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Relationship between Galactinol Synthase Activity and Sugar Composition of Leaves and Seeds of Several Crop Species

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Abstract. **Galactinol synthase was assayed from leaves of 24 different accessions (20 species), maturing seeds of soybean, and cotyledons of germinating cucumber seeds. Leaf tissue contained concentrations of raffinose ranging from not detectable to 0.36 mg/g fresh weight (gfw) and stachyose ranging from not detectable to 1.39 mg/gfw. Galactinol synthase activity from leaves was correlated with the proportion of total sugar present as raffinose saccharides. In maturing soybean seeds, the appearance of galactinol synthase coincided with the biosynthesis of the galactosyl-sugars. Cucumber seeds contained high levels of raffinose and stachyose which decreased in the cotyledon during germination to a steady-state level coincident with the appearance of galactinol synthase.**

The raffinose family of oligosaccharides occurs in various plant families, many of them of horticultural importance such as *Fabaceae*, *Cucurbitaceae*, and *Brassicaceae* (7, 17, 25, 35). In legumes, these sugars often accumulate in seeds during maturation, but are not found in detectable amounts in other parts of the plant (14). Levels of 4–5% stachyose and 1–2% raffinose have been reported in soybean seeds (4, 5), where stachyose levels can often equal those of sucrose (5–7%) (2, 5). These sugars are considered major transport sugars in cucurbits (12), and they also occur in many tree species (36).

Biosynthesis of raffinose saccharides occurs by sequential transfer of galactosyl units to sucrose mediated by specific transferase enzymes (18). This is believed to occur through the intermediate galactinol, a compound composed of galactose and myo-inositol in an alpha linkage (O-α-D-galactopyranosyl-myo-inositol). Galactinol was first isolated from sugar beet by Brown and Serro (3) and later shown to be associated with the biosynthesis of raffinose saccharides by Senser and Kandler (24). Through galactinol, galactose is transferred to sucrose in the formation of raffinose and to raffinose in the formation of stachyose (19, 31) (Fig. 1).

Galactinol is synthesized by the enzyme galactinol synthase (UDP-D-galactose:inositol galactosyltransferase) which catalyzes the following reaction: UDP-galactose + myo-inositol → galactinol + UDP. It was first isolated from maturing pea seeds (8) and later isolated from *Cucurbita* leaves (34). This enzyme has also been isolated and characterized from leaves of *Cucumis sativus* (11, 22) where it has been shown to be subject to UDP

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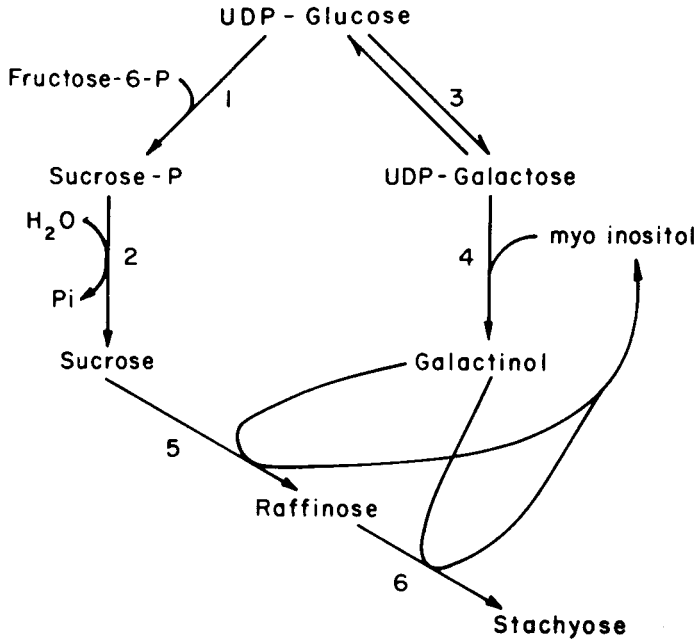

Fig. 1. Pathway of raffinose saccharide and sucrose formation in plants from UDP-glucose (9, 17, 18) Enzyme: 1, sucrose-6-phosphate synthase; 2, sucrose-6-phosphate phosphatase; 3, UDPglucose-4-epimerase; 4, galactinol synthase; 5, galactinol : sucrose 6-galactosyl-transferase; 6, galactinol : raffinose 6-galactosyl-transferase.

inhibition and affected by both Mn^{++} concentration and sulfhydryl reagents in *in vitro* experiments.

