A Bifunctional 3,5-Epimerase/4-Keto Reductase for Nucleotide-Rhamnose Synthesis in Arabidopsis

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l-Rhamnose is a component of plant cell wall pectic polysaccharides, diverse secondary metabolites, and some glycoproteins. The biosynthesis of the activated nucleotide-sugar form(s) of rhamnose utilized by the various rhamnosyltransferases is still elusive, and no plant enzymes involved in their synthesis have been purified. In contrast, two genes (rmlC and rmlD) have been identified in bacteria and shown to encode a 3,5-epimerase and a 4-keto reductase that together convert dTDP-4-keto-6-deoxy-Glc to dTDP-β-l-rhamnose. We have identified an Arabidopsis cDNA that contains domains that share similarity to both reductase and epimerase. The Arabidopsis gene encodes a protein with a predicted molecular mass of approximately 33.5 kD that is transcribed in all tissue examined. The Arabidopsis protein expressed in, and purified from, *Escherichia coli* converts dTDP-4-keto-6-deoxy-Glc to dTDP-β-l-rhamnose in the presence of NADPH. These results suggest that a single plant enzyme has both the 3,5-epimerase and 4-keto reductase activities. The enzyme has maximum activity between pH 5.5 and 7.5 at 30°C. The apparent $K_m$ for NADPH is 90 μM and 16.9 μM for dTDP-4-keto-6-deoxy-Glc. The Arabidopsis enzyme can also form UDP-β-l-rhamnose. To our knowledge, this is the first example of a bifunctional plant enzyme involved in sugar nucleotide synthesis where a single polypeptide exhibits the same activities as two separate prokaryotic enzymes.

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activity similar to the bacterial rmlB (Fig. 1). Since at least some plants generate dTDP-β-L-Rha (Milner and Avigad, 1965; Delmer and Albersheim, 1970) in addition to UDP-α-D-Glc, formation of dTDP-β-L-Rha from dTDP-α-D-Glc was reported as well (for review, see Feingold, 1982). Less is known about the subsequent reactions in the pathway, and it was suggested that the 3,5-epimerization and 4-reduction is mediated by a single enzyme (Kamsteeg et al., 1978). Which nucleotide-activated form(s) of rhamnose plants utilize for various rhamnosylation pathways (glycoproteins, polysaccharides, secondary metabolites, and arabinogalactan proteins) is still unknown.

Here we report that an Arabidopsis gene encodes a protein that contains amino acid motifs that are present in both nucleotide-sugar epimerases and reductases. The recombinant Arabidopsis protein converts dTDP-4-keto-6-deoxy-Glc to a product identified as dTDP-β-L-Rha. Thus, the Arabidopsis protein is a dTDP-rhamnose synthase that possesses 3,5-epimerase and 4-keto reductase activities that we named NRS/ER for nucleotide-rhamnose synthase/epimerase-reductase.

