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Cyclitol galactosides in low-raffinose, low-stachyose soybean embryos after feeding D-*chiro*-inositol, *myo*-inositol or D-pinitol

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Abstract

Sucrose, raffinose and stachyose accumulate as stored soluble carbohydrates in embryos during soybean [*Glycine max* L. (Merrill)] seed development and maturation. Raffinose and stachyose in soybean feed products are not digested by humans, chickens or pigs, resulting in flatulence and reduced nutritional value. Soybean lines selected for low raffinose and low stachyose (LRS) or low raffinose, low stachyose and low phytin (LRSP1, LRSP2) concentrations in mature seeds were compared to a CHECK line with normal raffinose, stachyose and phytin. To determine whether increasing the supply of free cyclitols to immature embryos of these lines results in increased accumulation of galactosyl cyclitols, isolated immature embryos free of maternal tissues were fed solutions containing either D-*chiro*-inositol, *myo*-inositol or D-pinitol, or a control solution without cyclitols, for 24 h. Embryos were precociously matured by slow drying for 14 d with daily transfers to stepwise lower relative humidities. Soluble carbohydrates were extracted from axis and cotyledon tissues of mature, dry embryos and analysed by high-resolution gas chromatography. Axis and cotyledons from LRS, LRSP1 and LRSP2 embryos had low concentrations of stachyose compared to CHECK embryos after feeding a control solution without cyclitols. Feeding D-*chiro*-inositol to isolated embryos increased fagopyritol B1 accumulation in embryos of all lines. Feeding *myo*-inositol increased stachyose accumulation in LRSP1 and LRSP2 cotyledons. Feeding D-pinitol increased free D-pinitol in cotyledons of all lines but increased galactopinitol A and galactopinitol B only in LRS cotyledons. Supplying additional D-*chiro*-inositol to immature embryos can enhance accumulation of fagopyritol B1 in mature embryos of low-raffinose and low-stachyose or low-raffinose, low-stachyose and low-phytin soybeans.

Keywords: fagopyritol, galactopinitol, *Glycine max*, D-*chiro*-inositol, D-pinitol, soybean embryo

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Abbreviations: CHECK, seed phenotype with normal raffinose, stachyose and phytin; Ciceritol, digalactopinitol A; DGMI, digalactosyl *myo*-inositol; gol, galactinol; GmGolS, *Glycine max* galactinol synthase (EC 2.4.1.123); GolS, galactinol synthase (EC 2.4.1.123); GPA, galactopinitol A; GPB, galactopinitol B; IMP, *myo*-inositol-phosphate monophosphatase (EC 3.1.3.25); IMT, *myo*-inositol 4-*O*-methyltransferase (EC 2.1.1.129); IPK, inositol polyphosphate kinases; LRS, seed phenotype with low raffinose and stachyose; LRSP1 and LRSP2, seed phenotype with low raffinose, stachyose and phytin; MIPS, *myo*-inositol-phosphate synthase (EC 5.5.1.4); *mips*, mutant form of *Mips* gene (Gm mI 1-PS-1A, AY038, 802); *myo*, *myo*-inositol; D-ononitol, (1D-4-*O*-methyl-*myo*-inositol); PAR, photosynthetically active radiation; D-pinitol, (1D-3-*O*-methyl-*chiro*-inositol); RH, relative humidity; RFOs, raffinose series oligosaccharides; RFS, raffinose synthase (EC 2.4.1.82); *stc1*, mutant form of *Stc1* gene; STS, stachyose synthase (EC 2.4.1.67); STS?, indicates stachyose synthase or similar enzyme but not confirmed experimentally; TGMI, trigalactosyl *myo*-inositol; TGPA, trigalactopinitol A; UDP, uridine diphosphate; UDP-gal, uridine diphosphate galactoside; VBS, verbascose synthase.

Introduction

Developing soybean [*Glycine max* (L.) Merrill] seeds accumulate sucrose, raffinose and stachyose as well as lesser amounts of galactopinitol A, galactopinitol B and fagopyritol B1 in axis and cotyledon tissues during seed maturation (Hsu *et al.*, 1973; Schweizer and Horman, 1981; Obendorf *et al.*, 1998, 2009, 2012; Obendorf and Kosina, 2011). Raffinose series oligosaccharides (RFOs) including raffinose, stachyose and verbascose are α-galactoside derivatives of sucrose. Galactosyl cyclitols, including galactinol, galactopinitol A and galactopinitol B, and fagopyritol B1,

are α-galactoside derivatives of the cyclitols *myo*-inositol, D-pinitol (1D-3-*O*-methyl-*chiro*-inositol) and D-*chiro*-inositol, respectively. The free cyclitols are synthesized in maternal tissues of soybean, transported to the seed coat where they are unloaded into free space surrounding the embryo, and loaded into embryo tissues where they are stored primarily as their respective galactosides (Gomes *et al.*, 2005; Obendorf *et al.*, 2008a, b, 2009; Kosina *et al.*, 2009, 2010; Obendorf and Kosina, 2011; Obendorf and Górecki, 2012) in mature seeds. Soybean galactinol synthase (GmGolS; EC 2.4.1.123) forms galactinol from *myo*-inositol and UDP-galactose and also forms fagopyritol B1 from D-*chiro*-inositol and UDP-galactose (Frydman and Neufeld, 1963; Obendorf *et al.*, 2004). Stachyose synthase (STS; EC 2.4.1.67) forms galactopinitols from D-pinitol and galactinol (Hoch *et al.*, 1999; Peterbauer and Richter, 2001). Raffinose, stachyose and sucrose serve as soybean seed reserves contributing to about 15% of the soybean seed dry mass (Hsu *et al.*, 1973). Raffinose and stachyose are one factor that has been proposed to contribute to seed desiccation tolerance and tolerance to heat or cold stresses (Koster and Leopold, 1988; Blackman *et al.*, 1992; Horbowicz and Obendorf, 1994; Obendorf, 1997; Hoekstra *et al.*, 2001; Farrant and Moore, 2011). Stachyose accumulates concomitantly with desiccation tolerance during seed maturation and drying, including precocious maturation (Blackman *et al.*, 1992; Obendorf *et al.*, 1998, 2009), but stachyose is not essential for germination (Blackman *et al.*, 1992; Dierking and Bilyeu, 2009). Galactosyl cyclitols have been proposed to function in the same role as raffinose and stachyose in seeds that normally do not accumulate raffinose and stachyose (Horbowicz and Obendorf, 1994; Horbowicz *et al.*, 1998). For this reason, the presence of raffinose and stachyose, or alternatively galactosyl cyclitols, is thought to be of central importance for seed quality, field emergence and agronomic crop yield.

The utilization of soybean as an excellent source of protein and fibre in feed applications is limited by its chemical composition. While raffinose and stachyose may be important for seed viability, these compounds are not digested by non-ruminant animals and are fermented by microflora in the lower gut, causing acidification and production of gas (Gitzelmann and Auricchio, 1965; Ruttloff *et al.*, 1967; Price *et al.*, 1988; Naczk *et al.*, 1997). Low-raffinose, low-stachyose soybean is desired as a metabolically efficient feed for chickens and pigs (Parsons *et al.*, 2000; Sebastian *et al.*, 2000) and for reduced flatulence in humans (Suarez *et al.*, 1999).

