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Soybean galactinol synthase forms fagopyritol B1 but not galactopinitols: substrate feeding of isolated embryos and heterologous expression

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Abstract

Soybean (*Glycine max* (L.) Merrill) seeds accumulate sucrose, raffinose, stachyose and lesser amounts of galactopinitol A, galactopinitol B and fagopyritol B1 in axis and cotyledon tissues as part of the seed maturation process. Somatic embryos appear to be deficient in D-pinitol and galactopinitols, indicating a lack of synthesis by embryo tissues *in vitro*. Isolated immature soybean zygotic embryos were fed *myo*-inositol, D-pinitol, D-*chiro*-inositol and sucrose, individually and in combination, to evaluate the role of substrate availability on galactosyl cyclitol accumulation during precocious maturation. Feeding *myo*-inositol transiently doubled galactinol accumulation with little effect on other soluble carbohydrates. Feeding D-pinitol increased free D-pinitol 8-fold, galactopinitol A 4.5-fold and galactopinitol B 4.2-fold. Stachyose concentration was 2-fold higher in cotyledons after feeding D-pinitol than after feeding D-*chiro*-inositol. Feeding D-*chiro*-inositol increased fagopyritol B1 17-fold in the axis and 7-fold in the cotyledons, but did not increase other soluble carbohydrates. Feeding D-pinitol and D-*chiro*-inositol together reduced uptake of D-*chiro*-inositol and steady-state accumulation of galactinol and galactopinitols by 50%, compared to feeding D-pinitol alone. Increasing sucrose concentration from 0 to 200 mM had no effect. Recombinant soybean galactinol synthase, heterologously expressed in *Escherichia coli*, catalysed the synthesis of fagopyritol B1 and galactinol, but not galactopinitols. These results were consistent with the following interpretations: D-pinitol and D-*chiro*-inositol were transported from maternal tissues and not synthesized in the embryo, D-*chiro*-inositol uptake into embryos may be reduced by D-pinitol, fagopyritol B1 was synthesized by galactinol synthase while galactopinitols were not, and fagopyritol B1 and galactopinitols accumulated in response to the supply of free D-*chiro*-inositol and D-pinitol to embryos.

Keywords: *Glycine max*, D-*chiro*-inositol, cyclitol substrate, fagopyritol synthesis, galactinol synthase, heterologous expression, soybean embryo

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Introduction

Soybean (*Glycine max* (L.) Merrill) seeds accumulate soluble carbohydrates, mostly sucrose, stachyose, raffinose and less galactopinitol A (α-D-galactopyranosyl-(1→2)-4-*O*-methyl-1D-*chiro*-inositol), galactopinitol B (α-D-galactopyranosyl-(1→2)-3-*O*-methyl-1D-*chiro*-inositol) and fagopyritol B1 (α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol) in axis and cotyledon tissues as part of the seed maturation process (Schweizer and Horman, 1981; Obendorf *et al.*, 1998b). By contrast, embryos of common buckwheat (*Fagopyrum esculentum* Moench) accumulate mostly sucrose and fagopyritols, but little raffinose and stachyose (Horbowicz and Obendorf, 1994; Horbowicz *et al.*, 1998). Buckwheat embryos do not accumulate pinitol and galactopinitols but do accumulate *myo*-inositol and galactinol. Total D-pinitol or total D-*chiro*-inositol (both free and its galactosyl derivatives) in soybean zygotic embryos (axis plus cotyledons), matured *in vitro*, did not exceed that present in embryos before culture (Obendorf *et al.*, 1998a, b), and soybean and alfalfa (*Medicago sativa* L.) somatic embryos also appear to be deficient in D-pinitol and galactopinitols (Horbowicz *et al.*, 1995; Obendorf *et al.*, 1996; Chanprame *et al.*, 1998), indicating a lack of D-pinitol and D-*chiro*-inositol synthesis by embryos *in vitro*. Instead of free cyclitols, seeds accumulate galactosyl cyclitols (Horbowicz and Obendorf, 1994; Obendorf, 1997).

Accumulation of galactopinitol A, galactopinitol B and fagopyritol B1 parallel the accumulation of raffinose and stachyose immediately following the accumulation of galactinol (Obendorf *et al.*, 1998b), the galactosyl donor for raffinose and stachyose synthesis (Peterbauer and Richter, 2001).

We hypothesize that D-pinitol and D-*chiro*-inositol are not synthesized in the soybean embryo, but are transported from the maternal tissues to embryos in the seeds. We also hypothesize that galactopinitols and fagopyritol B1 are synthesized by different enzymes in soybean embryos (see Fig. 1). If made by the same enzyme, product accumulation may be reduced due to competition between substrates. If made by different enzymes, product accumulation should be independent of substrate competition. The rationale behind these hypotheses is as follows.

Raffinose synthase (RFS, galactinol:sucrose galactosyltransferase, EC 2.4.1.82) (Peterbauer *et al.*, 2002a) and stachyose synthase (STS, galactinol: raffinose galactosyltransferase, EC 2.4.1.67) (Hoch *et al.*, 1999) form galactopinitols from D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) and galactinol (α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol). A partially purified STS from maturing soybean seeds formed stachyose using galactinol and raffinose as substrates, and formed galactopintol A using galactinol and D-pinitol as substrates (T.P. Lin and R.L. Obendorf, unpublished, 1998). This soybean STS preparation was not active with uridine (5′-)diphosphate (UDP)-galactose as the galactosyl donor and had no detectable galactinol synthase (GolS, UDP-D-galactose:*myo*-inositol galactosyltransferase, EC 2.4.1.123) activity. In addition, soybean STS and galactopinitol synthase activities co-eluted during protein purification (T.P. Lin and R.L. Obendorf, unpublished, 1998), an observation also described by Hoch *et al*. (1999). STS may use D-*chiro*-inositol and galactinol to form galactosyl D-*chiro*-inositol (Hoch *et al.*, 1999), and GolS may use D-*chiro*-inositol and UDP-galactose to form α-D-galactosyl D-*chiro*-inositol (Frydman and Neufeld, 1963). For both enzymes, product identification was not confirmed.

D-Pinitol, D-*chiro*-inositol and *myo*-inositol, but not D-ononitol or other free cyclitols, have been detected in soybean embryos and seeds (Horbowicz and Obendorf, 1994; Obendorf *et al.*, 1998a, b). Free D-pinitol concentration is highest in seed coats and lower in the axis and cotyledons of soybean seeds (Kuo *et al.*, 1997a), suggesting the transport of D-pinitol from leaves to seeds. Stachyose accumulates to amounts 20 times greater than galactopinitols or fagopyritol B1 (Obendorf *et al.*, 1998b), indicating that activities of both STS and GolS are abundant for galactosyl cyclitol synthesis in soybean embryos. Accumulation of galactosyl cyclitols may be limited

