An acyltransferase catalyzing the formation of diacylglycose is a serine carboxypeptidase-like protein

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1-O-β-acyl acetal donors have been identified. We report the identification of a cDNA encoding an 1-O-β-acyl glucose-dependent acyltransferase in Brassicaceae, similar to 1-O-β-acyl glucose. Chlorogenic acid, a high-energy acyl donor native to plants, is now recognized. Although the role of serine carboxypeptidases in the mechanism of catabolism and modified it to accommodate a wide range of group transfer reactions used in biosynthetic reactions of secondary metabolism. The abundance of serine carboxypeptidase-like proteins in plants suggests that this motif has been used widely for metabolic functions.

Activity and transacylation of many fatty acids and phenolic acids are known to proceed by means of a thioester-dependent mechanism. However, an alternative mechanism of activation involving the UDPglucose-dependent transglycosylation of phenolic and fatty acids as 1-O-β-acetyl acetal donors is now recognized. Although the role of activated acetal donors such as caffeoyl β-glucose in transacylation reactions of quinic acid was first proposed by Kojima and Uritani (2) for chlorogenic acid biosynthesis, it is now known that other 1-O-β-acyl acetals are also used in a wide range of transacylation reactions. In Brassicaceae, 1-O-sinapoyl-β-glucose serves as an acyl donor for the formation of sinapoylcholine, sinapoylmalate, and sinapoylglycerol (1, 3, 4). 1-O-galloyl-β-glucose (β-gluco/rallin) acts as a donor in biosynthesis of gallotannins (5). Hydroxycinnamoylglycose and its hydroxylated derivative caffeoyl glycosides are intermediates in the synthesis pathway for chlorogenic acid (3-O-caffeoylquinic) acid in Ipomoea (1, 6, 7) in an alternative route to the caffeoyl-CoA-dependent biosynthesis of this compound (8). Chlorogenic acid, a high-energy acyl donor similar to 1-O-acyl-β-glucose, serves as the caffeine donor for the transacylation of chlorogenic acid (3,5-di-O-caffeoylquinic acid), caffeoylglucic acid, and caffeoylglactonic acid (9–11). For the synthesis of a number of feruloylated betacyanins, 1-O-feruloyl-β-glucose is the feruloyl donor (12). In the conjugation of the plant growth hormone indoleacetic acid, a UDP-glucose-dependent reaction activates indoleacetic acid to form 1-O-indolylacetyl-β-glucose. Indolylacetyl-n-pyrrolo-glucoses are intermediates for the formation of NADH in the synthesis of glucose polyesters (16–19). Enzymatic disproportionation of two equivalents of 1-O-acyl-β-glucose results in the formation of 1,2-diacyl-β-glucoses. The acyltransferase as was recently purified to homogeneity (19). In this paper, we report the cloning and heterologous expression of this glucose acyltransferase and its relation to the serine carboxypeptidase gene family, and we suggest a mechanism for the evolution of acyl transfer by activated 1-O-β-acetals.

Materials and Methods

Cloning of the Acyltransferase cDNA. Reverse transcription–PCR (RT-PCR) was used to clone an internal fragment of the cDNA. DNA was extracted from L. pennelli as reported (27). mRNA was obtained by using PolyATtract mRNA isolation kit (Promega). Oligo(dT)15 was used as the first-strand cDNA synthesis. Superscript II reverse transcriptase from Gibco/BRL was used; reaction conditions were described as by the manufacturer. Primers were designed according to the cDNA sequences from the purified 34-kDa subunit of the acyltransferase (19). Two primers, pt50 and re24, were selected based on their low level of degeneracy, and tomato codon usage

Abbreviations: DFP, diisopropyl fluorophosphate; RT-PCR, reverse transcription–PCR; RACE, rapid amplification of cDNA ends.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF248647, AF006078, AF006079, and AF006080).