RESULTS

Isolation and Cloning of NRS/ER from Arabidopsis

The biosynthetic genes involved in the synthesis of dTDP-β-L-Rha in human pathogens such as Salmonella enterica (Marumo et al., 1992), Versinia enterocolitica (Zhang et al., 1993), Escherichia coli (Moraio et al., 1993; Marolda and Valvano, 1995), Shigella flexneri (MacPherson et al., 1994), and in plant-associated bacteria including Xanthomonas campestris pv campestris (Koplin et al., 1993) and Rhizobium meliloti (Becker et al., 1997) were discovered and characterized a decade ago. Concomitantly, we identified some Arabidopsis cDNA clones (T44775, R92767, T88368, and Z26952) obtained from the Expressed Sequence Tag (EST) projects at Michigan State University (Newman et al., 1994) and at Institut National de la Recherche Agronomique (INRA), France, that showed sequence similarity to some of these bacterial genes. This finding provided us with valuable initial information to identify biosynthetic genes involved in formation of activated nucleotide-rhamnose in plants. We subsequently isolated a full-length cDNA corresponding to the Arabidopsis gene that encodes a protein (GenBank accession no. AAF75813). This protein (301 amino acids long, which we named NRS/ER, see below) contains structural and functional motifs that are conserved in a large family of NAD-dependent epimerases, reductase, and dehydratases. The Arabidopsis NRS/ER shares approximately 40% amino acid sequence similarity to the functional rmlD from S. enterica and E. coli (Reeves et al., 1996). Multiple sequence alignments revealed several conserved amino acid regions and motifs in NRS/ER and RmlD from several bacteria (Fig. 2A). Two distinctive features of this family are a conserved catalytic triad composed of a Thr/Ser and a YXXXK (where X is any amino acid), and a modified Rossman motif GXXGXXG (X = any amino acid) involved in the coenzyme-binding domain (Wierenga et al., 1986). The YXXXK motif, likely located on NRS/ER between amino acid 144 and 148, is within a larger conserved domain (EXDX7XKXXGXXGX3EX2L) that appears to be conserved in NRS/ER and rmlD-like proteins from various bacteria (Fig. 2A). The GXXGXXG motif [that is predicted to bind NAD(P)H] is located between amino acids 19 and 26 at the N-terminal region of NRS/ER and is contained within a larger conserved domain: OLOXGXXGXXGXLL (where O = hydrophobic amino acid and X = any amino acid; Fig. 2A). Searches of the database of Expressed Sequence Tags (dbEST) revealed putative NRS/ER orthologs with amino acid sequence identities of approximately 80% over 280 amino acids in cotton, wheat, soybean, rice, maize, potato, tomato, sugar beet, and barley. NRS/ER orthologs with at least 80% amino acid sequence identity were also identified in grape, orange, pine, poplar, spruce, lettuce, and ice plant (Fig. 3). Thus NRS/ER is highly conserved in plants and is likely to carry out important functions in plants. A BLAST search (Altschul et al., 1997) of the Arabidopsis genomic database using the amino acid sequences corresponding to NRS/ER identified three additional coding regions with sequence identities of between 78% and 81% to NRS/ER over 290 amino acids (Fig. 2B).

In addition, a BLAST search identified a fungal (Podospora anserina; NCBI accession no. CAD60850) protein with greater than 70% sequence similarity (greater than 55% amino acid identity) to NRS/ER over 275 amino acids. This is interesting since, to the best of our knowledge, rhamnose biosynthesis in fungi has not...
been reported. However, the biochemical function of this fungal protein has not been determined.

In Arabidopsis, \( NRS/ER \) (AY513232; also annotated \( \text{At1g63000} \)) appears to be transcribed in leaves, roots, and flowers as judged by reverse transcription (RT)-PCR (Fig. 4). These expression data are consistent with information in the EST database, since multiple \( NRS/ER \) transcript entries are in cDNA libraries prepared from Arabidopsis flower buds, green siliques, shoots, leaves, and roots (http://www.ncbi.nlm.nih.gov/dbEST/), as well as callus (massively parallel signature sequencing database; http://dbixs001.dbi.udel.edu/MPSS4).

**Figure 2.** Amino acid sequence alignment of Arabidopsis NRS/ER. A, Comparing the conserved amino acid sequences between NRS/ER and functional bacterial rmlD proteins. Protein sequence alignment was performed using ClustalX (version 1.83; Jeanmougin et al., 1998). \( \text{E. coli} \) K-12 (GenBank accession no. AAB88399), \( \text{S. enterica} \) serovar Typhimurium (accession no. 21730455), \( \text{Mycobacterium tuberculosis} \) H37Rv (accession no. CAB07093), \( \text{Fusobacterium nucleatum} \) (accession no. NC_003454), and Arabidopsis NRS/ER (accession no. AAR99502). The putative conserved \( G_X2G_X2G \) motif and the putative catalytic triad \( Y_{X3K} \) (Giraud et al., 2000) are marked. B, Comparison of the conserved amino acid sequences between NRS/ER and three Arabidopsis proteins predicted from the genomic database (At3g14790, At1g53500, and At1g78570). Protein sequences were aligned with ClustalX software.

**Characterization of the Enzymatic Properties of Arabidopsis NRS/ER**

The coding region of NRS/ER, containing a tag at the N-terminal region with six His, was expressed in \( \text{E. coli} \), and the recombinant protein was purified by nickel-affinity column chromatography. The purified protein migrated as a single band on SDS-PAGE with an apparent mass of approximately 35 kD (Fig. 5A). Such a value is consistent with the molecular weight of 35,759 predicted from the amino acid sequence of the recombinant protein. Based on the similarity of the NRS/ER to the reductase, epimerase, and dehydratase (RED) family we predicted that this plant protein is an enzyme with dual function (epimerase/reductase), as suggested by Kamsteeg et al. (1978) and by our own work (Bar-Peled et al., 1991).