Soybean plants homozygous for the *stc1* mutant phenotype have low raffinose synthase (RFS; EC 2.4.1.82) activity (Hitz *et al.*, 2002; Dierking and Bilyeu, 2008) but normal galactinol synthase (GolS; EC 2.4.1.123) and stachyose synthase (STS; EC 2.4.1.67) activities (Hitz *et al.*, 2002) in seeds and produce seeds with low raffinose and low stachyose (Sebastian *et al.*, 2000; Hitz *et al.*, 2002; Dierking and Bilyeu, 2008). Plants homozygous for the *mips* mutant phenotype have reduced *myo*-inositol phosphate synthase (MIPS; EC 5.5.1.4) activity and produce seeds with low raffinose, low stachyose and low phytin (Sebastian *et al.*, 2000; Hitz *et al.*, 2002). Low-raffinose, low-stachyose seeds from plants homozygous for *stc1* had similar rates of field emergence as seeds with normal levels of raffinose and stachyose from normal (*Stc1*) plants (Neus *et al.*, 2005), and seeds expressing the mutant *stc1* phenotype were tolerant to imbibitional chilling (Obendorf *et al.*, 2008b). By contrast, seeds from plants homozygous for the mutant *mips* allele had reduced field emergence when compared to seed from normal (*Mips*) plants (Meis *et al.*, 2003), and seeds expressing the mutant *mips* phenotype were sensitive to imbibitional chilling (Obendorf *et al.*, 2008b).

Sucrose and *myo*-inositol concentrations affected RFO accumulation in pea (*Pisum sativum* L.) embryos (Karner *et al.*, 2004) and soybean embryos expressing the mutant *mips* phenotype (Hitz *et al.*, 2002). Similarly, feeding D-*chiro*-inositol or D-pinitol to isolated immature soybean embryos increased the accumulation of fagopyritols and galactopinitols, respectively, during embryo maturation (Obendorf *et al.*, 2004). The objective of this research was to determine if feeding *myo*-inositol, D-*chiro*-inositol or D-pinitol to isolated immature soybean embryos without maternal tissues results in increased accumulation of galactinol, fagopyritols or galactopinitols in maturing embryos expressing the mutant *stc1* phenotype or expressing the mutant *mips* phenotype in comparison to embryos from seeds expressing the *Stc1* and *Mips* phenotype with normal raffinose, stachyose and phytin.

Materials and methods

Plant material

Seeds for each of four proprietary soybean [*Glycine max* (L.) Merrill] lines with low raffinose and stachyose (LRS) seeds expressing the mutant *stc1* phenotype; low raffinose, stachyose and phytin (LRSP1, LRSP2) seeds expressing the mutant *mips* phenotype; and normal raffinose, stachyose and phytin (CHECK) seeds expressing the *Stc1* and *Mips* phenotype were provided by Steve Schnebly, Pioneer Hi-Bred. All were advanced breeding lines in related, but not isogenic, Group II maturity agronomic backgrounds developed by traditional breeding. The *stc1* and *mips* alleles in the breeding lines utilized in this study were described by Sebastian *et al.* (2000), Hitz *et al.* (2002) and Meis *et al.* (2003). Plants of each line were grown in a greenhouse

at 22°C nights (10 h) and 27°C days (14 h) with natural light supplemented 14 h with 640 μmol m^{-2} s^{-1} (photosynthetically active radiation, PAR) incandescent light from Sylvania 1000-watt metal halide lamps at Ithaca, New York, USA, 42° north latitude.

Standards and reagents

Fructose, glucose, maltose, sucrose, raffinose, stachyose, *myo*-inositol, galactinol, phenyl α-D-glucoside, trimethylsilylimidazole and pyridine were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). D-Pinitol, D-ononitol (1D-4-*O*-methyl-*myo*-inositol), and D-*chiro*-inositol were purchased from Industrial Research Limited (Lower Hutt, New Zealand). Fagopyritols, digalactosyl *myo*-inositol (DGMI) and trigalactosyl *myo*-inositol (TGMI) were extracted from buckwheat (*Fagopyrum esculentum* Moench) seeds and purified by carbon (Mallinckrodt Baker Inc., Phillipsburg, New Jersey, USA)–Celite (Supelco, Bellefonte, Pennsylvania, USA) column chromatography (Whistler and Durso, 1950) as described by Obendorf *et al.* (2000). Galactopinitols were extracted from seeds of hairy vetch (*Vicia villosa* L.) or chickpea (*Cicer arietinum* L.) and purified following the same procedures.

Embryo feeding

Embryos from each of four soybean lines were isolated from immature seeds (mid pod fill; 30–35 d after flowering) by surgical removal of seed coat and nucellus–endosperm tissues. Initial fresh weight averaged 237 mg per embryo ($N = 192$). Three isolated embryos were placed in 20-ml bottles containing 3 ml solutions for each of four feeding treatments: (1) 100 mM D-*chiro*-inositol plus 100 mM sucrose; (2) 100 mM *myo*-inositol plus 100 mM sucrose; (3) 100 mM D-pinitol plus 100 mM sucrose; or (4) 100 mM sucrose (a control solution without cyclitols) for 24 h at 25°C under light (300 μmol m^{-2} s^{-1} PAR, fluorescent) on oscillating shakers following the procedures described by Obendorf *et al.* (2004). After 24 h incubation, embryos were blotted to remove adhering solution and placed in small plastic Petri dishes. Embryos were precociously matured by slow drying for 14 d at 22°C by daily transfer to successive lower relative humidities (RH) controlled by saturated salt solutions (Blackman *et al.*, 1991, 1992): day 1, 92% RH (KNO_3); days 2 and 3, 87% RH (Na_2CO_3); day 4, 75% RH (NaCl); day 5, 51% RH [$Mg(NO_3)_2$]; day 6, 45% RH (K_2CO_3); day 7, 32% RH ($MgCl_2$); day 8, 12% RH (LiCl); and remained at 12% RH days 8–14. Precociously matured dry embryos (6% moisture) were separated into axis and cotyledons, weighed and stored at −80°C. For determination of soluble carbohydrates before incubation, immature embryos that were not placed in solutions were separated into axis and cotyledons, weighed and stored at −80°C. The experiment was replicated four times with three embryos per replication ($N = 12$) for each of four lines and four treatments.

Soluble carbohydrate extraction and analysis

Cotyledons and axes were frozen in liquid nitrogen and ground to a fine powder with a cold mortar and pestle chilled with liquid nitrogen. Soluble carbohydrates in the frozen powder of cotyledon tissues were extracted in 1800 μl ethanol:water (1:1, v/v) containing 300 μg of phenyl α-D-glucoside (internal standard) in a ground glass homogenizer. The frozen powder of axis tissues was extracted in 800 μl ethanol:water (1:1, v/v) containing 100 μg of phenyl α-D-glucoside (internal standard). Extracts were centrifuged at 14,000 × *g* for 20 min. Supernatants (500 μl) were passed through 10,000 molecular weight (MW) cutoff filters (NANOSEP 10K Omega, Pall Corp., East Hills, New York, USA) by centrifugation (14,000 × *g*). Filtrates (200 μl for cotyledons, 400 μl for axes) were dried under a stream of nitrogen gas and stored overnight above P_2O_5 in desiccators to remove traces of water. Dry residues were derivatized with 100 μl of 1-(trimethylsilyl)-imidazole (TMSI):pyridine (1:1, v/v) for 45 min at 80°C. After cooling to room temperature, soluble carbohydrates were analysed by high-resolution gas chromatography (Horbowicz and Obendorf, 1994). Results are expressed as mg per g dry weight of cotyledon or axis tissues. Values below the level of detection are presented as zero. Significant differences ($P < 0.05$) after a Tukey correction for multiple comparisons were determined between lines, feeding treatments, and line by feeding treatment interactions using JMP Statistical Discovery Software (SAS Institute Inc., Cary, North Carolina, USA). Statistical analyses were performed using a square root transformation of the response to correct for non-constant residual variance.

Gas chromatography

Trimethylsilyl-derivatives of soluble carbohydrates were analysed on a Hewlett-Packard 6890 Gas Chromatograph (Agilent Technologies, Palo Alto, California, USA) equipped with a flame ionization detector, split-mode injector (1:50) and a HP-1MS capillary column (15 m length, 0.25 mm internal diameter, 0.25 μm film thickness). Oven temperature was programmed to an initial temperature of 150°C, adjusted to 200°C at 3°C min^{-1}, adjusted to 325°C at 7°C min^{-1}, and held at 325°C for 20 min. The injector port was operated at 335°C and the detector at

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350°C. The carrier gas was nitrogen at 2.5 ml min^{-1}. Characterization of seed soluble carbohydrates by gas chromatography has recently been reviewed (Obendorf *et al.*, 2012).