Galactinol synthase is the first enzyme to commit carbon to the formation of raffinose and stachyose (Fig. 1). UDP galactose is not unique to this pathway since it is a substrate in other biosynthetic reactions in the plant cell such as cell wall and galactolipid formation. The only known function of galactinol is in the formation of the raffinose saccharides. Galactinol synthase, therefore, could play a key role in the regulation of the synthesis of these sugars.

Accordingly, this study was conducted to investigate the relationship between galactinol synthase activity and raffinose saccharide content in leaves. This involved surveying leaves of a number of known raffinose-stachyose- and sucrose-transporting species for enzyme activity and carbohydrate content. Studies of seed maturation in soybean and of seed germination in cucumber were also conducted to determine if this enzyme is correlated with the appearance of the raffinose-type sugars.

Materials and Methods

Plant materials and sampling procedures. Twenty-four different accessions representing 20 species (Table 1) were grown during the summer of 1981 in Raleigh, N.C. Nineteen accessions were grown under field conditions; 4 (*Stachys, Coleus, Ipomoea*, and *Nicotiana*) were grown in greenhouses. Recently fully expanded leaves were collected from each accession during the course of the season to be used for enzyme assays and carbohydrate determinations. One sample, consisting of 5 g of leaves collected from 3 or more plants, was taken from each accession. Leaves were kept in ice (< 1 hr) after harvesting before being processed. Major veins were removed before analysis.

'Ransom' soybean plants were grown in a greenhouse in 17.5-cm pots for seed development studies. Seeds were planted on June 18, 1981, and plants began flowering August 10, 1981, under natural photoperiods. Thirty-eight days after this date, developing pods were collected at 4-day intervals and their seeds were removed to be used as above. The most mature pods, estimated visually, were selected at each sampling. Triplicate samples were taken for both the enzyme assay and sugar samples. 'Calypso' cucumber seeds were sown in a mixture of 1 peat : 1 vermiculite in 17 × 21.5 cm flats and placed in a growth chamber at 26°C constant temperature, with a 16-hr photoperiod (157 $\mu E\ s^{-1}\ m^{-2}$ intensity) provided by fluorescent tubes and incandescent bulbs. Germinating seeds and seedlings were collected at 12-hr intervals. Unshed seedcoats were carefully removed, and only the 2 cotyledons of each seedling were used. The radicle or root and embryo axis were removed. Duplicate samples were taken for enzyme assay and sugar samples.

Galactinol synthase extraction and assay. Samples of leaves or developing soybean seeds (5g) were homogenized at 4°C in 25 ml 50 mM Na-phosphate buffer, pH 7.0, containing 0.5 mM DTT (Dl-dithiothreitol) and 0.2 M Na-ascorbate. The homogenate was decanted through 4 layers of cheesecloth into centrifuge tubes and spun at 27,000× *g* for 15 min. The supernatant was filtered through 1 layer of Miracloth and kept at 4°C until assayed.

Cucumber cotyledons (1 g) were homogenized in the same buffer without the Na-ascorbate. The homogenate was then processed as above.

Galactinol synthase activity was determined in the supernatant using an assay described previously (11, 22). The complete reaction mixture contained supernatant (10–30 µl), UDP-[^{14}C]-galactose (0.05 µmol, 1.00 µCi/µmol), myo-inositol (2 µmol), $MnCl_2$ (0.1 µmol), DTT (0.05 µmol) and Hepes-NaOH buffer at pH 7.0 (2.5 µmol) in a total volume of 100 µl. Reactions were initiated with enzyme in 1.5-ml Eppendorf Microfuge tubes and incubated for 10 min at 30°C. The reaction was terminated by the addition of 400 µl of 100% ethanol to each tube. Unreacted UDP-[^{14}C]-galactose was removed from the terminated reaction mixture by adding 200 µl of a 1:1 (w/v) slurry of water : Dowex-1 anion exchange resin to the tube and incubating for 20 min. The tubes were centrifuged for 2 min in an Eppendorf Microfuge. A 300-µl aliquot of the supernatant was pipetted into 15 ml of a scintillant composed of 2 parts of toluene; 1 part Triton X-100 containing 6.0 g/liter PPO and 0.3 g/liter POPOP. Radioactivity was measured using a Packard Tri-Carb scintillation spectrophotometer, model 300 CD. Minus-inositol counts (from tubes containing no inositol) were subtracted from plus-inositol counts so that enzyme activity was determined from inositol dependent counts and expressed as nmols of galactinol formed per gfw per minute. The limit of detection of this assay is 100 dpm above background or 0.5 nmoles galactinol ml enzyme^{-1} min^{-1}.