Since the potential substrate for NRS/ER (dTDP-4-keto-6-deoxy-Glc) is not available, we generated it using recombinant rmlB purified from \( \text{E. coli} \) (Graninger et al., 1999). The bacterial rmlB preferentially converts dTDP-\( \alpha-D\)-Glc to dTDP-4-keto-6-deoxy-Glc (Graninger et al., 1999). After incubating recombinant rmlB with dTDP-\( \alpha-D\)-Glc, the nucleotide-4-keto-6-deoxy-Glc eluted from a reverse-phase ODS2 HPLC column as a broad peak (Fig. 5B, 3), which is consistent with the results obtained by Nakano et al. (2000). The reason for this peak broadening has not been determined, although the presence of a 4-keto group may increase the hydrophobicity of the sugar nucleotide and thereby promote interaction with the column. 4-Keto sugars are likely to exist on the column as different keto-enol isomers that may also lead to peak broadening on the ODS column. Incubating dTDP-4-keto-6-deoxy-Glc with recombinant NRS/ER in the presence of NADPH resulted in the formation of a product that co-eluted with dTDP-\( \beta-L\)-Rha (Fig. 5B, 5). This major peak, which eluted from the HPLC column at 15.3 min, was collected and analyzed by \( ^1\text{H}\)-NMR spectroscopy. The \( ^1\text{H}\)-NMR spectrum of the product contained a doublet-of-doublet signal at 5.21 ppm (Table I) with coupling constants, \( ^3\text{J}_{1\_2} \) of 8.8 Hz and \( ^3\text{J}_{2\_3} \) of less than 2 Hz, diagnostic for proton H-1 of 4-keto-L-Rha. The signal at 1.30 ppm is diagnostic for the H-6 methyl protons of rhamnose. All other signals in the
1H-NMR spectra of the enzymatically synthesized compound had chemical shifts and coupling constants that are similar to dTDP-β-L-Rha reported by Nakano et al. (2000). For example, the coupling constant between protons 3' and 4', and protons 4' and 5' are 9.3 and 9.8 Hz, respectively, indicating the trans configuration that is expected for rhamnose (see Table I). We observed only one point of chemical shift discrepancy for proton 5', on the deoxy-ribose, from the published report (Nakano et al., 2000). Our data showed a chemical shift of 4.16 ppm (Table I), whereas Nakano et al. (2000) reported a value of 4.03 ppm for H-5' on deoxy-ribose. However, our assignment for this proton is in complete agreement with the assignment reported for other dTDP sugars (Amann et al., 2001) that indicated a chemical shift of 4.15 for proton 5' on deoxy-ribose. Thus, our data provide evidence that dTDP-β-1-Rha is formed when the recombinant NRS/ER is incubated with dTDP-4-keto-6-deoxy-Glc.

We further characterize NRS/ER properties using dTDP-4-keto-6-deoxy-Glc as substrate. The cofactor NADPH is required for enzymatic activity of the recombinant protein, and without it no conversion to dTDP-β-1-Rha is observed (Fig. 5B, 4). We found that NADH is also a hydride ion donor for the reduction of the 4-keto derivative, but only 40% of dTDP-4-keto-6-deoxy-Glc was converted to dTDP-β-1-Rha by NRS/ER when 1 mM NADH was used compared to 1 mM NADPH. The addition of up to 10 mM MgCl2 or CaCl2 had no discernible effect on the activity nor was NRS/ER activity inhibited by millimolar concentrations of the divalent cation chelator EDTA. In contrast to the plant epimerase/reductase, EDTA strongly inhibits the prokaryote 3,5-epimerase and 4-reductase activities, and the magnesium ion is essential for their activities (Graninger et al., 1999). The NRS/ER is stable for at least 30 months when stored as a crude extract at −20°C. However, more than 60% of NRS/ER activity is lost when the purified enzyme is subjected to repeated freezing and thawing. The recombinant NRS/ER is active between pH 4.5 and 10.5 with maximum activity between pH 5.5 and 7.5 (Fig. 6B). Maximum enzyme activity is obtained at 30°C, and approximately 35% of the activity is retained at 45°C (Fig. 6A). Under optimal conditions enzyme activity is linear for 30 min, and, thus, kinetic analyses were performed using 20-min

