Carmi et al., 2003; Fig. 3, subgroup II; 25% to 27% identical), nor with the galactinol-dependent RFO biosynthetic enzymes, raffinose synthases (Fig. 3, subgroup III; 29% to 35% identical), and stachyose synthases (Fig. 3, subgroup IV; 28% to 31% identical) of family 36.

ArGGT-I Was Functionally Expressed in Tobacco Protoplasts and the Recombinant Enzyme Showed Typical GGT Characteristics

To confirm that the cloned ArGGT-1 gene does indeed encode an acid galactosyltransferase, it was heterologously expressed in tobacco protoplasts under the control of the cauliflower mosaic virus 35S promoter. Protoplast extracts were prepared after 20 h of transfection and assayed for GGT activity. When recombinant GGT was incubated with 50 mM stachyose (DP = 4), the galactosyltransferase products verbascose (DP = 5) and raffinose (DP = 3) were clearly observed (Fig. 4A); the activity was 15 nkat 10⁻⁶ protoplasts (Table I). After 120 min of incubation, even some ajugose (DP = 6) appeared (Fig. 4A). Some hydrolyase activity was also present as indicated by the formation of free Gal (mainly from the substrate stachyose and the product raffinose). Control protoplasts, which were transfected with the empty vector, were virtually devoid of galactosyltransferase activity (Fig. 4B). They showed a small background of invertase activity, producing manninotriose and Fru (from stachyose) as well as traces of α-Gal activity, producing minimal amounts of Gal (from stachyose; Fig. 4B). The presence of intrinsic tobacco protoplast invertase activity is a likely explanation for the appearance of melibiose (from raffinose) in Figure 4A.
The identity of the recombinant GGT reaction products verbascose (from stachyose; Fig. 4A; Table I) and ajugose (from verbascose; Table I) was confirmed by (1) additional HPLC-pulsed-amperometric detection (PAD) using a different mode of carbohydrate separation (on a Dionex PA1 anion-exchange column; data not shown) and (2) collecting the corresponding peaks after HPLC separation on a Benson BC-200 column and subjecting them to enzymatic digestion by an \( \alpha \)-Gal from \textit{Aspergillus niger} (\textit{CaAGAL}; accession no. AAA33022). Numbers indicate codons of ArGGT-1. Underlined amino acid residues in ArGGT-1 indicate the identified peptide sequences from the purified common bugle GGT and were used to design degenerate DNA primers. The \( \alpha \)-Gal motif is marked with asterisks. Of the rice active site, the nucleophile Asp is marked by a bold arrow and the Asp functioning as the general acid/base catalyst by a normal arrow. A secretory signal peptide of the \textit{C. arabica} precursor peptide is indicated by bold italic letters; putative secretory signals of the other peptides are in italics. Amino acids identical to the ArGGT-1 sequence are boxes in black. Gaps, marked by dots, are included for a better match. The sequence reported here has been deposited in the GenBank database (ArGGT-1; accession no. AY386246). The alignment was generated using PileUp/GCG.
The catalytic properties of recombinant GGT were further investigated by incubation of protoplast extracts with various substrates (Table I). With raffinose (50 mM) as substrate, GGT produced mainly stachyose and with verbascose (50 mM), it produced mainly ajugose (Table I). Because the products formed by GGT can in return serve as galactosyl acceptors leading to the next higher DP RFOs, relatively short incubation times (90 min) were used for quantitative GGT assays. Prolonged incubation times (120 min) resulted in the formation of higher DP RFOs, such as verbascose, ajugose, and even DP7 RFO (unnamed), depending on the substrates used (Fig. 4A; data not shown).

The recombinant GGT transferase activities observed with the natural substrates raffinose, stachyose, and verbascose (at 50 mM concentrations) were quite similar (between 13.0 and 17.5 nkat 10^{-5} protoplasts; Table I). As expected and recently reported for the highly purified common bugle GGT enzyme (Inan Haab and Keller, 2002), galactinol (5 mM) inhibited the recombinant GGT reaction with raffinose as substrate, and it also showed some hydrolytic (α-galactolytic) activity (between 0.6 and 2.2 nkat 10^{-5} protoplasts; Table I).