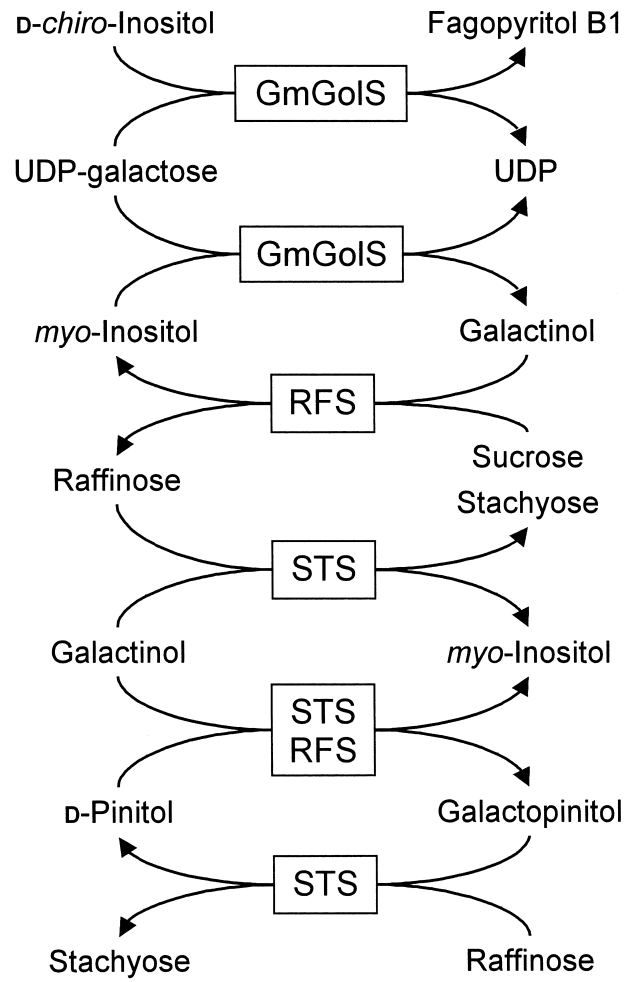

Figure 1. Proposed pathways for synthesis of fagopyritol B1, galactinol, raffinose, stachyose and galactopinitols (from Odorcic and Obendorf, 2003, with modifications). GmGolS, *Glycine max* galactinol synthase; RFS, raffinose synthase; STS, stachyose synthase.

by availability of free cyclitols to the embryo tissue, or STS (or GolS) may be less active with D-pinitol or D-*chiro*-inositol as galactosyl acceptors (Hoch *et al.*, 1999; Peterbauer *et al.*, 2002b). If feeding D-pinitol and D-*chiro*-inositol to soybean embryos markedly increased accumulation of galactopinitols and fagopyritol B1, respectively, we may deduce that D-pinitol and D-*chiro*-inositol are in limited supply in embryos, and are likely synthesized in maternal tissues and transported to embryos. If synthesized in the embryo, feeding exogenous D-pinitol and D-*chiro*-inositol should have less effect on galactosyl cyclitol accumulation, unless STS and/or GolS were markedly less active with D-pinitol or D-*chiro*-inositol, compared to raffinose as galactosyl acceptor. If D-pinitol and D-*chiro*-inositol are synthesized in the

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embryo, feeding *myo*-inositol should enhance their accumulation and also their galactosides.

Slow drying of immature soybean zygotic embryos induces the accumulation of stachyose (Blackman *et al.*, 1992), galactopinitol A, galactopinitol B and fagopyritol B1 (Obendorf *et al.*, 1998a, b) during precocious maturation. Exogenous feeding of free cyclitols, individually and in combination, to immature soybean embryos, followed by precocious maturation induced by slow drying of the embryos, should increase the level of free cylitols in the embryos for incorporation into galactosyl cyclitols, thereby providing a novel method to test our hypotheses. If D-pinitol and D-*chiro*-inositol are not synthesized in the embryo, exogenous feeding of D-pinitol and D-*chiro*-inositol should markedly increase the accumulation of galactopinitols and fagopyritol B1, respectively (Fig. 1).

Our objectives were: (1) to determine whether feeding free cyclitols to soybean embryos enhances the accumulation of galactosyl cyclitols; (2) to determine whether feeding D-*chiro*-inositol and D-pinitol in combination to soybean embryos reduces the accumulation of fagopyritol B1 and galactopinitols below the amounts observed after feeding D-*chiro*-inositol and D-pinitol individually; and (3) to determine if heterologously expressed recombinant GmGolS catalyses the synthesis of fagopyritol B1. Some of the observations have been included in a preliminary report (Odorcic and Obendorf, 2003) and a biology research Honours thesis (Odorcic, 2003).

Materials and methods

Plant materials

Soybean (*Glycine max* (L.) Merrill) plants were grown in the greenhouse at 27°C day (14 h) and 22°C night (10 h) under natural sunlight, supplemented 14 h daily with 640 µmol m^{-2} s^{-1} incandescent light from metal halide lamps (Sylvania 1000 watt BU) (Obendorf *et al.*, 1980, 1998b). Three embryos, isolated from immature seeds (250 ± 20 mg fresh weight, approximately 35 d after pollination) by removal of the seed coat and nucellus remnants, were incubated in 20-ml screw-capped vials containing 3 ml of substrate (cyclitol and/or sucrose) solutions for 24 h at 25°C under 200 µmol m^{-2} s^{-1} fluorescent light. Embryos were blotted, placed in small plastic Petri dishes, and subjected to slow drying at 22°C by daily transfer to successively lower relative humidity (RH) controlled by saturated salt solutions (Blackman *et al.*, 1992): day 1, 92% RH; day 2, 87% RH; day 3, 75% RH; day 4, 54% RH; day 5, 45% RH; day 6, 32% RH; day 7, 12% RH; and remained at 12% RH during days 8–14.

Embryo feeding experiments: substrate concentration series

Four substrate concentration experiments were conducted. Embryos for each experiment were incubated in each of the substrate solutions for 24 h, blotted and slow-dried for 14 d. Axis and cotyledon tissues were separated and analysed for soluble carbohydrates. In the sucrose concentration series, three replicates of three embryos (total of nine embryos/treatment) were incubated with 0, 25, 50, 75, 100 and 200 mM sucrose. The following concentration series experiments were adjusted to be a constant 100 mM (cyclitol plus sucrose). Four replicates of three embryos each (total of 12 embryos/treatment) were incubated in the *myo*-inositol–sucrose concentration series: (A) 0 mM *myo*-inositol + 100 mM sucrose; (B) 10 mM *myo*-inositol + 90 mM sucrose; (C) 25 mM *myo*-inositol + 75 mM sucrose; (D) 50 mM *myo*-inositol + 50 mM sucrose; (E) 100 mM *myo*-inositol + 0 mM sucrose; and (F) 0 mM *myo*-inositol + 0 mM sucrose. Six replicates of three embryos each (total of 18 embryos/treatment) were incubated in the D-*chiro*-inositol–sucrose concentration series, and three replicates of three embryos each (total of nine embryos/treatment) were incubated in the D-pinitol–sucrose concentration series. Treatments (A) through (F) were identical in both concentration series, except for the substitution of D-*chiro*-inositol or D-pinitol instead of *myo*-inositol.

Embryo feeding experiments: drying time series

Six slow-drying time experiments were conducted. In each experiment, three replicates of three embryos each (a total of nine embryos per treatment) were incubated in a different sucrose and/or cyclitol substrate solution for 24 h, blotted and slow-dried for 0, 1, 2, 3, 4 or 14 d. Axis and cotyledon tissues were separated and analysed for soluble carbohydrates. The substrate solutions for the six experiments were as follows: 30 mM *myo*-inositol + 100 mM sucrose; 100 mM D-*chiro*-inositol; 100 mM D-pinitol; 100 mM D-pinitol + 100 mM D-*chiro*-inositol; 50 mM D-pinitol + 50 mM D-*chiro*-inositol; and 100 mM D-pinitol + 100 mM D-*chiro*-inositol + 100 mM sucrose.