Figure 3. Phylogenetic analysis of NRS/ER proteins. NRS/ER-like protein sequences, translated from dbEST, were aligned with ClustalX software. Alignments were analyzed with the PAUP software (Swofford, 1998) to generate an unrooted tree. Percentage bootstrap values of 1,000 replicates are given at each branch point. Branch lengths are to scale. Accession numbers are in parentheses: cotton (Gossypium arboreum, BE052407); wheat (Triticum aestivum, CD875945); lobolly pine (Pinus taeda, CF386165); sweet potato (Ipomea batatas, CB330308); soybean (Glycine max, CA784305); orange (Citrus sinensis, CB292447); lettuce (Lactuca sativa, BG871942); grape (Vitis vinifera, CS12199); barley ( Hordeum vulgare, BG369398); medicago (Medicago truncatula, BG587952); brassica (Brassica napus, CD839832); sugarcane (Saccharum officinarum, CA258761); onion (Allium cepa, CF443605); ice plant (Mesembryanthemum crystallinum, BF479490); spruce (Picea mariana, AF051236); sugar beet (Beta vulgaris, BF011071); poplar (Populus tremulaoides, CF119362); rice (Oryza sativa, AK071766); corn (Zea mays, AY108467); and the conserved amino acid NRS/ER sequence domain within three Arabidopsis genes annotated At3g14790, At1g53500, and At1g78570.

Figure 4. NRS/ER transcript is expressed in all Arabidopsis tissues. Total RNA isolated from stems (S), roots (R), rosette leaves, of 3-week-old plants (L), and flowers (F) was used to amplify a NRS/ER specific 912-bp transcript (lanes 5–8). The AtUXS3 gene, encoding a UDP-GlcA decarboxylase whose RNA is expressed in all Arabidopsis tissues that have been examined (Harper and Bar-Peled, 2002), was used as an internal RT-PCR control and resulted in the predicted 1,046-bp transcript (lanes 1–4). The data are representative of at least three independent RT-PCR reactions.
reactions. The apparent $K_m$ for NADPH is 90 $\mu$M and 16.9 ± 0.7 $\mu$M for dTDP-4-keto-6-deoxy-Glc.

We tested the specificity of the NRS/ER toward other nucleotide-4-keto-6-deoxy-sugars. To determine if NRS/ER can utilize GDP-4-keto-6-deoxy-Man as a substrate we first purified recombinant GDP-Man 4,6-dehydratase (GMD) as described (Sturla et al., 1997) and incubated GMD with GDP-$\alpha$-D-Man. HPLC analysis was used to confirm full conversion of GDP-$\alpha$-D-Man to GDP-$\beta$-$\alpha$-6-deoxy-Man. The product was then incubated, in control experiments, with recombinant human GDP-Man 3,5-epimerase/4-keto-reductase (Tonetti et al., 1996) that readily converted GDP-4-keto-6-deoxy-Man to GDP-Fuc as determined by HPLC. However, no conversion to GDP-Fuc was evident when NRS/ER was incubated with GDP-4-keto-6-deoxy-Man (with or without NADPH).