Cold Treatment Induced GGT Transcripts and Enzyme Activities

To define the physiological roles of GGT more precisely, we performed cold-acclimation experiments. When leaves of warm-grown plants (20°C) were excised and subjected to cold growing conditions (8°C/3°C, day/night) for up to 14 d, both the GGT transcripts (Fig. 5A), RFO concentrations (Fig. 5, B and C), and GGT enzyme activities (Fig. 5E) increased concomitantly. The concentrations of galactinol, Suc, and the hexoses remained fairly constant or increased only transiently (Fig. 5D). As reported before (Inan Haab and Keller, 2002), long-chain RFOs (DP > 4) contributed considerably (approximately 50%) to the totality of the accumulated RFOs (Fig. 5B), which is in line with the observed up-regulation of GGT transcripts (Fig. 5A) and enzyme activities (Fig. 5E), respectively, as well as the proposed role of GGT in RFO chain elongation.

Similarly, GGT transcript levels were inducible in specific parts of whole common bugle plants, which were derived from cuttings, that had reached an eight-leaf stage. After chilling the plants for 6 weeks (4°C), GGT transcripts accumulated in mature, fully developed leaves and in roots. By contrast, young, still-developing leaves showed only very faint GGT transcripts (Fig. 6).

DISCUSSION

During the last few years, we have provided increasing evidence that common bugle runs on RFOs. It produces RFOs photosynthetically, translocates RFOs through the phloem, and stores RFOs in the vacuoles of the mesophyll (besides the roots; Bachmann et al., 1994; Bachmann and Keller, 1995; Braun and Keller, 2000; Peterbauer and Richter, 2001; Inan Haab and Keller, 2002). Vacuolar RFO storage deserves special mention because (1) it involves the production and accumulation of long-chain RFOs, which seem to be the exception rather than the rule in the plant kingdom (Bachmann et al., 1995), and (2) the long-chain RFOs are produced by a novel enzyme, GGT, which is a galactinol-independent galactosyltransferase and, thus, clearly deviates from the common raffinose synthase and stachyose synthase, which are galactinol-dependent galactosyltransferases (Bachmann et al., 1994; Inan Haab and Keller, 2002).

In this study, we cloned a cDNA encoding GGT from common bugle leaves on the basis of sequenced tryptic fragments of the purified GGT protein reported earlier (Inan Haab and Keller, 2002). The identity of the cDNA was verified by functional expression in tobacco protoplasts, which we found to be a useful eukaryotic system for heterologous expression of GGT because (1) wild-type tobacco synthesizes no or only traces of RFOs and galactinol (Haritatos et al., 2000 Ayre et al., 2003), (2) it shows only slight α-Gal activity (Fig. 4B), and (3) its acid invertase (compare with accession nos. 1380 Plant Physiol. Vol. 134, 2004

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Figure 2. DNA gel blot analysis of GGT in common bugle leaves. Genomic DNA of common bugle (10 μg per lane) was digested with different restriction enzymes, blotted, and hybridized with a 250-bp radiolabeled cDNA fragment from the GGT 3’ end, including 148 bp from the 3’ UTR. Lane 1, HindIII; lane 2, XhoI; lane 3, undigested; and lane 4, DNA Mr marker (kb).
CAA57428 and CAC83577) hydrolyzes mainly Suc and raffinose but hardly any stachyose (Fig. 4, A and B). Activity assays provided compelling evidence that the cDNA obtained does indeed encode a functional GGT enzyme with characteristics similar to those of the purified enzyme reported previously (Inan Haab and Keller, 2002). Like the native enzyme, the recombinant GGT had a pH optimum around 5 (data not shown) and displayed a fairly broad substrate spectrum, which includes all the RFO species tested, i.e. raffinose, stachyose, and verbascose (Table I). As expected, it also showed some hydrolase activity (Fig. 4; Table I). None of the investigated reactions required galactinol as the galactosyl donor. Rather, galactinol seemed to be an inhibitor of the transferase reaction. When lysates of transfected protoplasts were incubated with 50 mM raffinose and 5 mM galactinol, no GGT enzyme activity was detected, while in incubations with 50 mM raffinose alone, an activity of 13 nkat 10⁻⁶ protoplasts was measured (Table I). Such an inhibitory effect of galactinol on GGT-mediated RFO chain elongation was observed before with highly purified GGT enzyme preparations (Inan Haab and Keller, 2002). Theoretically, this finding could have potential physiological implications, i.e. GGT’s activity could be regulated by the galactinol level in the vacuole, the site of GGT’s action (Bachmann et al., 1994; Braun and Keller, 2000). Because galactinol and GGT do not seem to be located in the same subcellular site (cytosol and vacuole, respectively; Bachmann et al., 1994), such a function is not very likely in planta. However, only a careful reevaluation of the exact subcellular location of galactinol will allow us to settle this issue unequivocally.