Carbohydrate determination—species study. Leaf material (5g) were finely chopped and boiled in 50 ml of 80% ethanol for 10 min. This was cooled and stored at −20°C until further processed. The tissues in ethanol were then homogenized for 3 min. Half of the homogenate was removed and centrifuged at 27,000× *g* for 15 min. The supernatant was evaporated to dryness by shaking under reduced pressure at 40°C on a Buchler vortex-evaporator (Searle Analytic Inc.) and stored in desiccator jars overnight. The dry pellet was washed twice with 5 ml of anhydrous ether and then dissolved in 1 ml of water. This was applied to a combination anion-cation exchange column consisting of 5 ml each of a 1 : 1 slurry in water of Dowex 50 and Dowex 1 exchange resins. The column was then washed with 35–50 ml water. The eluant was lyophilized and redissolved in

0.5 ml 80% ethanol. Carbohydrate content was estimated using anthrone assays (13) with glucose as the standard.

A volume of each extract containing 200 μg of total carbohydrate was spotted on Whatman No. 1 chromatography paper for separation of neutral sugars. The papers were run for 40 hr in a solvent of n-butanol : benzene : pyridine : water (5 : 1 : 3 : 3), and the sugar spots were developed using a procedure employed by Pharr et al. (23). Identification of the neutral sugars in the leaf extracts was based on coloration and co-chromatography with authentic sugar standards. About 10 μg of each sugar (or 5% of total carbohydrate) could be visualized in this system.

Following paper chromatography, the samples were relyophilized and redissolved in water. Stachyose, raffinose, and sucrose were analyzed by high performance liquid chromatography (HPLC). The HPLC system consisted of a Waters 6000A pump and model 401 refractive index (RI) detector, a Rheodyne 7125 loop injector and a Spectra-Physics 4100 computing integrator. The oligosaccharides were separated by reverse phase using a Waters 8 × 100 mm $10_{\mu m}$, C_{18} Radial-Pak column. The elution solvent was 0.06 M phosphoric acid with the pH adjusted to 2.5 with concentrated NH_4OH. A 10-μl aqueous sample was injected. Concentrations were estimated based upon comparison to the peak heights of standard compounds. Concentrations of ≥ 10 μg/gfw of sucrose, raffinose, or stachyose could be detected by this system, based on the amounts of plant material used and the limit of resolution of the RI detector. Quadruplicate injections of a given sample generally agreed within 12 %.

Carbohydrate determinations—soybean study. Triplicate samples, each consisting of 5 g of seeds, were finely chopped and boiled in 50 ml of 80% ethanol for 10 min. These were stored at −20°C until the completion of all harvests. All samples were then homogenized for 2 min and brought up to 100 ml with 80% ethanol. Five ml were removed, centrifuged at 27,000 × *g* for 15 min and the supernatant was processed as above, omitting the 2 ether washes. The lyophilized samples were redissolved in 0.5 ml water and sucrose, raffinose, and stachyose concentrations estimated using HPLC analysis as described above with 20-μl injections.

Carbohydrate determinations—cucumber study. Cotyledons (3g) were taken for each duplicate sample, boiled in 30 ml 80% ethanol for 10 min, and stored at −20°C until homogenization. Samples were homogenized for 3 min and centrifuged at 27,000 × *g* for 15 min. The supernatant was evaporated using a Buchler vortex-evaporator at 40°C, redissolved in 6 ml water, and analyzed for total carbohydrate (13). Subsequently, aqueous samples were lyophilized to remove ethanol and redissolved in water, and the levels of the 3 oligosaccharides were estimated using HPLC analysis as described above with 20-μl injections.