Substrates

Sucrose, raffinose, stachyose, *myo*-inositol, *scyllo*-inositol, *epi*-inositol and UDP-galactose were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). D-Pinitol, D-*chiro*-inositol, L-*chiro*-inositol, D-ononitol and L-quebrachitol (1L-2-*O*-methyl-*chiro*-inositol) were purchased from Industrial Research Limited (Lower Hutt, New Zealand). Sequoyitol (5-*O*-methyl-*myo*-inositol) was purchased from Carl

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Roth GmbH & Co. KG (Karlsruhe, Germany). D-Bornesitol (1D-1-*O*-methyl-*myo*-inositol) was purified from seeds of *Lathyrus odoratus* L. Galactinol was purified from lemon balm (*Melissa officinalis* L.) leaves. Fagopyritols were purified from buckwheat seeds. Galactopinitols were purified from seeds of hairy vetch (*Vicia villosa* L.) or chickpea (*Cicer arietinum* L.). When needed, substrates were purified by carbon (Mallinckrodt Baker Inc., Phillipsburg, New Jersey, USA)–Celite (Supelco, Bellefonte, Pennsylvania, USA) column chromatography (Whistler and Durso, 1950).

Carbohydrate analysis

Soluble carbohydrates were extracted from two cotyledons or one axis for each embryo. Two cotyledons were extracted with 2.0 ml of ethanol:water (1:1, v/v), containing 300 µg of phenyl α-D-glucoside as the internal standard. One axis was extracted with 1.0 ml of ethanol:water (1:1, v/v), containing 100 µg of phenyl α-D-glucoside as the internal standard. Extracts were passed through a 10,000 MW cut-off filter (NANOSEP 10K Omega, Paul Filton Co., Northborough, Massachusetts, USA) by centrifugation, and 200 µl were dried in silylation vials under nitrogen gas, derivatized with 200 µl of trimethylsilylsylimidazole:pyridine (1:1, v/v) and analysed by high-resolution gas chromatography on an HP1-MS (Agilent Technologies, Palo Alto, California, USA) capillary column (15 m length, 0.25 mm internal diameter, 0.25 µm film thickness), as described by Horbowicz and Obendorf (1994).

Soybean EST clone corresponding to the gene encoding galactinol synthase (GolS)

cDNA sequences corresponding to the *GolS* gene in soybean were searched in the nucleotide and protein databases using the BLAST programs (http://www.ncbi.nlm.nih.gov) and a multiple sequence alignment program, CLUSTAL W (http://workbench.sdsc.edu). A soybean expressed sequence tag (EST) clone (Shoemaker *et al*., 1999; GenBank BE330777), sharing a very high level of DNA sequence identity with the *GolS* genes reported from other plant species, was identified and purchased from INCYTE GENOMICS (Palo Alto, California, USA; cat. no. Gm-c1041). Since only partial DNA sequence data were available for this EST clone in GenBank, the whole EST insert was re-sequenced (nucleotide sequence assigned to GenBank AY126715) at the DNA Sequencing Facility at the Resource Center at Cornell University (http://brcweb.tech.cornell.edu).

Bacterial expression and purification of recombinant soybean GolS (GmGolS) protein

The entire 987 bp protein coding sequence of *GmGolS* was polymerase chain reaction (PCR) amplified using the soybean EST clone template. Two primers, 5′-CATCACTGAGCATATGGCTGG-3′ and 5′-GGATC CAAAGACACTCTTAAGCAGCAGATGGGG-3′, containing the restriction enzyme recognition sites *Nde*I and *Bam*HI, respectively, were used in the PCR assays. After cloning into the pCRII-TOPO vector (Invitrogen, Carlsbad, California, USA) for sequencing and amplification in *Escherichia coli*, the *Nde*I/*Bam*HI fragment containing the entire protein-coding sequence was isolated and cloned into the pET-14b vector (Novagen, Madison, Wisconsin, USA) (T. Ueda, M.P. Coseo, T.J. Harrell and R.L. Obendorf, unpublished, Cornell, 2002) directly downstream from the poly-histidine codons. The pET-14b plasmid containing *GmGolS* cDNA was mobilized into *E. coli* strain BL21 (DE3) (Novagen). Expression of the recombinant GmGolS protein was induced in *E. coli* with 1 mM isopropylthio-β-D-galactoside (IPTG), following the manufacturer's protocol (Novagen). The bacterial cells were collected by centrifugation and resuspended in 10 mM Tris–HCl buffer (pH 8.0). The soluble protein fraction was extracted from the bacterial cells by the gentle disruption of their cell walls with BugBuster Protein Extraction Reagent (Novagen) containing Benzonase (Novagen). Poly-histidine-tagged recombinant proteins were purified from the extracts using His.Bind Quick 900 Cartridges (Novagen). Purification of proteins was verified by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE). Purified recombinant proteins were dialysed against 50 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid]–NaOH buffer, pH 7.0, containing 5 mM $MnCl_2$, immediately after elution from the His.Bind Quick 900 Cartridges and prior to enzyme assay.

Enzyme assays

Both crude soluble protein extracts from *E. coli* containing the recombinant GmGolS protein and purified recombinant GmGolS protein were used in enzyme assays. GmGolS activity assays included 20 mM UDP-galactose as the galactosyl donor, 20 mM *myo*-inositol as the galactosyl acceptor, 50 mM HEPES buffer, pH 7.0, 2 mM 1,4-dithiothreitol (DTT), 5 mM $MnCl_2$ and 1–5 µg of crude protein extract or purified GmGolS protein in 50 µl total volume. In fagopyritol synthase assays, *myo*-inositol was substituted with 20 mM D-*chiro*-inositol as the galactosyl acceptor. Assays were run at 30°C for 10–120 min (30–300 min for crude protein extracts). Reactions were stopped by addition of 50 µl of 100%

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ethanol. After addition of 25 µg of phenyl α-D-glucoside as the internal standard, the reaction mixture was heated at 80°C for 30 min, passed through a 10,000 MW cut-off filter (NANOSEP), and evaporated to dryness under a stream of nitrogen gas. Residues were dried, derivatized and analysed for fagopyritols or other soluble carbohydrate products by high-resolution gas chromatography, as described above.

Results

Substrate feeding

Cyclitols, including *myo*-inositol, D-*chiro*-inositol and D-pinitol, were fed to immature soybean embryos, followed by precocious maturation induced by slow-drying of the embryos and analysis of soluble carbohydrates in axis and cotyledon tissues. Exogenously fed free cyclitols were taken up by embryo tissues. In 250 mg fresh weight of embryos, initial concentrations of cyclitols in axis and cotyledon tissues, respectively, were 10.9 and 11.0 mg (g dry weight)$^{-1}$ for *myo*-inositol, 1.4 and 1.2 mg (g dry weight)$^{-1}$ for D-*chiro*-inositol, and 6.0 and 4.0 mg (g dry weight)$^{-1}$ for D-pinitol. After incubation with 30 mM *myo*-inositol for 24 h at 22°C, concentrations of free *myo*-inositol increased 1.8-fold in the axis and 2-fold in cotyledon tissues. After incubation with 100 mM D-*chiro*-inositol, free D-*chiro*-inositol increased 18-fold and 40-fold, and after incubation with 100 mM D-pinitol, free D-pinitol increased 6-fold and 11-fold, respectively.