Further, we have now additional experimental evidence that GGT is indeed involved in the RFO chain elongation process. When GGT was incubated with stachyose (DP = 4), GGT also produced some ajugose (DP = 6) as soon as the concentration of verbascose (DP = 5) had reached about 1 mM in the incubation medium (Fig. 4A). With verbascose as the substrate, a similar situation was observed, and finally DP7-RFO was 

Figure 3. Unrooted phylogenetic tree containing α-galactoside hydrolase and galactosyltransferase cDNA-derived amino acid sequences from plants. Phylogenetic analysis was performed by ClustalX [http://inn-prot.weizmann.ac.il/software/ClustalX.html], and the unrooted tree was drawn with theTreeView32 software [http://taxonomy.zoology.gla.ac.uk/rod/treviewview.html]. Four subgroups can be discerned. Group I, α-Gals and GGT, including C. arabica (Ca, AAA33022), Cyamopsis tetragonoloba (Ct, AAE69558), Glicyne max (Gm, AAA73963), P. vulgaris (Pv, AAA73964), Lycopersicum esculentum (Le, AAF04591), Helianthus annuus (Ha, BAC66445), rice (Os2, BAB12570/1UAS), and common bugle (Ar1, AY386246), as well as sequences homologous to α-Gal or GGT, respectively, including Arabidopsis (At1, CAC08338; At2, NP_568193; At3, CAC08337), C. papaya (Cp, AAP04002), and rice (Os1, AC023240). Group II, alkaline α-Gals, including Cucumis melo (Cm1, AY114164; Cm2, AY114165) and L. esculentum (Le, AAN32945), as well as seed imbition proteins (SIPs) of Arabidopsis (At1, NP_175970; At2, CAB66109), Brassica oleracea (Bo, CAA55893), Hordeum vulgare (Hv, AAD3975), Persea americana (Pa, CAB57245), and rice (Os1, CAD41092; Os2, BAC29698). Group III, raffinose synthase (RS), including sequences of Cucumis sativus (Cm, AAD02832) and pea (Ps, CAD20127), as well as sequences homologous to RS, including Arabidopsis (At, BAB11595) and rice (Os, BAB64768). Group IV, stachyose synthase (STS) and verbascose synthase (VS), including Alonsoa meridionalis (Am, CAD31704), pea (Ps1-STS_VS, CAC8094; Ps2-STS, CAD55555), Stachys affinis (Sa, CAC86963), and Vigna angularis (Va, CAB64363), as well as sequences homologous to STS of Arabidopsis (At, AAD22659). Family 27 of glycosylhydrolase (according to the CAZY database, http://afmb.cnrs-mrs.fr/CAZY/) includes the newly characterized GGT and eukaryotic acid α-Gals (group I). Family 36 of glycosylhydrolases includes eukaryotic alkaline α-Gals, SIPs, and RS and STS genes (groups II, III, and IV), respectively. Scale bar indicates distance value of 0.1 substitutions per site.
produced from the intermediary product ajugose (data not shown). However, we have no direct in vitro evidence that all the RFO species found in common bugle leaves (up to DP = 15; Bachmann et al., 1994) are synthesized by the GGT reported here. To answer this question, we will need to isolate the respective longer-chain RFOs in large enough quantities to test them as putative substrates. The possibility also exists for the presence of a second RFO chain elongation enzyme responsible for the synthesis of the higher DP RFOs in common bugle. In fact, Southern-blot analysis revealed two bands, which indicate the existence of a second gene highly homologous to GGT (Fig. 2).