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normal 3
enrichment of templates for the gene of interest was added to the
by the manufacturer, except for the following modification:
adapter primer provided by the kit. Different clones from
pool with higher abundance possessed sequences similar to the
BRL). The 5′
are described below:

GSP REV2: GCCTGTCACCAACTTCAATA,
GSP REV1: ATCTGTTAGAGCATTGCCTT,
GSP REV: AAGGATCCAATGGCGCGGGTCACACTGTT and

3′ primer: AGAATTCTTAACGTCAACCTCGAATGA.

Procedures were the same as described for RT-PCR. PCR
products were purified and cut by BamH1 and EcoRI and ligated to
pYES2 (Invitrogen) prepared with the same restriction en-
zeymes. Ligation was performed at 14°C overnight, and the
products were used to transform XL-1 Blue cells. Clones were
sequenced, and a clone with the acyltransferase cDNA, pY-
AGT2, was identified.

KT1115 (MATa leu2-3 leu2-112 ura-52) (31) was transformed
with pYAGT2. KT1115 was a gift from Ralph Dewey (North
Carolina State University, Raleigh, NC). Uracil dropout me-
dium [0.13% (wt/vol) amino acids without uracil, 0.17% (wt/
vol) yeast nitrogen base without amino acids and ammonium
sulfate, 0.5% (wt/vol) (NH4)2SO4, and 2% (wt/vol) glucose] was
used as selective medium. Baffled flasks were used to grow yeast
cultures for expression. Transformed yeast cells were inoculated in
uracil dropout medium with 5% (wt/vol) raffinose instead of
2% (wt/vol) glucose. After the culture density reached 0.4–0.5
OD600, galactose was added into the medium to a final concen-
tration of 2% (wt/vol) to induce transcription of the pYES2
insert. Cells were sampled at different time points after galactose
addition.

Functional expression of the acyltransferase was assayed by
using permeabilized cells. Ten milliliters of induced culture was
centrifuged, and the pellet was washed with 10 ml of ice-cold
H2O, and then with 10 ml of ice-cold freeze-thaw buffer [FT
buffer: 100 mM Hapes-NaOH, pH 7.5/20% (vol/vol) glycerol/0.1% (vol/vol) Triton X-100]. The pellet was resuspended in 50
μl of ice-cold FT buffer and frozen at −80°C overnight or longer
(32). Samples were thawed at 30°C, and 50 μl of the cell
suspension was removed for enzymatic assays and GC-MS
analyses. Immunoblot analyses were performed as reported (33)
with the following modification. Tris-buffered saline (50 mM
Tris-HCl, pH 7.5/1 mM MgCl2/10 mM NaCl) was used for all
incubations; and both primary and secondary antibodies were
used at 1:1000 dilution. The primary antibody was generated
against recombinant L. pennelli glucose acyltransferase ex-
pressed in Escherichia coli (unpublished work).

Disproportionation Activity Assay and Acyltransferase Characteriza-
tion. Disproportionation activity was detected by measuring the
formation of diacylglycerol from 1-O-[1-14C]isobutyryl-β-
glucose. Reactions were performed in a 15-μl solution containing
50 mM Hapes-NaOH at pH 7.5, 1 mM 1-O-[1-14C]isobutyryl-
β-glucose (105 cpm), and 5000-fold purified acyltransferase from
L. pennelli LA1376 leaves (19) or variable amounts of commer-
cial available serine carboxypeptidases. 1-
LA1376 leaves (19) or variable amounts of commer-
cial available serine carboxypeptidases. 1-

1H-NMR (500 MHz) analysis of the acyltransferase reaction
was performed in a 1 mM solution of synthetic 1-O-isobutyryl-
β-glucose (17) in 2H2O at 23°C. The reaction was initiated by the
addition of 50 μg of L. pennelli glucose acyltransferase (19). A
total of 120 transients per spectrum were collected for 1 h. This

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substrate, kindly provided by Gurdev Ghangas (Cornell University), contains small amounts of non-anoomerically substituted isobutrylglucose as an unavoidable consequence of nonenzymatic acyl migration during storage.