Both embryonic axis and cotyledon tissues were assayed. Embryonic axes matured (green to yellow at maximum dry mass) earlier than cotyledons and accumulated higher concentrations of soluble carbohydrates. Accumulation of products in axis tissues generally preceded accumulation of products in cotyledons, reflecting the differential in progression toward maturation. Feeding D-pinitol or combinations of D-pinitol and D-*chiro*-inositol resulted in yellowing of axis tissues 1–2 d earlier during precocious maturation than feeding D-*chiro*-inositol alone (data not shown).

Sucrose and myo-*inositol feeding*

Feeding sucrose at 0–200 mM resulted in a small decrease in galactinol and an increase in sucrose in axis tissues, but little change in concentrations of soluble carbohydrates in cotyledon tissues (data not shown). Feeding *myo*-inositol up to 50 mM doubled free *myo*-inositol concentrations in dry axis and cotyledon tissues after precocious maturation, with small increases in free D-pinitol and D-*chiro*-inositol (data not shown). Galactinol accumulation increased by 58% in cotyledons after feeding 50 mM *myo*-inositol, while fagopyritol B1 accumulation was reduced by 45%. There was little change in galactopinitol A, galactopinitol B, raffinose or stachyose concentrations in either axis or cotyledon tissues after feeding *myo*-inositol. In time-series experiments, feeding 100 mM *myo*-inositol ($n = 3$) or 30 mM *myo*-inositol + 100 mM sucrose ($n = 9$) resulted in doubling of free *myo*-inositol during day 1 of slow-drying, followed by a decline, and a transient increase in galactinol [6–8 mg (g dry weight)$^{-1}$] during days 2 and 3, followed by a decline as raffinose and stachyose accumulated (data not shown). Total *myo*-inositol (free *myo*-inositol plus galactinol) declined during precocious maturation in all experiments. There were no increases in total D-pinitol or total D-*chiro*-inositol.

D-chiro-*Inositol feeding*

Feeding D-*chiro*-inositol resulted in a 40- to 50-fold increase in free D-*chiro*-inositol concentrations in axis and cotyledons (Fig. 2A and D), a 17-fold increase in fagopyritol B1 concentration in axis tissues and a 7-fold increase in cotyledons (Fig. 2B and E), but did not increase D-pinitol, *myo*-inositol, galactopinitol A, galactopinitol B, galactinol, raffinose or stachyose concentrations (Fig. 2). Between days 1 and 3 (axes) and 2 and 4 (cotyledons) of slow-drying, there was a decrease in concentration of free D-*chiro*-inositol and a concomitant large increase in fagopyritol B1 (Fig. 3A and B, D and E). A transient accumulation of galactinol signalled an accumulation of raffinose and stachyose and modest accumulation of galactopinitol A and galactopinitol B (Fig. 3B and C, E and F).

D-*Pinitol feeding*

Feeding D-pinitol resulted in an 8-fold increase in free D-pinitol concentration (Fig. 4A and D) and greater than a 4-fold increase in both galactopinitol A and galactopinitol B concentrations (Fig. 4B and E). Concentrations of D-*chiro*-inositol, *myo*-inositol, fagopyritol B1, galactinol, raffinose and stachyose were not increased (Fig. 4). Feeding 100 mM D-pinitol resulted in high concentrations of free D-pinitol and a substantial increase in galactopinitol A and galactopinitol B between days 2 and 4 of slow-drying (Fig. 5A and B, D and E). A transient increase in galactinol occurred as raffinose and stachyose accumulated (Fig. 5B and C, E and F). Stachyose accumulation in cotyledons after feeding D-pinitol (Figs 4F and 5F) was double that after feeding D-*chiro*-inositol (Figs 2F and 3F).

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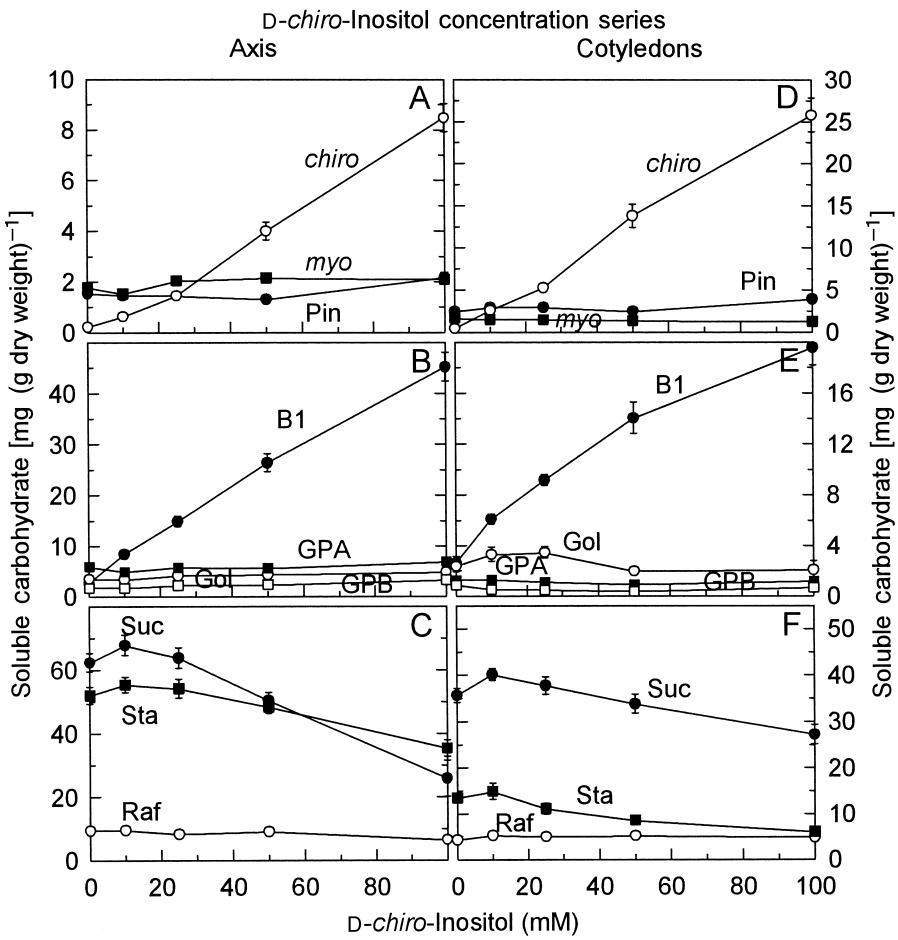

Figure 2. Accumulated soluble carbohydrates in axis and cotyledon tissues after precocious maturation of immature soybean embryos as a function of D-*chiro*-inositol concentration, after feeding D-*chiro*-inositol (0–100 mM) plus sucrose (100–0 mM) (100 mM total concentration) for 24 h at 25°C followed by 14 d precocious maturation in a slow-drying series of relative humidities. Values are mean ± SE (n = 18). Error bars are not shown if smaller than symbols. A–C, axis. D–F, cotyledons. A–F, *myo*-inositol (*myo*), D-pinitol (Pin), D-*chiro*-inositol (*chiro*), fagopyritol B1 (B1), galactinol (Gol), galactopinitol A (GPA), galactopinitol B (GPB), raffinose (Raf), stachyose (Sta), sucrose (Suc).