The amino acid sequence of GGT shares high homologies with acid plant α-Gals of family 27 of glycosylhydrolases. Phylogenetic analysis shows that GGT groups with acid α-Gals and clearly separates from raffinose synthases and stachyose synthases, which are more closely related to alkaline α-Gals/SIPs from family 36 of glycosylhydrolases (Carmi et al., 2003; Fig. 3). Further, it shows that common bugle GGT groups with sequences of Arabidopsis (CAC08338), C. papaya (AAP04002), and rice (AC023240) and seems to form a new subgroup within the acid α-Gals. Because the classification of these sequences as α-Gals is putative and purely based on their homologies to known α-Gals, it is tempting to speculate that these α-Gals might really be GGTs. Functional expression and characterization of the corresponding gene products will clarify this speculation.

**Figure 4.** HPLC-PAD chromatograms showing enzymatic activities of transfected tobacco protoplasts. Extracted and desalted enzyme samples were incubated with 50 mM stachyose for 30, 60, and 120 min in McIlvaine buffer, pH 5.0. A, Protoplasts transfected with GGT-1, producing mainly verbascose and raffinose. The melibiose and the Fru peaks originate from intrinsic tobacco protoplast invertase activity (high on raffinose and low on stachyose as substrates). B, Empty vector control, showing intrinsic invertase activity. IS, internal standard.
Classification into families of carbohydrate active enzymes is based on amino acid sequence similarities and was proposed a few years ago (Henrissat, 1991; Henrissat and Bairoch, 1993; Henrissat et al., 2001). This classification not only reflects substrate specificities and molecular mechanisms but also structural features of these enzymes. Hydrophobic cluster analysis of the GGT peptide sequence revealed that the catalytic domain of GGT comprised of an (α/β)₈-barrel and a C-terminal domain made up of eight β-strands containing a Greek key motif, just like the plant acid α-Gals from rice (Fujimoto et al., 2003). The active site of family 27 acid α-Gals was predicted to contain two carboxyl groups (Mathew and Balasubramaniam, 1987), one serving as the catalytic nucleophile and the other one as the general acid/base catalyst. Recently, crystallization of a rice α-Gal confirmed the identity of Asp in the conserved motif LKYDN DC after the fourth β-sheet as the catalytic nucleophile as shown previously for green coffee bean α-Gal (Ly et al., 2000; Fujimoto et al., 2002, 2003). Further, Asp in the motif DIXD was shown to be the acid/base catalyst, and Trp in the conserved motif TPPMGWNSW is discussed to function in hydrophobic substrate binding of the glycosyl oligosaccharids (Fujimoto et al., 2003). When GGT was modeled in the known structure of rice α-Gal (SWISS-MODEL; http://swissmodel.expasy.org/), Asp-162 and Asp-217 as well as Trp-45 of GGT precisely superimposed over the respective Asp and Trp residues of rice α-Gal mentioned above (Fig. 1, arrowheads; model not shown). We therefore assume that GGT operates by the same catalytic mechanism as plant acid α-Gals. However, we do not have any information on the molecular environment of the active center of GGT responsible for the preferred galactosyl transfer to RFOs rather than to water. Point mutation studies with heterologously expressed GGT are planned and should provide deeper insight into the structure-function relationship of GGT and α-Gal.