For GC-MS analysis of disproportionation products, the reaction was performed with 50 μL of permeabilized yeast cells and 25 μL of 12 mM O-isobutaryl-β-glucose at room temperature for 3 h. After the reaction, the cells were pelleted at 12,000 × g for 1 min, the supernatant was removed, and 500 μL of MeOH was added to the supernatant. The sample was dried under N2 and acetylated with 60 μL of pyridine and 30 μL of acetic anhydride overnight. The acetylated sample was dried under N2 at 40°C, and resuspended in 200 μL of formamide. One microliter was injected into a DB5 column (25 m × 0.22 mm) in a Hewlett-Packard 5890 gas chromatograph with an HP 5970 mass selective detector. The GC program was 80°C for 1 min, followed by an increase to 200°C at 12°C per min, to 260°C at 5°C per min, and was held at 260°C for another 15 min.

Inhibition of disproportionation by disopropyl fluorophosphate (DFP) and benzylsuccinic acid was studied. DFP or benzylsuccinic acid was dissolved in isopropyl alcohol at a final concentration of 150 μM (10–5 M) and used in the inhibition study. For GC-MS analysis of disproportionation products, the reaction was performed with 50 μL of permeabilized yeast cells and 25 μL of 12 mM O-isobutaryl-β-glucose. After the reaction, the cells were pelleted at 12,000 × g for 3 h. After the reaction, the cells were pelleted at 12,000 × g for 3 h. After the reaction, the cells were pelleted at 12,000 × g for 3 h. After the reaction, the cells were pelleted at 12,000 × g for 3 h. After the reaction, the cells were pelleted at 12,000 × g for 3 h.

Results

**cDNA Encoding Glucose Acyltransferase.** RT-PCR was used to amplify an internal fragment of the glucose acyltransferase cDNA (GenBank accession no. AF248647). Two peptide sequences derived from the 34-kDa subunit of the glucose acyltransferase were used as primers for RT-PCR to amplify a fragment of 632 bp possessing an ORF of 210 amino acids. Six of the seven peptides sequenced from the 34-kDa acyltransferase subunit (19) were present in the deduced amino acid sequence of this cDNA fragment. The entire 1548-bp sequence of the cDNA was obtained by 5′RACE and 3′RACE (29, 30) using sequences from the 632-bp cDNA fragment. The sequence begins with an ATG codon and has a 1392-bp ORF, encoding a 464-aa polypeptide with a predicted molecular mass of 53 kDa. The deduced protein sequence contains all of the seven polypeptide sequences from the 34-kDa subunit (Fig. 2). Because all of the seven polypeptide sequences were derived from tryptic digest, the deduced amino acid before each peptide in the cDNA sequence should be either R or K. This is true for all peptides except PT50 (Fig. 2). Hence, PT50 represents the mature N terminus of the 34-kDa protein. Therefore, the deduced sequence indicates that the enzyme is synthesized as a preprotein bearing a hydrophobic N-terminal 18-aa signal peptide; cleavage of the signal peptide at this site is also predicted (http://www.cbs.dtu.dk/services/SignalP/). The N-terminal sequence of the 24-kDa subunit is also present in the deduced amino acid sequence of the cDNA. Thus, the 34-kDa and 24-kDa subunits of the acyltransferase are encoded by the same gene and result from posttranslational processing of the primary translation product. Four possible N-glycosylation sites (NXT/S) are present in the deduced amino acid sequence (three on the 34-kDa subunit, one on the 24-kDa subunit); glycosylation of the enzyme is indicated by its affinity for Con A during purification (19). The site for posttranslational proteolytic processing should be between PT60 and N24 (Fig. 2). Thus, the deduced molecular mass for the large subunit of the acyltransferase is between 30 kDa and 33 kDa. The value for the small subunit is 20 KDa. Glycosylation may contribute to the differences between the molecular masses calculated from the gene and the estimated molecular mass from the purified protein.