Results

Cotyledons and axis prior to substrate feeding

Cotyledons of immature soybean embryos, before incubation in cyclitol solutions, contained sucrose, free cyclitols and reducing sugars; α-galactosides (galactosyl cyclitols or raffinose-family oligosaccharides) were detected in minute amounts (Table 1). Cotyledons of LRS, LRSP1 and LRSP2 initial embryos contained significantly more D-pinitol than the CHECK cotyledons (Table 1). LRSP1 cotyledons contained significantly less *myo*-inositol, galactinol and DGMI than CHECK cotyledons or LRS cotyledons, while LRSP2 embryos contained significantly less *myo*-inositol (Table 1). Cotyledons of LRSP1 initial embryos had less D-*chiro*-inositol than cotyledons of CHECK, LRS or LRSP2 (Table 1). Axes of immature soybean embryos, before incubation in cyclitol solutions, contained sucrose, free cyclitols and reducing sugars, but only minute amounts of RFOs and total α-galactosides (raffinose and galactinol) (Table 1).

Substrate feeding, cotyledons

RFOs and galactosyl cyclitols accumulated during precocious maturation after incubation in the control (sucrose without cyclitols) and cyclitol (sucrose plus cyclitol) feeding solutions. Feeding the control solution resulted in large accumulations of stachyose, total RFOs, and total α-galactosides in cotyledons of CHECK embryos, but significantly lower amounts in cotyledons of LRS, LRSP1 and LRSP2 embryos (Table 2). Cotyledons of the LRS line had 90% less RFO than the CHECK line, but accumulated significantly higher concentrations of total *myo*-inositol, galactinol, DGMI and ciceritol than CHECK cotyledons after feeding the control solution. LRS cotyledons accumulated significantly higher total *myo*-inositol, galactinol and DGMI concentrations but significantly lower raffinose and stachyose concentrations after all feeding treatments, consistent with low raffinose synthase activity (Hitz *et al.*, 2002; Dierking and Bilyeu, 2008) in LRS embryos (Table 2, Fig. 1). LRSP1 and LRSP2 cotyledons had 85% and 75% less total RFOs than CHECK cotyledons after feeding a control solution without cyclitols (Table 2). LRSP1 and LRSP2 cotyledons accumulated significantly more sucrose and D-pinitol but significantly less total *myo*-inositol, galactinol, DGMI, and galactopinitol A than CHECK cotyledons after feeding the control solution (Table 2).

As expected (Ma *et al.*, 2005), feeding D-*chiro*-inositol significantly increased the total D-*chiro*-inositol (free D-*chiro*-inositol plus that in fagopyritols) in cotyledons of CHECK, LRS, LRSP1 and LRSP2 embryos compared to feeding a control solution without cyclitols (Table 2). Feeding D-*chiro*-inositol to embryos increased fagopyritol B1 six- to eightfold in cotyledons of all lines (Fig. 2) compared to feeding a control solution, but did not significantly alter the concentration of RFOs (Table 2). Feeding D-*chiro*-inositol to LRS embryos increased fagopyritol B1 sixfold and fagopyritol B2 fivefold in cotyledons compared to feeding a control solution (Fig. 2). There were no significant line by feeding treatment interactions for total fagopyritols (Fig. 2). When the data were pooled across lines, feeding D-*chiro*-inositol to embryos resulted in significantly higher concentrations of fagopyritol B1 and total fagopyritols in cotyledons compared to feeding *myo*-inositol, D-pinitol or a control solution without cyclitols (Fig. 2). Raffinose and stachyose concentrations were significantly lower, but galactinol and DGMI concentrations were significantly higher in LRS cotyledons than in other lines after all feeding treatments (Table 2). Feeding D-*chiro*-inositol to LRSP1 and LRSP2 embryos increased free D-*chiro*-inositol and fagopyritol B1 eightfold, fagopyritol B2 sixfold, and total α-galactosides twofold in cotyledons compared to feeding a control solution (Table 2).

Feeding D-pinitol to embryos significantly increased free D-pinitol and total D-pinitol concentrations in cotyledons of all lines. Feeding D-pinitol to CHECK embryos increased free D-pinitol ninefold and galactopinitol A twofold in cotyledons compared to feeding a control solution (Table 2). Feeding D-pinitol to LRS embryos increased free D-pinitol tenfold and more than doubled galactopinitol A and galactopinitol B in cotyledons compared to feeding a control solution. Feeding D-pinitol to LRSP1 and LRSP2 embryos increased accumulation of free D-pinitol three- to fourfold and also increased total D-pinitol in cotyledons, but concentrations of galactopinitols were not increased (Table 2).

Feeding *myo*-inositol to CHECK embryos increased free *myo*-inositol threefold and free D-*chiro*-inositol 11-fold, but total *myo*-inositol and total D-*chiro*-inositol were not significantly different. Feeding *myo*-inositol caused significantly greater accumulation of galactinol in LRS cotyledons than after feeding D-*chiro*-inositol or D-pinitol (Table 2). Feeding *myo*-inositol to LRSP1 embryos increased stachyose eightfold, free *myo*-inositol 18-fold, and total RFOs fourfold in LRSP1 cotyledons compared to feeding a control solution (Table 2). Feeding *myo*-inositol to LRSP2 embryos

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Table 1. Soluble carbohydrates [mg (g dry weight)$^{-1}$] in cotyledon or axis tissues of CHECK, LRS, LRSP1 and LRSP2 soybean embryos before feeding

Soluble carbohydrate	Cotyledons				Axes			
	Before feeding				Before feeding			
	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2
Sucrose	55.53 a[†]	45.45 a	50.20 a	45.01 a	43.45 a	35.79 a	42.74 a	35.13 a
Raffinose	0.22 a	0.05 a	0.06 a	0.21 a	0.21 a	0.01 a	0.00 a	0.28 a
Stachyose	0.03 a	0.04 a	0.00 a	0.03 a	0 a	0 a	0 a	0 a
Verbascose	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
myo-Inositol	7.15 a	7.65 a	0.56 c	3.08 b	2.01 a	1.92 a	0.50 b	1.17 ab
Galactinol	2.64 b	2.74 a	0.66 c	1.59 bc	0.18 a	0.10 a	0.00 a	0.00 a
DGMI[‡]	0.11 ab	0.18 a	0.02 c	0.06 bc	0 a	0 a	0 a	0 a
TGMI[‡]	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
D-Pinitol	7.94 b	13.78 a	13.55 a	14.54 a	3.61 a	5.00 a	5.60 a	5.44 a
Galactopinitol A	0.05 a	0.04 a	0.02 a	0.08 a	0 a	0 a	0 a	0 a
Galactopinitol B	0.02 a	0.01 a	0.004 a	0.02 a	0 a	0 a	0 a	0 a
Ciceritol	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
TGPA[‡]	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
D-*chiro*-Inositol	2.11 a	2.80 a	1.79 b	2.80 a	0.46 a	0.71 a	0.58 a	0.72 a
Fagopyritol B1	0.02 a	0.01 a	0.04 a	0.03 a	0.00 a	0.00 a	0.05 a	0.00 a
Fagopyritol B2	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
Fagopyritol B3	0 a	0 a	0 a	0 a	0 a	0 a	0 a	0 a
Fructose	1.09 a	0.09 a	0.79 a	0.70 a	2.41 a	1.05 a	1.72 a	1.82 a
Glucose	0.92 a	0.42 a	0.73 a	0.70 a	3.98 a	3.19 a	3.37 a	4.11 a
Maltose	2.15 a	1.67 a	1.56 a	1.64 a	2.46 a	4.94 a	3.34 a	2.51 a
Total D-*chiro*-inos	2.12 ab	2.80 a	1.80 b	2.81 a	0.46 a	0.71 a	0.61 a	0.72 a
Total *myo*-inositol	7.21 a	7.74 a	0.57 c	3.10 b	2.10 a	1.97 a	0.50 b	1.17 ab
Total D-pinitol	8.0 b	13.8 a	13.6 a	14.6 a	3.61 a	5.00 a	5.60 a	5.44 a
Total sol carb	77.33 a	72.20 a	69.32 a	68.90 a	58.77 a	52.71 a	57.90 a	51.17 a
Total RFOs[‡]	0.25 a	0.09 a	0.06 a	0.24 a	0.21 a	0.01 a	0.00 a	0.28 a
Total α-galactoside	0.44 a	0.33 a	0.14 a	0.44 a	0.40 a	0.11 a	0.05 a	0.28 a
Ratio (suc:RFOs)	492.1 a	526.2 a	736.9 a	583.4 a	33.1 b	125.9 a	–	27.1 b
Ratio (suc:α-gal)	193.2 a	211.8 a	342.4 a	183.6 a	22.9 a	55.2 a	98.0 a	55.2 a