### Table 1. Substrate specificity of recombinant common bugle GGT determined in lysates of transfected tobacco protoplasts

<table>
<thead>
<tr>
<th>Donor</th>
<th>Acceptor</th>
<th>Product</th>
<th>Enzyme Activity (nkat 10⁻⁶ ppl)</th>
<th>Relative Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raffinose</td>
<td>Raffinose</td>
<td>Stachyose</td>
<td>13.0</td>
<td>87.0</td>
</tr>
<tr>
<td>Stachyose</td>
<td>Stachyose</td>
<td>Verbascose</td>
<td>15.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Verbascose</td>
<td>Verbascose</td>
<td>Ajugose</td>
<td>17.5</td>
<td>116.0</td>
</tr>
<tr>
<td>Raffinose</td>
<td>Water</td>
<td>Galactose</td>
<td>2.2</td>
<td>14.7</td>
</tr>
<tr>
<td>Stachyose</td>
<td>Water</td>
<td>Galactose</td>
<td>0.6</td>
<td>4.0</td>
</tr>
<tr>
<td>Verbascose</td>
<td>Water</td>
<td>Galactose</td>
<td>0.9</td>
<td>6.0</td>
</tr>
<tr>
<td>Galactinol</td>
<td>Raffinose</td>
<td>Stachyose</td>
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<td>ND</td>
</tr>
</tbody>
</table>

Reaction mixtures contained galactosyl donors and acceptors at concentrations of 50 mM (sugars) and approximately 50 μ (water), respectively. Empty vector controls were used to correct for the endogenous activities of the tobacco protoplasts. Products were analyzed by HPLC-PAD on a Benson BC-200 column. ND, Not detected; ppl, protoplasts.

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**Figure 5. GGT mRNA levels, carbohydrate concentrations, and GGT enzyme activities from pools of cold-grown excised leaves of common bugle plants.**

A. Radiosignals of ³²P-labeled GGT hybridized on RNA gel blots and ³²P-labeled 400-bp fragment of 26S rRNA as a control of the total RNA loaded. B–E. Carbohydrate concentration and extractable GGT activity of cold-grown (8/3°C day/night, 12 h photoperiod) excised leaves. Concentrations of carbohydrates were determined by HPLC-PAD. Enzyme activity was measured in desalted crude enzyme extracts incubated with 50 mM stachyose as the substrate. Values are means ± SE of four replicates with pools of 12 leaves each. Leaf size ranged from 4 to 9 cm².
The close relationship between GGT and α-Gal at both the biochemical and molecular level is reminiscent of that between β-fructosyltransferases and β-fructosidases (invertases) in fructan-containing tissues. It has long been established that fructan biosynthetic enzymes such as Suc:Suc 1-fructosyltransferase and fructan:fructan 1-fructosyltransferase show some invertase activity (transfer of fructosyl residues to water rather than to Suc or fructan). Conversely, many invertases are able to produce short-chain fructans when incubated at very high Suc concentrations (for review, see Vijn and Smeekens, 1999; Van Laere and Van den Ende, 2002). At the molecular level, fructosyltransferases showed very high homologies to vacuolar-type acid invertases (Van Laere and Van den Ende, 2002; Van den Ende et al., 2003), similar to GGT showing high homologies to different plant acid α-Gals (Fig. 3). Whether these acid α-Gals are vacuolar (like GGT and the fructosyltransferase-invertase pair) is actually not known. Interestingly, structure-function studies by site-directed mutagenesis showed that one single point mutation changed a bacterial fructosyltransferase (levansucrase) into an invertase (Chambert and Petit-Glatron, 1991).

Additional similarities between RFO and fructan metabolisms are indicated by their reported roles in stress protection and carbohydrate storage. Recent in vitro studies have provided direct evidence that both RFOs and fructans may be involved in cell membrane stabilization (Hincha et al., 2002, 2003; Vereyken et al., 2003). In vivo studies showed that down-regulation of α-Gal activities resulted in both enhanced raffinose content and freezing tolerance in transgenic petunia (Petunia hybrida; Pennycooke et al., 2003).