We also tested the activity of NRS/ER toward UDP-4-keto-6-deoxy-Glc. For these experiments we used a recombinant purified rmlB (Hegeman et al., 2002). Although the recombinant rmlB has preference for dTDP-$\alpha$-D-Glc, Hegeman et al. (2002) reported that partial conversion of UDP-$\alpha$-D-Glc to UDP-4-keto-6-deoxy-Glc was achieved using the rmlB. Our initial trials to convert UDP-$\alpha$-D-Glc to UDP-4-keto-6-deoxy-Glc using recombinant rmlB failed to produce a stable major product, and only trace amounts of putative UDP-4-keto-6-deoxy-Glc were produced after 60 min.
Incubation of rmlB with UDP-α-D-Glc for longer periods of time (up to 20 h) in an attempt to achieve more conversion to UDP-4-keto-6-deoxy-Glc resulted in partial degradation of the product, preventing quantitation and characterization of UDP-4-keto-6-deoxy-Glc. However, co-incubation of UDP-α-D-Glc and rmlB with NRS/ER and NADPH resulted in an HPLC peak at retention time 9.3 min, which was collected. This conversion was NADPH dependent. Since the amount of product converted was low (presumably due to the low activity of rmlB toward UDP-α-D-Glc), we pooled the peaks from several reactions and analyzed the product by NMR. The 1H-NMR spectrum of the enzymatic product contained a doublet-of-doublet signal at 5.21 ppm (Table II) with coupling constants, $J_{1^0, P}$ of 8.8 Hz and $J_{1^0, 2^0}$ of less than 2 Hz, diagnostic for the H-1 of β-L-Rha. The signal at 1.30 ppm is diagnostic of the H-6 methyl protons of rhamnose. Other rhamnose signals in the 1H-NMR spectra of the NRS/ER-derived compound had chemical shifts and coupling constants similar to those for dTDP-β-L-rhamnose (Table I). The uridine proton assignments in the 1H-NMR spectra are characteristic as those uridines of other UDP-sugars (for example, UDP-Xyl; Harper and Bar-Peled, 2002). Thus, our data provide evidence that UDP-β-L-Rha is formed when the recombinant NRS/ER protein is incubated with UDP-4-keto-6-deoxy-Glc. We therefore named the protein NRS/ER for nucleotide rhamnose/3,5-epimerase;4-reductase. Unfortunately, due to low conversion of UDP-α-D-Glc to UDP-4-keto-6-deoxy-Glc by rmlB, complete enzymatic characterization of NRS/ER was not possible.

### DISCUSSION

The Arabidopsis gene NRS/ER (also annotated At1g6300) encodes an approximately 33.5-kD enzyme (NRS/ER) which, in the presence of NADPH, converts dTDP-4-keto-6-deoxy-Glc and UDP-4-keto-6-deoxy-Glc to dTDP-β-L-Rha and UDP-β-L-Rha, respectively (Tables I and II). NRS/ER is similar to other plant enzymes involved in the formation of UDP-β-L-Rha (Barber and Chang, 1967; Kamsteeg et al., 1978; Bar-Peled et al., 1991) since all these enzymes have

### Table I. Proton chemical shifts and coupling constants of dTDP-β-L-Rha synthesized from dTDP-α-D-Glc using rmlB and NRS/ER

<table>
<thead>
<tr>
<th>Proton</th>
<th>Thymine</th>
<th>Deoxyribose</th>
<th>dTDP-β-L-Rha</th>
<th>Rhamnose</th>
<th>Rha coupling constants</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Chemical shifts, $\delta$</td>
<td>dTDP-β-L-Rha</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ppm$^a$</td>
<td>Rhamnose</td>
<td>$Hz$</td>
<td>$J_{1^0, P}$</td>
</tr>
<tr>
<td>1</td>
<td>—</td>
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<td>5.21 (5.21)</td>
<td>8.8</td>
<td>$&lt; 2$</td>
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<tr>
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<td>4.08 (4.08)</td>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>—</td>
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<td></td>
</tr>
<tr>
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<td>—</td>
<td>4.16 (4.19)</td>
<td>3.35 (3.36)</td>
<td>9.8</td>
<td></td>
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<tr>
<td>5 (CH$_3$)</td>
<td>1.92 (1.95)</td>
<td>4.16 (4.03$^b$)</td>
<td>3.43</td>
<td>6.3</td>
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<tr>
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<td>7.74 (7.74)</td>
<td>1.30</td>
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$^a$Chemical shifts are in ppm relative to acetone signal. Rhamnose proton-proton coupling constants in Hz are indicated as well as the $J_{1^0, P}$ coupling values between phosphate and the H-1 proton of Rha. Values in parentheses are chemical shifts of published data (Nakano et al., 2000). $^b$The chemical shift for this proton is 4.15 ppm in other dTDP-sugars derivatives (Amann et al., 2001).
In the synthesis of GDP-L-Fuc, GDP-β-L-Rha or UDP-β-L-fucose from GDP-β-D-Man is first converted to GDP-β-L-fucose from GDP-β-D-Man (Bonin and Reiter, 2000). The 4-keto-6-deoxy-β-Man is then converted to GDP-Fuc by a single bifunctional enzyme (Bonin et al., 1997). The 4-keto-6-deoxy-β-Man is first converted to the 4-keto-6-deoxy-derivative by a GMD (Bonin et al., 1997). While it is assumed that UDP-β-L-Rha has an equatorial hydroxyl at C-4, or to yield dTDP-6-deoxy-L-talose, which has an axial hydroxyl at C-4. Indeed, a gene from *Actinobacillus actinomycetemcomitans* has been identified and shown to encode a dTDP-4-keto-rhamnose reductase that converts dTDP-4-keto-β-L-Rha to dTDP-6-deoxy-L-Tal (Nakano et al., 2000); 6-deoxy-L-talose is a component of the capsular polysaccharide synthesized by this bacterium. To our knowledge, no 6-deoxy-β-L-talose-containing nucleotides and polysaccharides have been identified in plants.