**Acyltransferase Expression in S. cerevisiae.** The full-length acyltransferase cDNA was cloned into the BamHI and EcoRI sites of pYES2 (Invitrogen) to form pYAGT2, and transferred to S. cerevisiae KT1115 (ura3). Raffinose was substituted for glucose as the carbon source to avoid the inhibition effect of GAL1 by glucose. Immunoblotting of the yeast cell extract using acyltransferase antisera revealed 59-kDa, 30-kDa, and 24-kDa immunoreactive bands (Fig. 3), indicating the unprocessed and proteolytically processed forms of the protein. Triton X-100 permeabilized cells (32) were used to study enzyme activity in situ. S. cerevisiae expressing the acyltransferase cDNA has the ability to synthesize diacylglycerol when permeabilized and provided 1-O-acyl-β-glucose. Although total
ion chromatograms are extremely complex because of the diversity of yeast carbohydrate metabolism, extracted ion chromatograms selected for display of components fragmenting to the isobutyryl ion, m/z 71, provide a facile display of isobutyrylgucose-related metabolism (Fig. 4). The tetraacetate derivatives of the substrate, 1-O-isobutyryl-β-glucose eluted at 18.1 min, and the triacetate derivatives of the product, diisobutyrylglucose, eluted at 19.95 min. Yeast cells expressing the L. pennellii glucose acyltransferase disproportionate 1-O-isobutyryl-β-glucose to form diisobutyrylglucose (Fig. 4A). Vector-only control cells do not disproportionate 1-O-isobutyryl-β-glucose (Fig. 4B). Hence, it is clear that this cDNA encodes an acyltransferase that catalyzes disproportionation reactions characteristic of glucose polyester synthesis in L. pennellii glandular trichomes.

In addition, the disproportionation reaction catalyzed by this enzyme is regiospecific. Both the yeast-expressed (Fig. 4) and native enzyme catalyze the formation of a single diacylglycerol product. In a 1H-NMR time-course study (Fig. 5), the reaction of 1-O-isobutyryl-β-glucose with the native acyltransferase is characterized initially by the appearance of an anomeric proton signal (δ 5.7 ppm, J = 8.4 Hz) downfield shifted from the anomeric proton of the starting material (δ 5.45, d, J = 8.4 Hz) by 0.25 ppm, and a new dd (δ 4.8, J = 8.4 Hz) downfield shifted from the clustered signals derived from H-2, -3, and -4. The appearance of these signals is consistent only with the acylation of the H-2 hydroxyl by isobutyrate, leading to formation of 1,2-di-O-isobutyryl-β-glucose and deshielding of the proton at H-2. Concomitant with the transfer of the isobutyryl moiety from one equivalent of 1-O-isobutyryl-β-glucose, one equivalent of β-glucose is liberated, leading to the appearance of the anomeric proton of α-glucose (5.15 ppm, J = 4 Hz) as the β-glucose anomerizes. After 1 h, the newly formed 1,2-di-O-isobutyryl-β-glucose accumulates sufficiently to act as an acyl donor, consistent with previous observations (17). Acyl transfer from 1,2-di-O-isobutyryl-β-glucose leads to the formation of α- and β-2-O-isobutyrylglucoses and regenerates 1-O-isobutyryl-β-glucose when β-glucose is the acceptor; consequently, the spectra become difficult to interpret after this time (not shown).

However, it is clear that the initial reaction catalyzed by this acyltransferase shows a strong preference for acyl transfer to the 2-hydroxy of 1-O-isobutyryl-β-glucose.

**Glucose Acyltransferase Is a Serine Carboxypeptidase-Like Protein.**

The deduced acyltransferase protein sequence shows sequence similarities to serine carboxypeptidases, such as barley carboxypeptidase I, II, and III, wheat serine carboxypeptidase II, and yeast carboxypeptidase Y when BLASTP is performed (http://www.ncbi.nlm.nih.gov). Amino acid similarity and identity reach 76% and 35%, respectively, when the acyltransferase is compared with barley carboxypeptidase I (Fig. 2 and ref. 34).