To confirm the roles of GGT (and RFOs) in carbohydrate storage and possibly frost tolerance proposed earlier (Bachmann et al., 1994; Inan Haab and Keller, 2002), cold-acclimation experiments were conducted. When excised leaves of warm-grown common bugle plants were cold acclimated, the GGT gene expression, enzyme activities, and RFO concentrations increased concomitantly (Fig. 5). Interestingly, the RFO increase could be ascribed to about 50% to the contribution of longer-chain RFOs, which is in agreement with the proposed chain elongation properties of GGT. A similar parallel increase of GGT activity and long-chain RFO concentrations was described before for common bugle (Inan Haab and Keller, 2002). The results of our northern-blot analyses now provide evidence for the first time, to our knowledge, that the GGT up-regulation operates at the transcriptional level. Similar conclusions were drawn when galactinol synthase, which catalyzes the first step of the RFO biosynthetic pathway, was considered. Expression studies in common bugle (Sprenger and Keller, 2000), Arabidopsis (Taji et al., 2002), and alfalfa (Medicago sativa; Cunningham et al., 2003) showed that cold acclimation caused a parallel increase of both galactinol synthase gene expression, enzyme activities, and RFO concentrations.

The tissue-specific GGT expression pattern presented in Figure 6 provides yet another piece of evidence for the importance of GGT during cold acclimation. GGT transcripts were very high in mature leaves and roots of cold-grown plants, which are both known to store large amounts and long chains of RFOs; they were low or absent in leaves and roots of warm-grown plants, which are known to be low in RFO contents (Bachmann et al., 1994; Sprenger and Keller, 2000; Inan Haab and Keller, 2002). In this context, it is worth mentioning that not only leaves but also roots have RFO-accumulating functions in common bugle. In midwinter, when temperatures had been below 0°C for about 2 months, wild-grown common bugle plants from the Zurich area had twice as much RFOs in their roots than in their leaves, and the ratios of long-chain (DP > 4) to short-chain (DP ≤ 4) RFOs were 2 and 10 for leaves and roots, respectively (data not shown). This indicates a strong RFO-accumulating capacity of the roots and clearly needs more attention in future research.

In conclusion, we have cloned, functionally expressed, and characterized GGT from common bugle, showing that it is indeed an acid α-galactosyltransferase rather than an α-Gal, despite its high homology to plant acid α-Gals. It is also clearly distinct from the galactinol-dependent α-galactosyltransferases, raffinose synthase and stachyose synthase, both on molecular and biochemical grounds. Functionally, GGT was shown to be able to elongate RFOs in vitro, at least up to DP = 7, and its physiological behavior was positively related to long-chain RFO accumulation. GGT’s role in the acquisition of frost tolerance is only indirectly indicated by an increase of its transcripts and enzyme activities during cold acclimation. Unequivocal evidence for such a role is, however, still missing, and experiments to this end using a transgenic approach are under way.

Figure 6. GGT mRNA levels in different parts of common bugle plants grown for 6 weeks in the warm (22°C) or the cold (4°C). Radiosignals of 32P-labeled GGT hybridized on RNA gel blots and 32P-labeled 400-bp fragment of 26S rRNA as a control of the total RNA loaded.
MATERIALS AND METHODS

Chemicals

Chemicals were obtained from Fluka or Sigma (both in Buchs, Switzerland), if not noted otherwise. Verbascose was purchased from MegaZyme (Bray, Wicklow, Ireland).

Plant Material and Cold-Acclimation Conditions

Field-grown common bugle (Ajuga reptans) plants were collected in the Zurich Botanical Garden area and cultivated hydroponically on Perlite (Samen Mauser, Dübendorf, Switzerland) covered with gravel. Plants were watered weekly with 0.3% standard liquid fertilizer (Wuxal; Maag, Dübendorf, Switzerland) and treated with a 0.1% benlate solution (DuPont, Dübendorf, Switzerland) against fungal infection as required. The plants were grown in several growth chambers differing only in their temperature regimes as indicated in the text. In all chambers, the same light intensity of 50 to 90 μmol m⁻² s⁻¹, supplied by a mixture of incandescent and cool-white fluorescent lights, photoperiod of 12 h, and relative humidity of 60%, respectively, were used.