D-Pinitol plus D-chiro-inositol feeding

Feeding a combination of 100 mM D-pinitol and 100 mM D-*chiro*-inositol resulted in high concentrations of both free D-pinitol and free D-*chiro*-inositol. Initial uptake of free D-pinitol (0 d, Fig. 6) in the presence of free D-*chiro*-inositol was comparable to initial uptake of D-pinitol when fed alone (0 d, Fig. 5). Uptake of free D-*chiro*-inositol (0 d, Fig. 6) in the presence of D-pinitol was 50% (cotyledons) to 60% (axis) of the uptake of D-*chiro*-inositol when fed alone (0 d, Fig. 3). Free D-*chiro*-inositol declined with elevated concentrations of fagopyritol B1; D-pinitol decreased only slightly and galactopinitol A, galactopinitol B, stachyose and raffinose all increased between days 2 and 3 in embryo cotyledon tissues (Fig. 6). Galactinol concentration peaked by day 1 (axis) or day 2 (cotyledons) and declined as raffinose, stachyose and galactopinitols accumulated. Accumulation of fagopyritol B1 appeared to be independent of accumulation of galactopinitols, raffinose and stachyose. Feeding a combination of D-pinitol and D-*chiro*-inositol (Fig. 6) resulted in a 50% decrease (14 d) in steady-state galactinol concentration and a 50% decrease (14 d) in galactopinitol A plus galactopinitol B concentration in cotyledons (Fig. 6E), compared to feeding D-pinitol alone (Fig. 5E), but only a 10–15% decrease (4 d) in fagopyritol B1 concentration (Fig. 6E) compared to feeding D-*chiro*-inositol alone (Fig. 3E) [14 versus 16 mg (g dry weight)$^{-1}$ at day 4 of slow-drying]. In axis tissues, galactinol, galactopinitol A, galacto-

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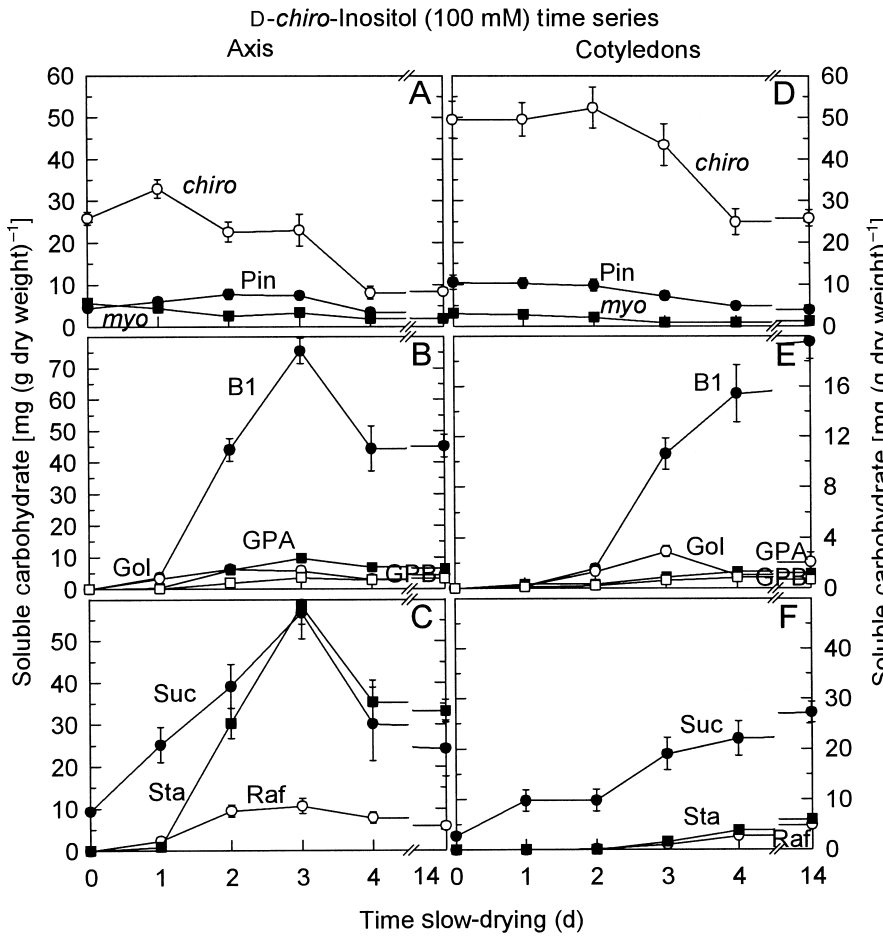

Figure 3. Accumulated soluble carbohydrates in axis and cotyledon tissues after precocious maturation of immature soybean embryos as a function of time of slow-drying, after feeding 100 mM D-*chiro*-inositol for 24 h at 25°C, followed by 0–14 d precocious maturation in a slow-drying time series of relative humidities. Values are mean ± SE (n = 9). Error bars are not shown if smaller than symbols. A–C, axis. D–F, cotyledons. A–F, *myo*-inositol (*myo*), D-pinitol (Pin), D-*chiro*-inositol (*chiro*), fagopyritol B1 (B1), galactinol (Gol), galactopinitol A (GPA), galactopinitol B (GPB), raffinose (Raf), stachyose (Sta), sucrose (Suc).

pinitol B, raffinose and stachyose were not decreased by feeding a combination of D-pinitol and D-*chiro*-inositol (Fig. 6B and C), compared to feeding D-pinitol alone (Fig. 5B and C). Both initial uptake of free D-*chiro*-inositol (0 d, Fig. 6A) and accumulation of fagopyritol B1 (14 d, Fig. 6B) in axis tissues were reduced 40% after feeding a combination of D-pinitol and D-*chiro*-inositol compared to feeding D-*chiro*-inositol alone (Fig. 3A, B). In all cases, fagopyritol B1 was maximum in axis tissues on day 3 of slow-drying, while in cotyledons fagopyritol B1 continued to increase during day 4 of slow-drying. Axis tissues yellowed (matured) 1–2 d sooner during precocious maturation after feeding D-pinitol or combinations of D-pinitol and D-*chiro*-inositol than after feeding D-*chiro*-inositol alone (data not shown). Feeding a combination of 50 mM D-pinitol plus 50 mM D-*chiro*-inositol (data not shown) resulted in patterns identical to those with 100 mM. Feeding a combination of 100 mM D-pinitol, 100 mM D-*chiro*-inositol and 100 mM sucrose resulted in patterns identical to those without sucrose (Fig. 6), except that sucrose concentrations were higher initially (data not shown).