Serine carboxypeptidases possess a characteristic Ser-His-Asp catalytic triad. The Ser residue is unusually reactive because of its interaction with the His imidazole nitrogen. In the catalytic mechanism of serine carboxypeptidases, this Ser undergoes acylation as a consequence of its nucleophilic attack on the carbonyl carbon of the peptide substrate, forming a tetrahedral acyl-enzyme intermediate. This intermediate is then decom-

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**Fig. 3.** L. pennellii acyltransferase expression in yeast. (A) Immunoblot of yeast cells expressing unprocessed (59 kDa) and processed (30 kDa and 24 kDa) forms of the acyltransferase. (C) Immunoblot of control yeast cells harboring vector only.

**Fig. 4.** GC-MS analysis of 1-O-isobutyryl-β-glucose disproportionation in yeast cells. Permeabilized cells were provided with 1-O-isobutyryl-β-glucose for 3 h at room temperature, and the supernatant was peracetylated. (A) m/z 71 extracted ion chromatogram from cells expressing the acyltransferase. (B) m/z 71 extracted ion chromatogram from reaction performed with control cells harboring vector only. The 18.1-min peak in A and B is the tetraacetate derivative of the substrate 1-O-isobutyryl-β-glucose. The 19.95-min peak in A is the triacetate derivative of diisobutyrylglucose.

**Fig. 5.** 1H-NMR of the disproportionation reaction catalyzed by L. pennellii glucose acyltransferase. The zero-time spectrum was acquired immediately after addition of acyltransferase to the NMR tube. Acylation at H-2 is indicated by the time-dependent appearance of a downfield-shifted anomeric proton (H1), and appearance of the downfield-shifted H-2 (in the nonacylated substrate, the signal for H-2 is present in the complex cluster of signals around δ4.6).
posed by water, liberating the bound peptide and regenerating the reactive Ser. The Ser-His-Asp catalytic triad is also responsible for the activities of endopeptidases, such as trypsin and subtilisin, as a result of convergent molecular evolution. Additionally, some lipases possess Ser-His-Asp or similar Ser-His-Glu catalytic triad centers (35, 36).

The sequences around the Ser, His, and Asp catalytic triad residues are conserved between the glucose acyltransferase and barley serine carboxypeptidases I (Fig. 2). The heterotetrameric composition of the acyltransferase is also similar to that of barley serine carboxypeptidase I (37, 38). This suggests that the acyltransferase is a serine carboxypeptidase-like enzyme that utilizes the Ser-His-Asp catalytic motif to catalyze acyl transfer and exchange reactions. In the glucose acyltransferase, the putative active Ser is present on the 34-kDa subunit, and the active His and Asp are on the 24-kDa subunit.

DFP is a transition state inhibitor of serine carboxypeptidases. DFP reacts irreversibly with the active Ser residue, forming a covalent DFP-enzyme complex. If transacylation catalyzed by the glucose acyltransferase employs an active serine-based catalytic mechanism, DFP should inhibit the disproportionation activity and covalently modify the active serine in the acyltransferase (Ser-174 in the 34-kDa subunit). Disproportionation of 1-O-acyl-β-glucose is inhibited 50% by 170 μM DFP and is completely inhibited by 1 mM DFP (Fig. 6). [14C]DFP specifically labeled the 34-kDa subunit in a 1200-fold purified acyltransferase preparation (Fig. 7). Therefore, an active Ser residue appears to play a critical function in catalysis of acyl transfer from 1-O-acyl-β-glucose. A minor 59-kDa DFP-labeled band is likely to represent the unprocessed form of the acyltransferase. This suggests that the unprocessed enzyme also adopts a conformation sufficient to activate Ser by a proximal His.

Benzylosuccinic acid is a competitive inhibitor of serine carboxypeptidases, such as yeast carboxypeptidase Y, barley serine carboxypeptidases, and wheat serine carboxypeptidase II (39–41), and is widely used as an affinity support for purification of plant serine carboxypeptidases. Although benzylosuccinic acid is a potent inhibitor of wheat serine carboxypeptidase II, it has no effect on disproportionation activity up to 10 mM (Fig. 6), suggesting that charge or conformational differences at active sites exist between the glucose acyltransferase and general serine carboxypeptidases. Similarly, the glucose acyltransferase does not exhibit activity toward model substrates reported for a wound-induced tomato serine carboxypeptidase (not shown) (42). Furthermore, wheat and yeast carboxypeptidases are incapable of disproportionating 1-O-acyl-β-glucose even when assayed at very high concentrations of this substrate (Fig. 8).