For single-leaf cold-acclimation experiments, leaves of 7 to 9 cm² area from 8-week-old warm-grown plants were excised and placed upright in glass test tubes filled with tap water. They were exposed to the cold with a day/night temperature regime of 8°C/3°C for 14 d. For whole-plant cold-acclimation experiments, cuttings that were derived from runners and consisted of one leaf pair and one internode each were placed into pretreated commercial potting soil and kept at 22°C for 6 weeks. Cold acclimation was performed at 4°C for 6 weeks, with cuttings having formed 5 to 6 new leaf pairs.

RNA Library Isolation, cDNA Synthesis, and λ-Library Production

Unless otherwise stated, standard protocols were used for molecular procedures (Sambrook et al., 1989). Total RNA was extracted from fully developed common bugle source leaves according to Wadsworth et al. (1988). Messenger RNA was isolated from 1.3 mg of total RNA using the poly(U) Sepharose 4B kit (Amersham Biosciences, Dübendorf, Switzerland). The cDNA synthesis from 5 μg of mRNA and preparation of the λ-phage cDNA library was performed with the HybriZAP-2.1 two-hybrid cDNA cloning kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. After determining the size of the library (1.2 × 10⁶ independent plaque forming units [pfu]), it was amplified once, yielding a titer of 2.3 × 10¹⁰ pfu/μl λ-phage suspension.

Molecular Cloning and Sequencing of GGT cDNA and 26S Ribosomal Probe

The cDNA prepared from common bugle source leaves as described above, together with degenerate primers FR10 (5'-AAC GCN YTN GCN GAY Tay GTN CA-3') and FR12V (5'-GG GTC NAC YTT RTG YTC CCA-3'), which were designed according to tryptic peptide sequences obtained by Inan Haab and Keller (2002), was used to amplify a 774-bp GGT fragment. After verification by sequencing, this fragment was used to screen the λ-cDNA library. Two plaques covering the 3' and 5' ends, respectively, were identified. The primers GTGTFR (5'-ATG GAG GCC TCA TGC TTC TTC AC-3') and GTGT6 (5'-AGT CCA GAA CAC GCT TAA TGC-3'), respectively, were used in a PCR reaction employing the proofreading DNA polymerase (Deep Vent, NEB, Beverly, MA) to amplify the entire open reading frame, including 148 bp from the 3' UTR. After ligation into pGEM-T Easy (Promega, Madison, WI), the nucleotide sequence from several bacterial colonies was determined, and a cDNA sequence identical with the two λ-plaques was identified and designated ArGGT-1.

For the amplification of a 26S rRNA fragment, primers 26S-A and 26S-B were designed, and the resulting fragment had a length of 407 bp and was identified with a large number of plant 26S rDNA genes as determined by BLAST search. It was used as positive controls in northern-blot analyses.

DNA Extraction and Southern-Blot Analysis

Genomic DNA was extracted according to Granner et al. (1990), and 10 μg of DNA was digested with HindIII or XhoI, respectively, separated in a 0.8% agarose gel, and blotted onto Hybond N+ membranes (Amersham Biosciences). The blot was hybridized with the ArGGT-1-5' UTR cDNA fragment (250-bp 3' end, including 148 bases of the 3' UTR), which was 32P-labeled using the Random Primer Labeling Kit (NEB). Hybridization was carried out as described by Granner et al. (1990). Subsequently, the membrane was washed at 65°C with 6× SSC and 1% SDS for 10 min, with 2× SSC and 0.1% SDS for 30 min, and with 0.5× SSC and 0.1% SDS for 30 min. Signals were detected on an x-ray film (Medical RX; Fuji Photo Film, Tokyo).

RNA Extraction and Northern-Blot Analysis

Total RNA was isolated from frozen samples as described above. RNA samples (25 μg per lane) were separated in a 1% (w/v) agarose formaldehyde denaturing gel and capillary blotted onto Hybond N membranes (Amersham Biosciences). Radioactive 32P-labeled probes of a 1.2-kb GGT-1 or a 0.4-kb 26S rRNA gene fragment, respectively, were prepared, hybridized, and the blots developed as described above.