The Arabidopsis genome contains three additional genes (At1g53000, At1g78570, and At3g14790) that each encode large proteins (approximately 670 amino acids) having two domains: an N-terminal domain (approximately 330 amino acids long) with amino acid sequence similarity to 4,6-dehydratase followed by a C-terminal domain (greater than 320 amino acids) having two domains: an N-terminal domain (approximately 330 amino acids long) with amino acid sequence similarity to 4,6-dehydratase followed by a C-terminal domain (greater than 320 amino acids) that shares over 80% amino acid sequence identity to the functional NRS/ER described in this report. It is likely that these three Arabidopsis genes are also involved in the synthesis of activated Rha. Indeed, we have generated recombinant At1g78570 C-terminal domain in *E. coli* and found that the recombinant protein is able to convert dTDP-4-keto-6-deoxy-Glc to dTDP-β-β-L-Rha (data not shown). Additional studies are required to determine the catalytic activity of the full products of At1g78570, At1g53000, and At3g14790 and to establish if Arabidopsis has several genes encoding the same catalytic activities (i.e. isoforms) for the synthesis of activated nucleotide-rhamnose.

In this report we identified a gene product, NRS/ER, and showed that in vitro it forms both UDP-β-L-Rha and dTDP-β-β-L-Rha. While it is assumed that UDP-β-L-Rha is the activated sugar used for the synthesis of flavonoids, we cannot exclude the possibility that plants convert dTDP-α-β-Glc to dTDP-β-β-L-Rha for synthesis of other macromolecules. Isolation of the various RhaTs involved in the synthesis of pectic polysaccharides and glycoproteins, and characterization of their nucleotide-rhamnose preference, is thus essential.

### Table II. Proton chemical shifts and coupling constants of UDP-β-β-L-Rha synthesized from UDP-α-β-Glc using rmlB and NRS/ER

<table>
<thead>
<tr>
<th>Proton</th>
<th>Uricid</th>
<th>Ribose Chemical shifts, δ</th>
<th>UDP-β-β-L-Rha</th>
<th>Rhamnose</th>
<th>Rha coupling constants</th>
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<td>5.21 (5.21)</td>
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<td>4.08 (4.08)</td>
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<td>5.96</td>
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<td>1.30 (1.30)</td>
<td>J₁,₂ &lt; 9.8</td>
<td>J₁,₂ &lt; 6.3</td>
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</table>

*Chemical shifts are in ppm relative to acetone signal. Rhamnose proton-proton coupling constants in Hz are indicated as well as J₁,₂ coupling values between phosphate and the H-1 proton of Rha. Values in parentheses are chemical shifts of published data for dTDP-Rha (Nakano et al., 2000); chemical shifts for uridine protons are the same as for UDP-sugars.*
The identification of all the genes and the characterization of the proteins involved in the formation of rhamnose in plants are required to determine how many activated forms of rhamnose are used in plants.