Soybean galactinol synthase (GmGolS)

To confirm that GolS catalyses the synthesis of fagopyritol B1, a soybean galactinol synthase (*GmGolS*) cDNA was cloned (GenBank AY126715) and heterologously expressed in *Escherichia coli*. Purified recombinant GmGolS catalysed the

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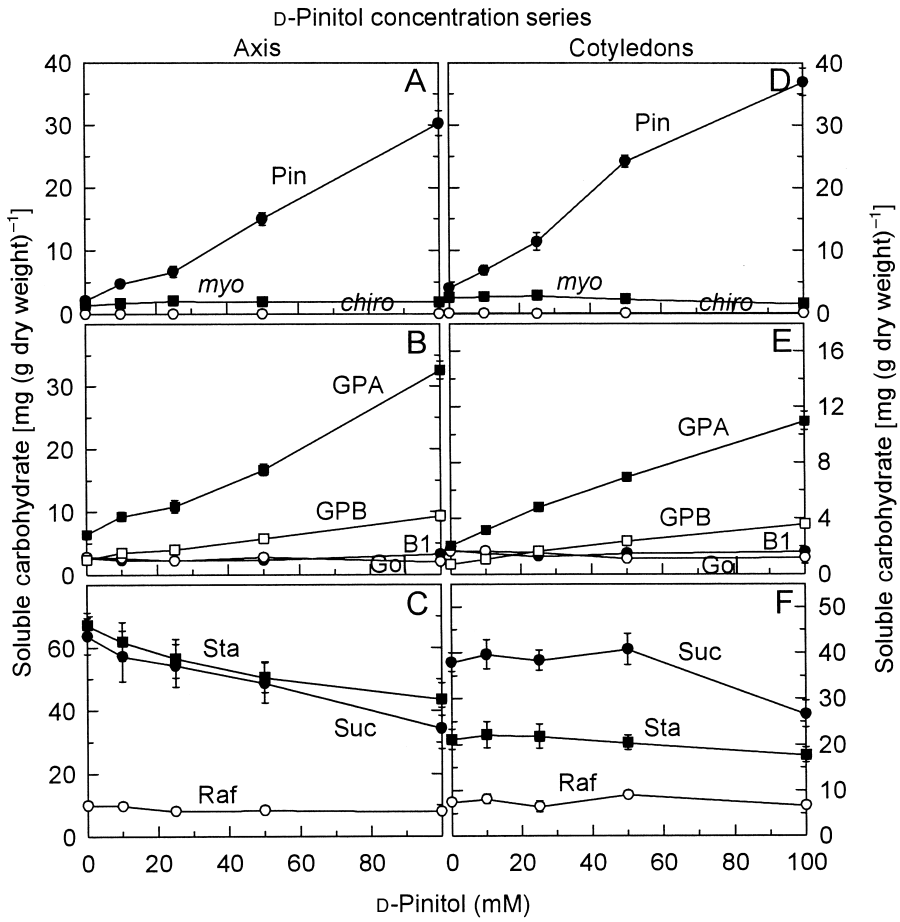

Figure 4. Accumulated soluble carbohydrates in axis and cotyledon tissues after precocious maturation of immature soybean embryos as a function of D-pinitol concentration, after feeding D-pinitol (0–100 mM) plus sucrose (100–0 mM) (100 mM total concentration) for 24 h at 25°C, followed by 14 d precocious maturation in a slow-drying series of relative humidities. Values are mean ± SE (n = 9). Error bars are not shown if smaller than symbols. A–C, axis. D–F, cotyledons. A–F, *myo*-inositol (*myo*), D-pinitol (Pin), D-*chiro*-inositol (*chiro*), fagopyritol B1 (B1), galactinol (Gol), galactopinitol A (GPA), galactopinitol B (GPB), raffinose (Raf), stachyose (Sta), sucrose (Suc).

synthesis of galactinol with UDP-galactose as the galactosyl donor and *myo*-inositol as the galactosyl acceptor (Fig. 7A), but also catalysed the synthesis of fagopyritol B1 with UDP-galactose as the galactosyl donor and D-*chiro*-inositol as the galactosyl acceptor (Fig. 7B). GmGolS activity was not detected with galactinol as the galactosyl donor. Using UDP-galactose as the galactosyl donor, GmGolS was also active with L-*chiro*-inositol and *scyllo*-inositol as galactosyl acceptors, but activity was not detected with *epi*-inositol, *O*-methylated cyclitols (D-pinitol, D-ononitol, sequoyitol, D-bornesitol or L-quebrachitol) or galactosyl cyclitols (fagopyritol B1 or galactinol) as galactosyl acceptors, using UDP-galactose as the galactosyl donor (Table 1).

Discussion

myo-Inositol, D-pinitol and D-*chiro*-inositol were the only cyclitols detected in soybean embryos (Horbowicz and Obendorf, 1994; Obendorf *et al.*, 1998a, b). Total D-*chiro*-inositol (free D-*chiro*-inositol plus galactosyl derivatives in the embryo, axis plus cotyledons) or total D-pinitol did not increase in the absence of exogenous feeding of the corresponding cyclitols, consistent with our previous results with soybean zygotic embryos matured *in vitro* (Obendorf *et al.*, 1998a, b), and indicating a lack of synthesis of both D-*chiro*-inositol and D-pinitol during precocious maturation of soybean zygotic embryos, even when fed *myo*-inositol, the precursor to synthesis of D-*chiro*-inositol and D-pinitol (Dittrich and Brandl, 1987; Obendorf, 1997).

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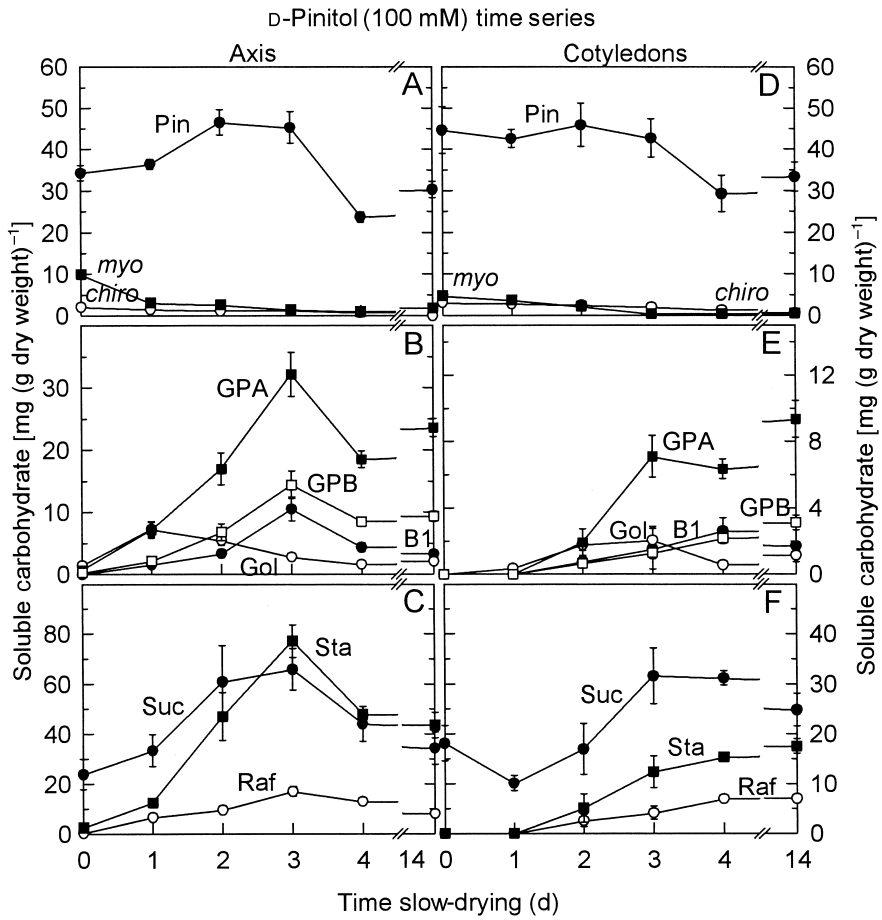

Figure 5. Accumulated soluble carbohydrates in axis and cotyledon tissues after precocious maturation of immature soybean embryos as a function of time of slow-drying, after feeding 100 mM D-pinitol for 24 h at 25°C, followed by 0–14 d precocious maturation in a slow-drying time series of relative humidities. Values are mean ± SE ($n = 9$). Error bars are not shown if smaller than symbols. A–C, axis. D–F, cotyledons. A–F, *myo*-inositol (*myo*), D-pinitol (Pin), D-*chiro*-inositol (*chiro*), fagopyritol B1 (B1), galactinol (Gol), galactopinitol A (GPA), galactopinitol B (GPB), raffinose (Raf), stachyose (Sta), sucrose (Suc).