D-*chiro*-inos, D-*chiro*-inositol; α-gal, α-galactoside; sol carb, soluble carbohydrate; suc, sucrose.

[†] For comparisons between columns within a row for cotyledons or axes, means not connected by the same letter are significantly different ($P<0.05$; $N=12$) after a Tukey correction for multiple comparisons. Statistical analyses were performed using a square root transformation of the response.

[‡] DGMI, digalactosyl *myo*-inositol; TGMI, trigalactosyl *myo*-inositol; TGPA, trigalactosyl pinitol A; RFOs, raffinose family oligosaccharides. Total D-*chiro*-inositol, total *myo*-inositol and total D-pinitol are calculated from each free cyclitol plus their respective galactosyl cyclitols.

increased stachyose, total RFOs and total α-galactosides more than twofold.

Substrate feeding, axes

During precocious maturation after feeding a control solution (sucrose without cyclitols), CHECK axes accumulated high concentrations of stachyose and total RFOs and detectable but lower concentrations of galactopinitols and fagopyritols compared to stachyose (Table 3). Axes of the LRS line had 75% less RFO but substantially more galactinol and DGMI than axes of the CHECK line after all feeding treatments (Table 3). LRS axes accumulated significantly more di- and trigalactosides of *myo*-inositol and D-pinitol than CHECK line axis tissues after feeding a control solution without cyclitols. Axes of the LRSP1 line had 80% less RFO than the CHECK line after feeding a control solution (Table 3). Axes of the LRSP2 line had RFO values between those of LRSP1 axes and CHECK axes after feeding a control solution (Table 3). LRSP1 and LRSP2 axes had significantly less galactinol than axes of the LRS line after all feeding treatments. Galactinol concentrations were similar in axes of CHECK, LRSP1 and LRSP2.

Feeding D-*chiro*-inositol to CHECK embryos increased fagopyritol B1 sixfold in CHECK axes

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Table 2. Soluble carbohydrates in cotyledon tissues [mg (g dry weight)$^{-1}$] after precocious maturation following feeding free cyclitols to isolated embryos of CHECK, LRS, LRSP1 and LRSP2 soybean

Soluble carbohydrate	Control				D-*chiro*-Inositol				*myo*-Inositol				D-Pinitol			
	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2
Sucrose	28.6 bcd[†]	53.5 ab	72.3 a	65.0 a	25.3 cd	49.0 abc	50.8 ab	44.0 a-d	30.7 bcd	55.7 ab	51.2 abc	45.9 a-d	22.2 d	49.4 abc	68.2 a	49.1 abc
Raffinose	5.20 b	0.35 c	6.97 ab	4.83 b	7.01 ab	0.39 c	12.58 a	9.70 ab	9.38 ab	0.29 c	8.31 ab	5.95 b	5.05 b	0.26 c	5.79 ab	6.59 ab
Stachyose	79.2 a	3.2 gh	4.8 fgh	16.6 e-h	45.7 a-d	3.6 gh	15.2 e-h	21.3 c-f	48.6 ab	3.4 h	41.8 b-e	48.9 abc	47.2 abc	2.8 h	8.0 fgh	21.7 d-g
Verbascose	2.66 a	0.57 bc	0.00 c	0.95 abc	1.29 abc	0.55 bc	0.47 bc	0.92 bc	0.98 abc	0.66 bc	1.69 abc	1.98 ab	1.41 abc	0.40 bc	0.21 c	0.78 bc
myo-Inositol	0.85 bc	0.91 b	0.11 f	0.19 c-f	1.05 b	0.70 bcd	0.22 ef	0.19 c-f	2.82 a	0.64 b-e	1.95 b	0.42 b-f	0.96 b	0.75 bc	0.15 ef	0.20 c-f
Galactinol	3.63 de	9.28 ab	0.18 hi	0.09 i	1.64 fg	7.12 bc	0.66 hi	0.09 i	4.55 d	11.95 a	1.25 fgh	0.69 ghi	2.02 ef	5.40 cd	0.06 i	0.11 hi
DGMI[‡]	1.37 bc	4.68 a	0.12 de	0.09 de	0.61 cde	3.83 a	0.38 de	0.10 de	0.78 cd	5.64 a	0.43 cde	0.23 cde	0.58 cde	3.25 ab	0.08 e	0.15 de
TGMI[‡]	0.33 a-d	0.30 ab	0.00 d	0.03 cd	0.09 bcd	0.32 a	0.02 d	0.03 d	0.09 bcd	0.35 a	0.03 d	0.03 d	0.09 bcd	0.24 abc	0.00 d	0.00 d
D-Pinitol	1.17 cd	0.32 d	3.51 b	4.45 b	1.98 bc	0.39 d	1.59 cd	2.95 bc	1.98 bc	0.24 d	2.04 bc	3.01 bc	10.85 a	3.27 bc	14.22 a	14.41 a
Galactopinitol A	4.41 cde	7.57 bc	2.08 e	2.51 e	3.29 de	7.67 bc	3.67 cde	3.73 cde	2.32 e	6.72 bcd	2.52 e	3.46 cde	10.65 b	19.03 a	3.03 de	4.90 cde
Galactopinitol B	1.72 bcd	2.63 bc	1.03 cd	1.38 cd	1.50 cd	2.79 bc	2.28 bcd	2.20 bcd	0.88 d	2.35 bcd	1.50 cd	1.69 bcd	3.77 ab	5.83 a	1.59 cd	2.35 bcd
Ciceritol	1.25 cd	5.25 ab	0.18 d	0.56 d	0.68 d	5.11 ab	0.59 d	0.63 d	0.45 d	4.98 abc	0.87 d	0.57 d	1.93 bcd	9.76 a	0.27 d	1.01 d
TGPA[‡]	0.15 bc	0.55 ab	0.00 c	0.08 c	0.07 c	0.57 a	0.03 c	0.06 c	0.03 c	0.65 a	0.08 c	0.03 c	0.12 c	0.82 a	0.00 c	0.09 c
D-*chiro*-Inositol	0.16 ef	0.03 f	0.07 ef	0.17 ef	4.13 a	0.30 ef	1.97 bc	1.35 bcd	1.84 b	0.48 c-f	0.94 b-e	0.68 cde	0.59 cde	0.17 ef	0.40 def	0.42 c-f
Fagopyritol B1	3.64 c	4.10 c	4.40 c	3.66 c	26.1 ab	24.5 b	36.6 a	31.3 ab	2.36 c	3.73 c	2.11 c	2.96 c	3.30 c	3.99 c	2.95 c	3.91 c
Fagopyritol B2	0.61 b-f	1.70 bcd	0.19 ef	0.47 c-f	1.80 b	9.51 a	2.07 b	2.66 b	0.20 f	1.63 b-e	0.41 ef	1.63 c-f	0.35 def	1.80 bc	0.18 f	0.37 def
Fagopyritol B3	0.17 b-e	0.26 abc	0.00 e	0.07 cde	0.13 b-e	0.48 a	0.09 cde	0.11 b-e	0.06 cde	0.30 ab	0.03 de	0.03 de	0.09 cde	0.23 a-d	0.00 e	0.08 cde
Fructose	0.24 ab	0.32 ab	0.17 ab	0.18 ab	0.19 ab	0.22 ab	0.18 ab	0.18 ab	0.35 a	0.19 ab	0.31 ab	0.31 ab	0.20 ab	0.10 b	0.19 ab	0.19 ab
Glucose	0.22 ab	0.30 a	0.19 ab	0.22 ab	0.17 ab	0.18 ab	0.21 ab	0.19 ab	0.21 ab	0.15 ab	0.21 ab	0.18 ab	0.20 ab	0.09 b	0.16 ab	0.16 ab
Maltose	1.07 a	1.43 a	1.88 a	1.59 a	1.20 a	0.91 a	1.12 a	1.42 a	1.35 a	0.91 a	1.54 a	1.22 a	1.22 a	1.18 a	1.45 a	2.21 a
Total D-*chiro*-inos	2.23 c	2.71 c	2.33 c	2.17 c	17.8 ab	15.8 b	21.0 a	17.9 ab	3.10 c	3.00 c	2.14 c	2.27 c	2.38 c	2.82 c	1.93 c	2.52 c
Total *myo*-inositol	3.21 def	7.18 ab	0.24 h	0.27 h	2.09 fgh	5.62 bc	0.68 gh	0.28 h	5.37 bcd	8.58 a	2.73 efg	0.85 fgh	2.18 fgh	4.59 cde	0.21 h	0.31 h
Total D-pinitol	4.7 b	7.3 b	5.1 b	6.6 b	4.6 b	7.5 b	4.8 b	6.1 b	3.7 b	6.6 b	4.4 b	5.8 b	18.7 a	19.2 a	16.6 a	18.4 a
Total sol carb	136.7 a	97.2 a	98.1 a	103.1 a	124.0 a	118.1 a	130.7 a	123.2 a	109.9 a	100.9 a	119.2 a	118.4 a	112.8 a	108.8 a	107.0 a	108.7 a
Total RFOs[‡]	87.1 a	4.2 f	11.7 ef	22.4 def	54.0 abc	4.6 f	28.3 b-e	31.9 b-e	58.9 ab	4.3 f	51.8 a-d	56.8 abc	53.6 abc	3.5 f	14.0 ef	29.1 cde
Total α-galactoside	104.4 a	40.5 bc	19.9 c	31.4 c	89.9 a	66.5 ab	74.6 ab	72.8 ab	70.6 ab	42.6 bc	61.0 abc	66.8 ab	76.6 ab	53.8 abc	22.2 c	42.0 bc
Ratio (suc:RFOs)	0.4 e	16.4 ab	8.4 bcd	10.7 bcd	0.6 e	15.1 abc	2.8 de	1.7 de	0.8 e	19.0 ab	8.4 cde	1.7 de	0.5 e	23.61 a	9.4 bcd	3.2 de
Ratio (suc:α-gal)	0.3 d	1.5 a-d	4.6 ab	6.4 a	0.3 d	0.8 cd	0.8 cd	0.7 cd	0.6 cd	1.5 bcd	5.1 abc	1.2 cd	0.3 d	1.1 cd	5.2 ab	1.9 a-d