MATERIALS AND METHODS

Cloning and RT-PCR Analysis of Arabidopsis NRS/ER

Arabidopsis expressed tag cDNA databases (dbEST) were searched to identify cDNA with amino acid sequence similarity to bacterial rmb, rmc, and rmd. Several ESTs (T44775, R92567, TW3688, and Z66952) that showed similarity to these bacterial gene products were identified and used to design primers to obtain the corresponding Arabidopsis genes by RT-PCR. Briefly, total RNA from Arabidopsis ecotype Columbia plants was isolated using Trizol reagent (Chomczynski, 1993). RNA was reverse transcribed into cDNA at 42°C for 60 min using 1 μM oligo(dT) primer (5’TTCCTGAGAATTCGCGGCCGCCTAGTTTCTTT-TTV) in a 20-μL total reaction consisting of 50 μL Tris-HCl (pH 8.4), 75 μM KCl, 3 mM MgCl₂, 0.2 mM of each deoxynucleotide triphosphates (dNTPs), 10 μM diethiohitol, and 200 units SuperScript II RNase H⁻ reverse transcriptase (Gibco-BRL, Gaithersburg, MD). Following the reaction two reaction products was used as a template for PCR using 1 unit of high fidelity Platinum Taq DNA polymerase mixed with GB-D proofreading DNA polymerase and Platinum antibody (Gibco-BRL, 0.2 μM of the sense primer [80AATF513851/3′] 5′CATATGGTCGAGCCCGAACGTTATC-ATC, 0.2 μM of the antisense primer [80AA7F513853/2′] 5′GGCGGCGCTCCAAAATCTAGTCGTTCTTGTGTT, and 0.2 μM of each dNTPs (Roche, Indianapolis, IN)) in buffer containing 60 μM Tris-SO₄ (pH 8.9), 50 mM ammonium sulfate, and 1 mM MgSO₄. RT-PCR reaction product was separated by agarose-gel electrophoresis, purified, and cloned into pCR2.1 TOPO plasmid (Invitrogen, Carlsbad, CA). The cloned RT-PCR product was sequenced and the nucleotide sequence submitted to GenBank (accession no. AY513122, NRS/ER). The Nde I-Not I DNA fragment from pCR2.1-81.5, consisting of the coding region for NRS/ER, was subcloned into a PET28b E. coli expression vector (Novagen, Madison, WI). The resulting clone, PET28b/81.5.3, was constructed to give an in-frame N-terminal 6His tag-NRS/ER gene fusion. The mass of the recombinant NRS/ER (approximately 35 kDa) is less than the native protein (approximately 33 kDa) due to the presence of the 6His amino acid tag.

For NRS/ER expression studies in Arabidopsis Columbia, total RNA from flowers, fully expanded rosette leaves and stems of 6-week-old plants, rosette leaves of 3-week-old plants, or roots of 4-week-old plants grown in liquid media as described (Bar-Peled and Raikher, 1997) was reverse transcribed into cDNA in 20-μL reactions using 200 units of SuperScript II reverse transcriptase (Invitrogen) and 1 μM oligo(dT) primer using the manufacturer’s recommended buffer. One-twentieth of each of the reverse-transcribed products was used as a template for PCR reactions using 0.5 units Taq DNA polymerase (Roche, Basel, Switzerland), the manufacturer’s buffer, 0.2 mM dNTPs, 1.5 mM MgCl₂, and 0.2 μM NBS/ER gene specific sense and anti-sense primers (see above). As internal RT-PCR controls, we used Actin3 (UDP-GlcA decarboxylase) gene primer [101-T5 5′GAGATTTCCCATGCGCCA-TCAAAGTGAAGAACACAG, and 101-2AS 5′GCGGCGGCTCTTAGTTTCTTGCGACGCTTAAGCG] as described (Harper and Bar-Peled, 2002). PCR conditions were one cycle at 95°C for 2 min, 30 cycles (95°C, 30 s; 55°C, 30 s; 70°C, 1 min), and a final extension at 70°C for 5 min. One-tenth of each sample and the DNA marker (1KB plus, Invitrogen) were resolved on a 1% agarose gel and visualized by staining with ethidium bromide.