Table 1. Substrate specificity of recombinant GmGolS with each galactosyl acceptor at 20 mM and 20 mM UDP-galactose as galactosyl donor

Galactosyl acceptor	Relative activity (%)
Inositols	
D-*chiro*-Inositol	94
myo-Inositol	100
L-*chiro*-Inositol	63
scyllo-Inositol	107
epi-Inositol	ND
Inositol-*O*-methyl ethers	
D-Pinitol (1D-3-*O*-methyl-*chiro*-inositol)	ND
D-Ononitol (1D-4-*O*-methyl-*myo*-inositol)	ND
Sequoyitol (5-*O*-methyl-*myo*-inositol)	ND
D-Bornesitol (1D-1-*O*-methyl-*myo*-inositol)	ND
L-Quebrachitol (1L-2-*O*-methyl-*chiro*-inositol)	ND
Galactosyl cyclitols	
Fagopyritol B1 (α-D-galactopyranosyl-(1→2)-1D-*chiro*-inositol)	ND
Galactinol (α-D-galactopyranosyl-(1→1)-1L-*myo*-inositol)	

ND, not detected.

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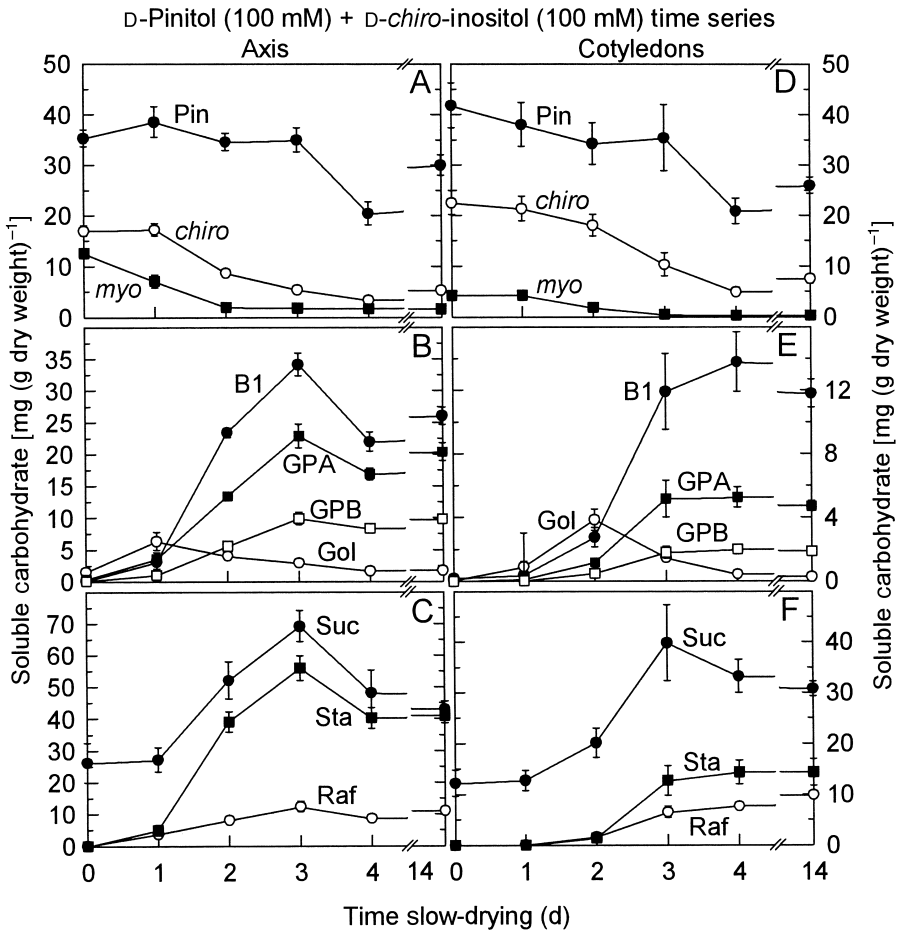

Figure 6. Accumulated soluble carbohydrates in axis and cotyledon tissues after precocious maturation of immature soybean embryos as a function of time of slow-drying, after feeding 100 mM D-*chiro*-inositol + 100 mM D-pinitol for 24 h at 25°C, followed by 0–14 d precocious maturation in a slow-drying time series of relative humidities. Values are mean ± SE (n = 9). Error bars are not shown if smaller than symbols. A–C, axis. D–F, cotyledons. A–F, *myo*-inositol (*myo*), D-pinitol (Pin), D-*chiro*-inositol (*chiro*), fagopyritol B1 (B1), galactinol (Gol), galactopinitol A (GPA), galactopinitol B (GPB), raffinose (Raf), stachyose (Sta), sucrose (Suc).

Exogenous feeding of *myo*-inositol (up to 100 mM) only doubled the concentration of free *myo*-inositol in axis and cotyledon tissues, consistent with *myo*-inositol synthesis in embryos (Hegeman *et al.*, 2001; Hitz *et al.*, 2002) and implying a rapid metabolic regulation of *myo*-inositol concentration in embryos. The decrease in total *myo*-inositol (free *myo*-inositol plus galactinol in axis and cotyledons) after the first day of slow-drying indicates the metabolism of *myo*-inositol to other products, including phytin and cell walls, within the embryo (Loewus and Murthy, 2000; Hegeman *et al.*, 2001; Hitz *et al.*, 2002). By contrast, the large increase in accumulated fagopyritol B1 and galactopinitols after exogenous feeding of free D-*chiro*-inositol and D-pinitol, respectively, was consistent with the absence of D-*chiro*-inositol and D-pinitol synthesis in embryo tissues. The *myo*-inositol 6-*O*-methyltransferase (mI6OMT or IMT, *S*-adenosyl-L-methionine:*myo*-inositol *O*-methyltransferase, EC 2.1.1.129) enzyme that forms D-ononitol is located in leaves and stems (Wanek and Richter, 1997; Streeter *et al.*, 2001). Soybean somatic embryos transformed with *IMT* formed D-ononitol but not D-pinitol (J.J. Finer and J.G. Streeter, personal communication, Ohio, 2002), indicating that soybean somatic embryos do not express genes encoding the enzymes for D-pinitol synthesis. The lack of increased D-pinitol or D-*chiro*-inositol (or their galactosyl derivatives), after exogenous feeding of *myo*-inositol, indicates that isolated soybean zygotic embryos also lack the enzymes that form D-pinitol and D-*chiro*-inositol.