D-*chiro*-inos, D-*chiro*-inositol; α-gal, α-galactoside; sol carb, soluble carbohydrate; suc, sucrose.

[†] For comparisons between columns within a row, means not connected by the same letter are significantly different ($P < 0.05$; $N = 12$) after a Tukey correction for multiple comparisons.

[‡] DGMI, digalactosyl *myo*-inositol; TGMI, trigalactosyl *myo*-inositol; TGPA, trigalactosyl pinitol A; RFOs, raffinose family oligosaccharides. Total D-*chiro*-inositol, total *myo*-inositol and total D-pinitol are calculated from each free cyclitol plus their respective galactosyl cyclitols.

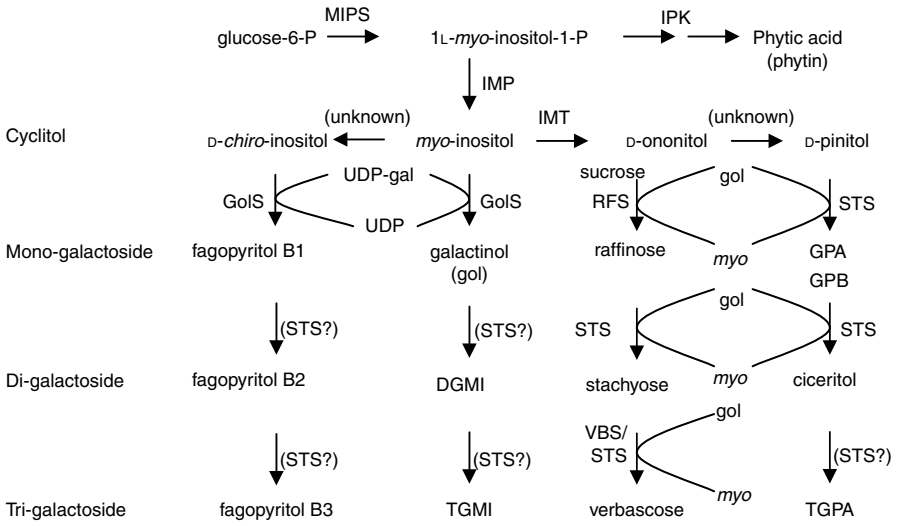

Figure 1. Proposed pathways for synthesis of cyclitols, cyclitol galactosides and raffinose family oligosaccharides (modified from Ma *et al*., 2005; Obendorf and Górecki, 2012). Parentheses (unknown) by an arrow indicate that an enzyme catalysing the reaction has not been identified. Some reactions may be reversible. Ciceritol, digalactopinitol A; DGMI, digalactosyl *myo*-inositol; gol, galactinol; GolS, galactinol synthase (EC 2.4.1.123); GPA, galactopinitol A; GPB, galactopinitol B; IMP, *myo*-inositol-phosphate monophosphatase (EC 3.1.3.25); IMT, *myo*-inositol 4-*O*-methyltransferase (EC 2.1.1.129); IPK, inositol polyphosphate kinases; MIPS, *myo*-inositol-phosphate synthase (EC 5.5.1.4); *myo*, *myo*-inositol; RFS, raffinose synthase (EC 2.4.1.82); STS, stachyose synthase (EC 2.4.1.67); (STS?), indicates STS is proposed by extrapolation but has not been demonstrated experimentally; TGMI, trigalactosyl *myo*-inositol; TGPA, trigalactopinitol A; UDP, uridine diphosphate; UDP-gal, uridine diphosphate galactoside; VBS, verbascose synthase. For chemical structures, see Obendorf (1997) and Obendorf and Górecki (2012).