Heterologous Expression of Recombinant ArGGT-1 in Tobacco Protoplasts

ArGGT-1 cDNA was cloned into pARF-7 (Gleave, 1992) and transiently expressed in tobacco (Nicotiana plumbaginifolia) protoplasts by means of 40% polyethylene glycol 6000 transfection (Goodall et al., 1990; Sprenger et al., 1995) using 20 μg of plasmid DNA for 10⁶ protoplasts. Controls were transfected with the empty vector. After 20 h of incubation at 26°C, protoplasts were collected by centrifugation and taken up in extraction buffer (EB) containing 50 mM Na citrate, pH 5.0, 200 mM dithiothreitol, 5 mM MgCl₂, 2% (w/v) PEG 20,000, 2% (w/v) polyvinylpyrrolidone K30, 1 mM EDTA, and 0.2% Triton X-100 (100 μL of EB/1.8 × 10⁶ protoplasts). Protoplasts were lysed on ice by intermittently vortexing for 1 h. The slurry was centrifuged desalted into McIlvaine buffer (McIlvaine, 1921), pH 5.0, using Sephadex G-50 fine (Amersham Biosciences) according to Helmerhorst and Stokes (1980). GGT activity was assayed for 30 to 90 min as described below.

Enzyme Extraction from Fresh Plant Material

Fresh common bugle plant material from cold-acclimation experiments was finely chopped and ground on ice in a mortar with three volumes of precooled extraction buffer EB (see above). The homogenate was centrifuged for 10 min at 13,000g and 4°C, and the supernatant transferred to a new tube. After centrifugation desalting (Helmerhorst and Stokes, 1980), 10 μL were assayed for GGT activity for 30 min with 50 mM stachyose as substrate as detailed below.

GGT Enzyme Assays

Aliquots of 10 μL of desalted protein extracts were incubated with 10 μL of 100 mM substrate (either raffinose, stachyose, or verbascose) in McIlvaine buffer, pH 5.0, at 30°C. After 30 to 120 min, the reaction was stopped with 5 μL of 0.5 M NaOH. The samples were denitized (Bachmann et al., 1994) and the carbohydrates analyzed by HPLC-PAD using a Benson BC-200 Ca-moderated ion partitioning carbohydrate column (7.8 × 300 mm; Benson Polymeric, Reno, NV) operated at 90°C and eluted with 0.005% (w/v) Ca/Na₂EDTA at a flow rate of 0.3 mL min⁻¹ (Bachmann et al., 1994). For confirmation of the reaction products, random samples were also analyzed using a CarboPac PA1 anion-exchange column (4 × 250 mm; Dionex, Sunnyvale, CA) operated at room temperature and eluted with 0.15 M NaOH at 1 mL min⁻¹. For additional product identification of the recombinant GGT assays, the putative verbascose and stachyose peaks were collected after separation on the BC-200 column and subsequently hydrolyzed at 40°C for 60 min with α-Gal from Aspergillus niger (MegaZyme) dissolved in 50 mM Na-acetate buffer, pH 4.5. The hydrolysis products were analyzed by HPLC-PAD using a Benson BC-100 column (7.8 ×
were used to correct for the endogenous activities of the tobacco protoplasts.

Extraction and Quantification of Carbohydrates

Water-soluble carbohydrates were prepared from 50 mg of fresh plant material by ethanol extraction as described (Sprenger et al., 1995), with the following modifications. The plant material was extracted successively at 80°C with three 0.5-mL portions, one portion each of 80% (v/v) ethanol, 50% (v/v) ethanol, and water for 10 min each extraction. The portions were pooled and 0.15-mL aliquots were column-deionized, dried by vacuum centrifugation, and redissolved in 1 mL of water. Carbohydrates were analyzed by HPLC-PAD on a BC-200 column as detailed above. For quantification, authentic Gc, Gal, Fru, myoinositol, galactinol, Suc, melibiose, manninotriose, raffinose, stachyose, and verbascose standards were employed. RFOs with DP > 5 were quantified by using the response factor of verbascose.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession number AY386246.

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