The reduction in fagopyritol B1 accumulation, while galactinol accumulation doubled, in cotyledons after feeding *myo*-inositol indicated a competition

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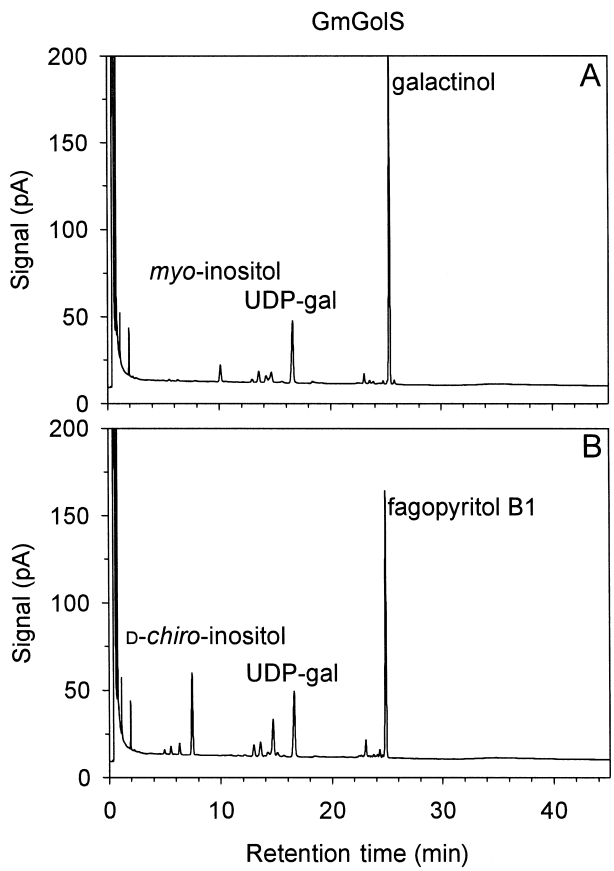

Figure 7. GmGolS products on gas chromatograms. A, Galactinol (retention time 25.8 min) accumulation after enzyme incubation with 25 mM *myo*-inositol, 25 mM UDP-galactose, 5 mM $MnCl_2$ and 2 mM dithiothreitol (DTT) at 30°C. B, Fagopyritol B1 (retention time 25.3 min) accumulation after enzyme incubation with 25 mM D-*chiro*-inositol, 25 mM UDP-galactose, 5 mM $MnCl_2$ and 2 mM DTT at 30°C. Reactions were run to near completion to emphasize the products.

between the synthesis of fagopyritol B1 and galactinol, and implied that both may be synthesized by GolS. Feeding D-pinitol and D-*chiro*-inositol together reduced galactinol and galactopinitol concentrations in cotyledons by 50% compared to feeding D-pinitol alone. The 50% reduction in the synthesis of galactopinitols reflects the 50% reduction in galactinol, the galactosyl donor for galactopinitol synthesis by STS (T.P. Lin and R.L. Obendorf, unpublished, 1998; Peterbauer and Richter, 2001). The small decrease in fagopyritol B1 synthesis after feeding D-pinitol and D-*chiro*-inositol together, compared to feeding D-*chiro*-inositol alone, probably reflects the reduced uptake of D-*chiro*-inositol by embryos in the presence of D-pinitol. The doubling of stachyose accumulation after feeding D-pinitol, compared to feeding D-*chiro*-inositol, implied that galactopinitols (but not fagopyritol B1) may also serve as galactosyl donors for stachyose synthesis in soybean, as Hoch *et al*. (1999) reported for lentil STS. The fact that fagopyritol B1 accumulation appeared to be independent of galactopinitol and stachyose accumulation suggests they are formed by different enzymes, and provided additional support for the hypothesis that fagopyritol B1 is formed by GolS, at least in soybean (Fig. 1), a hypothesis confirmed by product analysis using recombinant GmGolS.

The recombinant soybean GmGolS is a multi-functional enzyme with both GolS activity and fagopyritol synthase activity, but GmGolS does not exhibit galactopinitol synthase activity. The deduced amino acid sequence of GmGolS (AY126715) obtained from the public soybean EST project (Shoemaker *et al*., 1999; BE330777) differed by only one amino acid (isoleucine instead of methionine at position 223) (T. Ueda, M.P. Coseo, T.J. Harrell and R.L. Obendorf, unpublished, Cornell, 2002) from a reported soybean seed GolS (I55634), sequence number 6 (Kerr *et al*., 1997). GolS activity in maturing soybean seeds was associated with stachyose accumulation and remained high throughout seed maturity (Handley *et al*., 1983; Saravitz *et al*., 1987; Lowell and Kuo, 1989; Kuo *et al*., 1997b). During soybean seed development *in planta*, *GolS* mRNA was first detected in axis tissues at 44 d post anthesis (DPA) and in cotyledons at 46–48 DPA (Volk, 1998), coincident with galactinol and fagopyritol B1 accumulation at the onset of stachyose and galactopinitol accumulation (Obendorf *et al*., 1998b). *GolS* transcripts remained high during seed desiccation (Volk, 1998).

Substrate specificities of GmGolS and STS are different. The lack of GmGolS activity with D-pinitol, D-ononitol and sequoyitol as galactosyl acceptors contrasts with the activity of STS with these *O*-methylated cyclitols (Peterbauer and Richter, 1998; Hoch *et al*., 1999; Peterbauer *et al*., 2002b). Soybean STS (T.P. Lin and R.L. Obendorf, unpublished, 1998) and lentil *(Lens culinaris* Medic.) STS (Hoch *et al*., 1999) catalyse the synthesis of galactopinitols; the lentil enzyme had low activity with D-*chiro*-inositol and no activity with L-*chiro*-inositol (Hoch *et al*., 1999). By contrast, adzuki bean (*Vigna angularis* Ohwi *et* Ohashi) STS had only a trace of activity with D-pinitol, and no activity with D-*chiro*-inositol or L-*chiro*-inositol (Peterbauer and Richter, 1998). A recombinant RFS from pea (*Pisum sativum* L.) seeds was active with D-ononitol and D-pinitol to form galactosyl ononitol and galactosyl pinitol, using galactinol as the galactosyl donor (Peterbauer *et al*., 2002a). This pea RFS also exhibited a neutral α-galactosidase activity (Peterbauer *et al*., 2002a), consistent with its amino acid sequence similarity to a family of alkaline α-galactosidases (seed imbibition proteins, SIPs) (Carmi *et al*., 2003). A multi-functional pea seed STS had low

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activities for synthesis of galactopinitol and verbascose (Peterbauer *et al.*, 2002b). Collectively, these observations demonstrate substrate specificity of these multi-functional enzymes to be species-specific, and product accumulation to be dependent upon the availability of specific cyclitol substrates to the embryo tissues. Clearly, GmGolS can catalyse the synthesis of fagopyritol B1, but not galactopinitols. Soybean, like other plants, probably has multiple alleles encoding GolS. Since the purchased *GmGolS* cDNA was obtained from a different soybean cultivar, we cannot be certain that *GmGolS* is *the* allele or the only allele encoding the GolS enzyme that catalysed the accumulation of fagopyritol B1 in isolated soybean embryos in response to the exogenous supply of D-*chiro*-inositol.

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