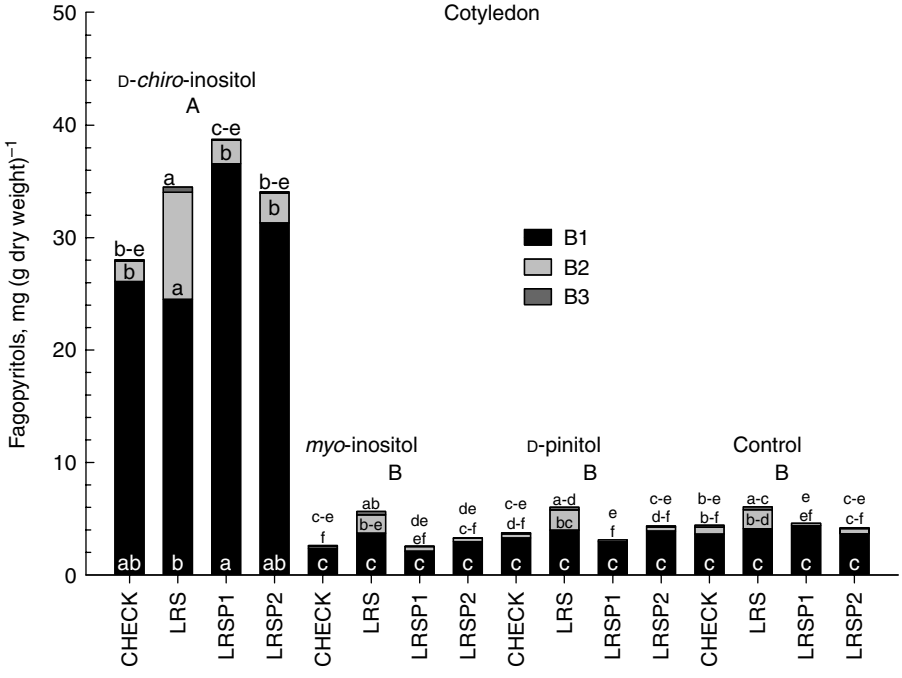

Figure 2. Fagopyritol B1, fagopyritol B2, fagopyritol B3 and total fagopyritols (fagopyritol B1 + fagopyritol B2 + fagopyritol B3) in cotyledon tissues of isolated embryos as a function of line (CHECK, LRS, LRSP1 and LRSP2) by feeding treatment (100 mM D-*chiro*-inositol plus 100 mM sucrose; 100 mM *myo*-inositol plus 100 mM sucrose; 100 mM D-pinitol plus 100 mM sucrose; or a control solution of 100 mM sucrose without cyclitols) followed by precocious maturation of embryos by slow drying. Bars not connected by the same letter are significantly different ($P < 0.05$, $N = 12$) after a Tukey correction for multiple comparisons. Use lower-case letters to compare fagopyritol B1, fagopyritol B2 or fagopyritol B3 across treatments. Use upper-case letters to compare total fagopyritols across treatments.

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Table 3. Soluble carbohydrates in axis tissues [mg (g dry weight)$^{-1}$] after precocious maturation following feeding free cyclitols to isolated embryos of CHECK, LRS, LRSP1 and LRSP2 soybean

Soluble carbohydrate	Control				D-*chiro*-Inositol				*myo*-Inositol				D-Pinitol			
	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2	CHECK	LRS	LRSP1	LRSP2
Sucrose	38.8 a†	65.6 a	45.7 a	54.8 a	30.2 a	55.0 a	54.8 a	43.8 a	56.8 a	65.6 a	44.4 a	32.4 a	49.0 a	51.0 a	57.4 a	44.6 a
Raffinose	4.7 a-e	2.2 b-e	4.1 a-e	6.3 a-d	4.3 a-e	2.0 e	8.0 ab	7.4 a-d	9.9 a	2.6 cde	6.2 a-e	4.1 a-e	8.2 ab	1.8 de	8.1 ab	6.8 abc
Stachyose	95.8 abc	19.2 de	17.5 e	52.4 a-e	74.3 a-d	11.6 e	30.6 cde	58.1 a-e	107.1 ab	10.7 e	49.0 a-e	57.4 a-e	113.2 a	10.8 e	24.0 de	43.8 b-e
Verbascose	8.1 a	4.4 a-d	0.45 d	3.9 a-d	5.8 a-d	2.0 a-d	1.4 cd	4.3 a-d	6.4 abc	2.0 a-d	2.4 a-d	3.1 a-d	8.3 ab	1.5 a-d	0.67 bcd	2.0 bcd
myo-Inositol	0.6 abc	1.1 ab	0.3 bc	0.4 bc	0.6 abc	0.9 ab	0.3 bc	0.3 bc	1.0 ab	1.8 a	0.8 ab	0.3 bc	0.7 abc	0.9 ab	0.1 c	0.3 bc
Galactinol	2.0 def	8.7 ab	0.5 fg	0.9 efg	1.7 ef	5.8 bc	0.6 fg	0.8 efg	3.4 cde	10.9 a	1.6 ef	0.5 fg	1.4 efg	5.7 bcd	0.2 g	0.6 efg
DGMI‡	0.6 bc	5.4 a	0.3 c	0.2 c	0.6 c	3.4 ab	0.6 c	0.2 c	0.9 bc	5.2 a	0.5 c	0.5 c	0.8 bc	4.0 a	0.2 c	0.1 c
TGMI‡	0.0 b	1.5 a	0.0 b	0.0 b	0.9 ab	1.1 a	0.0 b	0.0 b	0.0 b	1.1 a	0.0 b	0.0 b	0.0 b	0.6 ab	0.0 b	0.0 b
D-Pinitol	1.2 c	3.6 bc	2.0 c	4.4 bc	1.7 c	2.0 c	1.2 c	2.5 c	2.0 c	2.1 c	1.3 c	1.3 c	7.3 ab	4.3 bc	8.4 a	7.0 ab
Galactopinitol A	4.4 c-f	10.1 abc	2.9 f	5.6 c-f	4.8 c-f	7.1 b-e	4.4 def	6.7 c-f	5.8 c-f	7.8 bcd	3.6 def	3.8 def	13.1 ab	16.7 a	4.2 def	5.9 c-f
Galactopinitol B	1.5 c	3.7 abc	1.2 c	2.8 bc	2.0 c	2.8 bc	2.1 c	3.5 abc	1.8 c	2.9 bc	1.8 c	1.6 c	5.7 ab	6.6 a	2.2 c	2.6 bc
Ciceritol	0.8 cde	6.2 ab	0.3 e	1.2 cde	0.9 de	3.6 bcd	0.6 de	1.4 cde	0.7 de	4.5 abc	0.5 e	0.5 de	2.64 b-e	10.45 a	0.45 e	0.78 de
TGPA‡	0.0 d	2.2 ab	0.0 d	0.1 cd	0.0 d	1.1 a-d	0.0 d	0.0 d	0.4 bcd	1.2 abc	0.0 d	0.0 d	0.93 a-d	1.96 a	0.00 d	0.21 cd
D-*chiro*-Inositol	0.02 bc	0.51 abc	0.03 bc	0.03 c	0.74 ab	2.39 a	0.49 abc	0.56 abc	0.26 abc	0.69 abc	0.06 bc	0.02 c	0.16 bc	0.30 abc	0.14 bc	0.06 bc
Fagopyritol B1	2.93 b	4.65 b	2.31 b	3.74 b	17.8 a	19.6 a	20.8 a	22.6 a	9.3 b	4.2 b	2.1 b	2.1 b	3.8 b	4.0 b	2.6 b	2.9 b
Fagopyritol B2	0.4 b-f	2.1 a-e	0.2 f	0.7 c-f	2.5 abc	6.3 a	2.0 bcd	3.2 ab	0.3 def	1.6 b-f	0.4 def	0.5 c-f	0.5 c-f	2.1 b-e	0.4 ef	0.4 f
Fagopyritol B3	0.4 ab	2.4 a	0.6 ab	0.3 ab	1.4 ab	1.2 ab	0.8 ab	1.2 ab	0.7 ab	1.3 ab	0.4 ab	0.5 ab	0.5 ab	0.6 ab	0.2 b	0.5 ab
Fructose	0.01 b	1.84 ab	0.08 b	0.15 b	0.09 b	1.56 ab	0.25 b	0.08 b	0.16 b	2.51 a	0.18 b	0.14 b	0.27 b	0.39 ab	0.03 b	0.02 b
Glucose	1.75 ab	2.76 a	1.56 ab	1.72 ab	1.63 ab	2.51 ab	1.22 ab	1.57 ab	1.57 ab	1.94 ab	1.57 ab	1.38 ab	0.87 b	1.31 ab	1.17 ab	1.70 ab
Maltose	1.63 a	3.15 a	2.85 a	2.07 a	1.25 a	2.27 a	2.45 a	1.78 a	1.25 a	1.65 a	2.14 a	1.35 a	1.30 a	2.42 a	3.10 a	1.80 a
Total D-*chiro*-inos	1.7 d	4.1 cd	1.4 d	2.2 d	10.8 abc	14.6 a	11.8 ab	13.2 a	5.2 bcd	3.7 d	1.3 d	1.4 d	2.3 d	3.1 d	1.6 d	1.7 d
Total *myo*-Inositol	1.77 d	7.63 ab	0.62 d	0.91 d	1.85 d	5.23 bc	0.75 d	0.82 d	2.98 cd	9.24 a	1.76 d	0.70 d	1.67 d	5.25 bc	0.29 d	0.62 d
Total D-pinitol	4.4 def	13.1 abc	4.2 f	9.0 c-f	5.4 def	8.4 c-f	4.7 ef	8.0 c-f	6.1 c-f	9.3 c-f	4.2 f	4.2 f	17.8 ab	19.9 a	11.8 b-d	11.5 b-e
Total sol carb	165.7 ab	151.2 ab	82.8 b	141.8 ab	153.2 ab	134.0 ab	132.5 ab	159.9 ab	209.7 ab	132.3 ab	118.8 ab	111.7 ab	218.5 a	127.3 ab	113.5 ab	122.0 ab
Total RFOs‡	108.6 ab	25.8 cd	22.1 d	62.6 a-d	84.4 abc	15.5 d	40.0 bcd	69.8 a-d	123.4 ab	15.3 d	57.5 a-d	64.6 a-d	129.6 a	14.1 d	32.7 cd	52.6 bcd
Total α-galactoside	121.8 abc	72.5 a-d	30.3 d	78.2 a-d	117.0 ab	67.3 a-d	71.9 a-d	109.3 a-d	146.7 ab	56.1 bcd	68.3 a-d	74.8 a-d	159.9 a	66.8 a-d	43.0 cd	66.6 bcd
Ratio (suc:RFOs)	0.4 d	5.0 abc	3.1 a-d	2.2 bcd	0.5 d	4.3 ab	1.8 bcd	1.5 bcd	0.8 cd	6.7 a	2.0 a-d	1.1 cd	0.6 d	4.7 ab	2.7 a-d	2.1 bcd
Ratio (suc:α-gal)	0.4 b	1.2 ab	2.1 a	1.5 ab	0.3 b	1.0 ab	0.8 ab	0.6 ab	0.5 ab	1.4 ab	1.3 ab	0.8 ab	0.4 b	0.9 ab	1.9 a	1.2 ab