**Discussion**

Heterologous expression of the *L. pennellii* glucose acyltransferase cDNA results in disproportionation of 1-O-β-acylglucoses by yeast cells. The reaction catalyzed by the *L. pennellii* acyltransferase is regiospecific. The deduced sequence of the cDNA shows high sequence similarity to serine carboxypeptidases, and a highly conserved around the active site Ser, His, and Asp residues characteristic of serine carboxypeptidases. Inhibition of disproportionation activity by DFP, a transition state inhibitor of active Ser-based enzymes, and covalent DFP labeling of the enzyme demonstrate the involvement of an active serine residue in the transacylation process. Hence, the glucose acyltransferase utilizes a serine carboxypeptidase-like catalytic triad to accomplish the synthesis of glucose polyesters. By analogy to serine proteases, the mechanism of acyl transfer is likely to involve nucleophilic attack of the active Ser hydroxyl on the ester carbonyl carbon of 1-O-β-acylglucose, forming a tetrahedral enzyme-bound acyl intermediate and releasing free glucose. This transition state would then be subject to attack by the 2-hydroxyl of a second 1-O-β-acylglucose, resulting in formation of 1,2-di-O-β-acylglucose, and regenerating the active Ser. Attack of the acyl-enzyme intermediate by β-glucose or water would result in the anomeric acyl exchange and esterase activities, respectively, also reported to be catalyzed by preparations of this enzyme (16, 17, 19).

Despite the similarity between glucose acyltransferase and serine carboxypeptidases, no carboxypeptidase activity could be detected with a range of substrates used by many serine carboxypeptidases, including a wound-induced carboxypeptidase reported from tomato leaves (42). Furthermore, yeast and wheat serine carboxypeptidases do not possess the ability to disproportionation activity toward model substrates reported for a wound-induced tomato serine carboxypeptidase (not shown) (42). Furthermore, wheat and yeast carboxypeptidases are incapable of disproportionating 1-O-acyl-β-glucose even when assayed at very high concentrations of this substrate (Fig. 8).
portionate 1-O-β-glucoses. Therefore, although closely related to and apparently derived from serine carboxypeptidases, this glucose acyltransferase does not appear to retain carboxypeptidase function.

Difference in residues involved in substrate binding sites may reveal the reasons for the lack of carboxypeptidase activity from the acyltransferase. Three-dimensional structure (43) and site-directed mutagenesis studies of yeast carboxypeptidase Y (44) indicate that the side chains of Trp-49, Asn-51, Glu-65, and Glu-145 are involved in the recognition of the C-terminal carboxylate of peptide substrates by forming a hydrogen bond network. Mutations at these sites, especially on Asn-51 and Glu-145, dramatically decrease the $k_{cat}/K_m$ value for carboxypeptidase activity and may even eliminate the ability of the enzyme to interact with the carboxylate of the peptide substrate. Serine carboxypeptidases share conserved sequence around the sites of Asn-51 and Glu-145 analogous to those in carboxypeptidase Y (44); these residues are not conserved in this glucose acyltransferase or in the deduced sequences of three other serine carboxypeptidase-like acyltransferases isolated from Solanum berthaultii (GenBank accession nos. AF006078, AF006079, and AF006080). In general, sequence comparison of serine-carboxypeptidase-like acyltransferases with bonta fide proteinases of the same class does not immediately reveal characteristics that can be used to ascertain the functional activity of their products.

Serine carboxypeptidases are primarily known for catabolic or processing functions, e.g., protein turnover or modification of processed functions, e.g., protein turnover or modification of precursors. However, recruitment of serine carboxypeptidase motifs for other functions—primarily hydrolytic—have been reported. For example, a cyanogenic hydroxynitrile lyase of indoleacetic acid, phenolic, and other biosynthetic activities.