Protein Expression and Purification

Fifteen milliliters of an overnight culture of E. coli strain BL21(DE3)-pLysS (Novagen), carrying the PET28b/81.5 (NRS/ER) or control PET28b vector alone were inoculated to 0.5 L Luria-Bertani broth supplemented with 50 μg/mL kanamycin and 30 μg/mL chloramphenicol. Cells were grown at 37°C with shaking (200 rpm) until a cell density of OD₆₀₀ ≈ 0.6, and then induced by addition of isopropylthio-β-D-galactoside (final concentration, 1 mM). After approximately 3 h at 30°C, cells were collected by centrifugation (10 min at 6,000g at 4°C). Cells were washed with cold water, resuspended in 20 mL extraction buffer (50 mM MOPS-NaOH, pH 7.6, 0.5 mM EDTA) supplemented with fresh 1 mM diethiohitol and 0.5 mM phenylmethylsulfonyle fluoride, and ruptured by 10 sonication pulses (10-s pulse followed by 10-s rest) on ice, using a microtip probe and a Fisher model 550 sonicator (Fisher Scientific, Pittsburgh, PA) set at power of 4.5. The suspension was centrifuged (20,000g, approximately 30 min, 4°C), and the supernatant was collected and loaded onto a Sepharose CL-6B-nickel column (2 mL resin volume [NTA-Ni, Qiagen USA, Valencia, CA] packed in a 0.6-cm [i.d.] × 5-cm polystyrene column [Bio-Rad, Hercules, CA]) that was pre-equilibrated with buffer A (50 mM sodium phosphate, 0.3 M NaCl, pH 8). The recombinant protein was purified as described (Harper and Bar-Peled, 2002) and stored at −20°C until analysis. All chromatography steps were performed at 4°C. Protein concentration was determined using the Bradford dye-binding assay (Bio-Rad), using bovine serum albumin as a standard. Proteins were separated by 0.1% SDS-12% PAGE as previously described (Bar-Peled et al., 1991) alongside M markers (Bio-Rad) and visualized by staining with SimplyBlue (Invitrogen).

Enzyme Assays

The substrate dTDP-4-keto-6-deoxy-glucopyranose was generated from dTDP-α-D-Glc using recombinant dTDP-Glc 4,6-dehydratase (the product of the rmb gene; a gift from Paul Messner [University of Vienna, Austria] and Perry Frey [University of Wisconsin, Madison, WI]) as described (Graninger et al., 1999; Hegeman et al., 2002).

The assays for dTDP-4-keto-6-deoxy-Glc epimerase-reductase activity (final reaction volume of 75 μL) were performed in two steps. First, dTDP-4-keto-6-deoxy-Glc was produced by incubating dTDP-α-D-Glc (1.3 μM) with 5 μg recombinant rmlB in 50 μL of sodium phosphate, pH 8.5, for 10 min at 30°C. The recombinant dTDP/UDP-4-keto-6-deoxy-Glc epimerase-reductase (NRS/ER; 5 μg) and NADPH (1.3 μM) were added, and after a 20-min incubation at 30°C, the reactions were terminated by the addition of 20 μL chloroform. The mixture was vortexed and centrifuged at 16,000g for 5 min at room temperature. The aqueous phase was retained, and the organic phase was extracted with 80 μL water. The aqueous phases were combined and analyzed by HPLC using a Phenomenex-ODS2 column (250 × 4.6 mm; Phenomenex, Torrance, CA) or a Spheresorb-OdS2 column (250 × 4.6 mm; Waters, Milford, MA) eluted at 1 mL min⁻¹ with 0.5 mM KH₂PO₄ (A) for 15 min, followed by a gradient to 80% A and 20% methanol over 14 min at a flow rate of 0.7 mL min⁻¹. Peaks eluted from the ODS2 column were injected onto a Phenomenex-SAX ion exchange column (250 × 4.6 mm; Phenomenex) and eluted at 1 mL min⁻¹ with a linear 2- to 600-mM ammonium-formate gradient formed over 25 min (Bar-Peled et al., 2001). Nucleotides and nucleotide-sugars were detected by UV absorbance using a Waters photodiode array detector. The ArPs for thymidine and uridine were detected at 267 nm and 261 nm, respectively. The columns were calibrated with authentic nucleotide-sugars (Sigma, St. Louis).

1H-NMR Spectroscopic Analysis of the Products Formed by Incubating dTDP- and UDP-4-Keto-6-Deoxy-Glc with NRS/ER

UV-absorbing peaks eluting from the SAX column were collected and lyophilized to remove the ammonium formate. The residues were dissolved in water, relyophilized twice, and then exchanged twice with 99.96% D₂O. Proton NMR spectroscopy was performed at 25°C on Varian Inova spectrometers (Palo Alto, CA) operating at 500 MHz and 600 Hz (Bar-Peled et al., 2001). The chemical shifts are in ppm and were normalized using acetone (2.22 ppm) as external standard.

Sequence data from this article have been deposited with the EMBL/GenBank data libraries under accession numbers 21730455, AAB88399, AAR99502, AF051236, AK071766, AY108467, AY513322, BE052407, BF011071, BF479490, BG369398, BG587952, BC871492, CA258761, CA784305, CAD6080, CA96239, CB29447, CB30308, CD593832, CD579549, CF119362, CF863165, CF443605, CF512199, and NC_003454.

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