D-*chiro*-inos, D-*chiro*-inositol; α-gal, α-galactoside; sol carb, soluble carbohydrate; suc, sucrose.

† For comparisons between columns within a row, means not connected by the same letter are significantly different ($P < 0.05$; $N = 12$) after a Tukey correction for multiple comparisons.

‡ DGMI, digalactosyl *myo*-inositol; TGMI, trigalactosyl *myo*-inositol; TGPA, trigalactosyl pinitol A; RFOs, raffinose family oligosaccharides. Total D-*chiro*-inositol, total *myo*-inositol and total D-pinitol are calculated from each free cyclitol plus their respective galactosyl cyclitols.

compared to feeding a control solution without D-*chiro*-inositol (Table 3, Fig. 3). Feeding D-*chiro*-inositol to embryos increased fagopyritol B1 in axes of all lines compared to the other feeding treatments (Fig. 3). Feeding D-*chiro*-inositol to LRS embryos increased fagopyritol B1 fourfold in axes of precociously matured embryos compared to feeding a control solution. Feeding D-*chiro*-inositol to LRSP1 embryos increased fagopyritol B1 and fagopyritol B2 nine- to tenfold compared to feeding a control solution (Table 3). Feeding D-*chiro*-inositol to LRSP2 embryos increased fagopyritol B1 sixfold and fagopyritol B2 fivefold in precociously matured axes of LRSP2 compared to feeding a control solution (Table 3).

Feeding D-pinitol to CHECK embryos increased free D-pinitol sixfold, galactopinitol A threefold and galactopinitol B threefold in CHECK axes compared to feeding a control solution without D-pinitol (Table 3). RFOs and galactinol series oligosaccharides were not significantly increased in axis tissues after feeding the free cyclitols D-pinitol or *myo*-inositol (Table 3). Feeding D-pinitol had no significant effect on the accumulation of soluble carbohydrates in LRSP2 axes compared to feeding a control solution, and feeding D-pinitol to LRSP1 embryos significantly increased only free D-pinitol in LRSP1 axes compared to feeding a control solution (Table 3).

Feeding *myo*-inositol to CHECK, LRS, LRSP1 and LRSP2 embryos resulted in no significant changes in soluble carbohydrates in precociously matured axes compared to feeding a control solution (Table 3).

Discussion

LRS soybean embryos expressing the mutant *stc1* phenotype have low raffinose synthase (RFS) activity (Hitz *et al.*, 2002; Dierking and Bilyeu, 2008), but normal *myo*-inositol phosphate synthase (MIPS) activity and normal stachyose synthase activity (Hitz *et al.*, 2002), resulting in reduced raffinose and stachyose accumulation but normal to elevated concentrations of galactopinitols and fagopyritols during precocious maturation (Sebastian *et al.*, 2000; Hitz *et al.*, 2002; Dierking and Bilyeu, 2008; Obendorf *et al.*, 2009) (Fig. 1) compared to CHECK embryos after feeding a control solution without cyclitols. LRS embryos also have higher galactinol and digalactosyl *myo*-inositol (DGMI), probably due to the decreased accumulation of RFOs resulting in higher accumulations of galactinol (Fig. 1). Low raffinose synthase activity in LRS embryos results in less accumulation of raffinose and stachyose (Fig. 1) which, in turn, results in an increase in galactinol and a higher concentration of DGMI (Fig. 1). LRSP1 and LRSP2 soybean embryos expressing the mutant *mips* phenotype have low *myo*-inositol phosphate synthase (MIPS) activity (Hitz *et al.*, 2002) but normal raffinose synthase and

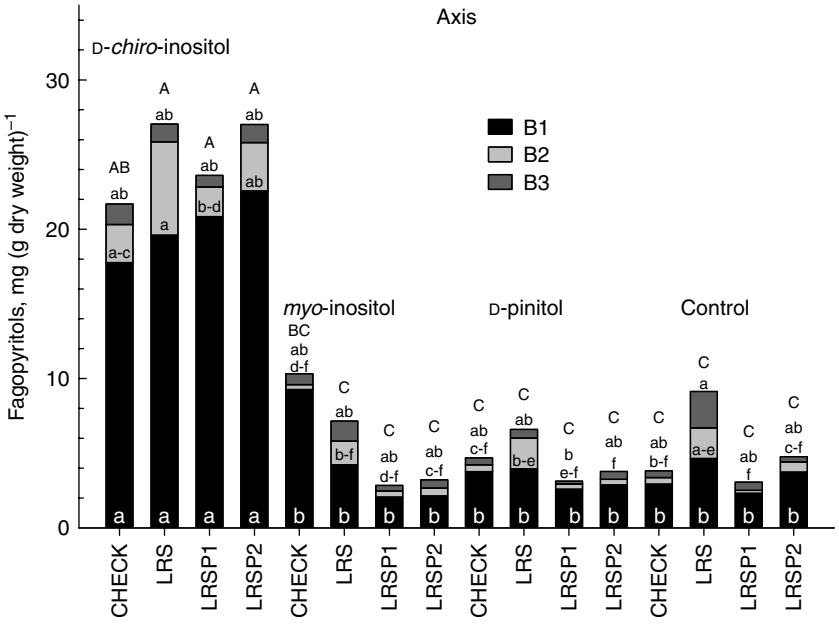

Figure 3. Fagopyritol B1, fagopyritol B2, fagopyritol B3 and total fagopyritols (fagopyritol B1 + fagopyritol B2 + fagopyritol B3) in axis tissues of isolated embryos as a function of line (CHECK, LRS, LRSP1 and LRSP2) by feeding treatment (100 mM D-*chiro*-inositol plus 100 mM sucrose; 100 mM *myo*-inositol plus 100 mM sucrose; 100 mM D-pinitol plus 100 mM sucrose; or a control solution of 100 mM sucrose without cyclitols) followed by precocious maturation of embryos by slow drying. Bars not connected by the same letter are significantly different ($P < 0.05$, $N = 12$) after a Tukey correction for multiple comparisons. Use lower-case letters to compare fagopyritol B1, fagopyritol B2 or fagopyritol B3 across treatments. Use upper-case letters to compare total fagopyritols across treatments.

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stachyose synthase activities (Hitz *et al.*, 2002), resulting in reduced galactinol, raffinose, stachyose, total RFOs and phytin (Sebastian *et al.*, 2000; Hitz *et al.*, 2002) (Fig. 1). CHECK soybean embryos expressing the normal *Stc1* and normal *Mips* phenotype have normal enzyme activities and accumulate normal concentrations of raffinose, stachyose, total RFOs and phytin, and smaller but normal concentrations of galactopinitols and fagopyritols (Fig. 1).

Sucrose, *myo*-inositol, D-*chiro*-inositol and D-pinitol are synthesized in soybean leaf (maternal) tissues, transported to the seed, unloaded by the seed coat to the embryo, and may be stored as sucrose, RFOs, fagopyritols and galactopinitols in mature seeds (Gomes *et al.*, 2005; Obendorf *et al.*, 2008a, b, 2009; Kosina *et al.*, 2009, 2010). About 70% of RFOs accumulate in soybean seeds after physiological maturity (maximum dry mass) during the period of seed drying (Obendorf *et al.*, 2009). The development of desiccation tolerance occurs concomitantly with the accumulation of stachyose (Blackman *et al.*, 1992; Buitink *et al.*, 2003; Rosnoblet *et al.*, 2007; Obendorf *et al.*, 2009). Galactosyl cyclitols, such as fagopyritols, have been proposed to function in the same role as raffinose and stachyose in seeds that normally do not accumulate raffinose and stachyose (Horbowicz and Obendorf, 1994; Horbowicz *et al.*, 1998). Kosina *et al.* (2010) proposed that upregulation of maternally synthesized D-*chiro*-inositol may be effective for increasing unloaded D-*chiro*-inositol to embryos and increasing the accumulation of fagopyritols during seed maturation of soybean lines with reduced raffinose and stachyose or with reduced raffinose, stachyose and phytin. Soybean plants normally unload sucrose, *myo*-inositol, D-*chiro*-inositol and D-pinitol from seed coats to the embryos during seed development (Gomes *et al.*, 2005; Kosina *et al.*, 2009). Feeding D-*chiro*-inositol to stem–leaf–pod explants of CHECK, LRS, LRSP1 and LRSP2 soybean increased the unloading of D-*chiro*-inositol from seed coats (Kosina *et al.*, 2010) and increased the accumulation of fagopyritol B1 in mature, dry seeds of all four lines in the presence of maternal tissues (Obendorf *et al.*, 2008a). The results of the present study demonstrate that feeding D-*chiro*-inositol to CHECK, LRS, LRSP1 and LRSP2 isolated embryos (free of maternal tissues) significantly increased the accumulation of fagopyritol B1 in both cotyledon and axis tissues during precocious maturation of embryos of all four lines. These results support the proposal (Kosina *et al.*, 2010) that increasing the supply of maternal D-*chiro*-inositol to soybean embryos may increase the accumulation of fagopyritol B1 in mature seeds expressing the mutant *stc*1 phenotype with low raffinose and stachyose, expressing the mutant *mips* phenotype with low raffinose, stachyose and phytin, or expressing the normal *Stc1* and *Mips* phenotype with normal raffinose, stachyose and phytin.

Feeding D-pinitol to isolated embryos increased free D-pinitol in cotyledons of all four lines and increased galactopinitol A in cotyledons of CHECK and LRS embryos, but galactopinitols were not increased in LRSP1 and LRSP2 embryos, probably due to low availability of galactinol in these embryos (Fig. 1). Feeding *myo*-inositol (with sucrose) to LRSP1 and LRSP2 embryos, expressing a mutant *mips* gene, resulted in increased stachyose accumulation, most likely by increasing the supply of galactinol for use as the galactosyl donor to form stachyose (Fig. 1), in cotyledons, as previously reported (Hitz *et al.*, 2002; Karner *et al.*, 2004).

The *myo*-inositol feeding experiments are complicated and difficult to interpret. *myo*-Inositol is synthesized in soybean leaves (and other maternal tissues) and transported to seeds (Gomes *et al.*, 2005; Kosina *et al.*, 2009, 2010). Since the *Mips* gene is also expressed in embryos of soybean seeds (Hitz *et al.*, 2002; Chappell *et al.*, 2006), it is likely that *myo*-inositol is also synthesized in embryos. Additionally, *myo*-inositol is used to form many products (Loewus and Murthy, 2000; Raboy, 2009) including membranes (phosphatidyl inositol phosphates), cell walls (pectin), phytic acid (phytin), galactinol, RFOs, cyclitols (including D-*chiro*-inositol, D-ononitol and D-pinitol) and galactosyl cyclitols (Fig. 1). We did not analyse all possible products of *myo*-inositol in cotyledons and axis tissues, including membranes, cell walls and phytic acid; this work focused on the low molecular weight soluble carbohydrates present in maturing soybean seeds. Therefore, it is not surprising that the concentrations of free *myo*-inositol or total *myo*-inositol measured after feeding *myo*-inositol and sucrose to soybean embryos were not as markedly increased as noted for D-*chiro*-inositol or D-pinitol after feeding these cyclitols. However, LRSP1 and LRSP2 embryos expressing a mutant *mips* gene accumulated significantly higher concentrations of stachyose and total RFOs in cotyledons (Table 2) after *myo*-inositol feeding, as expected (Fig. 1); this result has also been reported by others (Hitz *et al.*, 2002; Karner *et al.*, 2004).

Fagopyritol B1 is formed by galactinol synthase (GolS) using D-*chiro*-inositol as the galactosyl acceptor and UDP-galactose as the galactosyl donor (Fig. 1; Obendorf *et al.*, 2004). Enhanced accumulation of fagopyritol B1 in maturing embryos after feeding D-*chiro*-inositol has been demonstrated widely in different species (Obendorf and Górecki, 2012) and in soybean lines expressing different mutant genes (Obendorf and Kosina, 2011). Embryos of smooth tare [*Vicia tetrasperma* (L.) Schreb.] (Lahuta *et al.*, 2005) and garden pea (*Pisum sativum* L.) (Lahuta and Dzik, 2011), which do not normally have D-*chiro*-inositol or fagopyritols, can take up D-*chiro*-inositol into

immature embryos and form fagopyritol B1 during precocious maturation. In soybean, we have demonstrated an enhanced accumulation of fagopyritol B1 after feeding D-*chiro*-inositol to immature embryos expressing the mutant *stc1* gene and the mutant *mips* gene (Tables 2 and 3). Because excessively high concentrations of D-*chiro*-inositol may result in shrivelled seeds (Gomes *et al.*, 2005), increasing the conversion of *myo*-inositol to D-*chiro*-inositol in maternal tissues, followed by transport to and unloading by seed coats, may be a preferred option for increasing fagopyritol B1 in mature seeds toward the improvement of agronomic performance of soybean lines with low raffinose, stachyose and phytin in seeds.

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