Results

Analysis of sugars by HPLC from leaves of the different accessions showed a wide range of levels of raffionose saccharides from not detectable to 0.94 mg/gfw for raffinose and not detectable to 1.39 mg/gfw for stachyose (Table 1). When both raffinose and stachyose were present, stachyose was the predominant form. Sucrose levels also varied greatly among the species by a factor of more than 20-fold.

Paper chromatography showed the presence of sucrose, glucose, and fructose in all species (data not shown). Raffinose and stachyose were detected by paper chromatography in all species in Table 1 except the last 7. HPLC, being a more sensitive assay, detected traces of raffinose not found by paper chromatography in all except the last 2 accessions. Plants of *Cucurbitaceae* and *Lamiaceae* previously have been reported to contain raffinose sugars (25) which was confirmed by our data. Three species (*Vigna, Capsicum*, and *Phaseolus*), which have not been reported to contain raffinose saccharides in their leaves, showed traces of raffinose and stachyose by paper chromatography as well as HPLC.

Table 1. Concentration of the oligosaccharides sucrose, raffinose, and stachyose determined by HPLC in fully expanded leaves of 24 plant accessions.

Accessions	Concn (mg/g fresh wt)		
	Sucrose	Raffinose	Stachyose
Cucurbita pepo var. *ovifera*	1.17	0.36	1.39
Stachys byzantina	3.67	0.94	1.32
Cucurbita pepo var. *melopepo*	1.00	0.24	1.03
Citrullus lanatus	1.99	0.33	0.92
Lagenaria siceraria	1.04	0.21	0.92
Cucumis anguria	0.44	0.14	0.49
Cucumis sativus var. *hardwickii*	0.52	0.17	0.49
Cucumis myriocarpus	0.26	0.08	0.48
Coleus blumei	1.13	0.14	0.44
Cucumis sativus 'Little Leaf'	0.20	0.08	0.42
Cucumis melo	0.33	0.10	0.41
Cucumis sativus 'Calypso'	1.03	0.12	0.41
Luffa cylindrica	0.66	0.09	0.39
Lamium maculatum	0.69	0.10	0.26
Vigna unguiculata	4.18	0.20	ND
Capsicum annuum var. *annuum*	1.38	0.08	ND
Phaseolus vulgaris	0.93	0.06	ND
Zea mays	3.21	0.04	ND
Ipomoea setosa	1.81	0.04	ND
Triticum aestivum	0.68	0.03	ND
Lycopersicon esculentum 'Floramerica'	0.78	0.02	ND
Lycopersicon esculentum 'Manapal'	0.48	0.02	ND
Nicotiana tabacum	3.53	ND[z]	ND
Glycine max	1.37	ND	ND

[z]ND = not detected.

Plants are ranked in Table 2 according to galactinol synthase activity. Percentages of the total soluble carbohydrate pool present as sucrose and as raffinose saccharides are given. Coefficients were calculated for correlations between galactinol synthase activity values and the percentage values of sucrose and of total raffinose saccharides. Where sugars were not detected (ND), a value of zero was assigned for these calculations. There was a significant correlation ($r = +0.84$) between galactinol synthase activity (X) and the proportion of total sugar present as raffinose saccharide (Y) ($Y = 0.016X + 10.43$) and a significant negative correlation ($r = -0.73$) between activity (X) and proportion as sucrose (Y); $Y = -0.012X + 50.43$.

Correlations between galactinol synthase activity and absolute concentration of sucrose or raffinose saccharides (Table 1) ($r = +0.67$ for galactinol synthase vs. raffinose + stachyose; $r = -0.62$ for galactinol synthase vs. sucrose) were lower than between galactinol synthase activity and the concentration of these sugars as percentage of soluble carbohydrate ($r = +0.84$ or -0.73) (Table 2). In some cases (Table 2), leaves are shown to contain low levels of galactinol synthase and no detectable raffinose saccharides or a low percentage of raffinose and no apparent galactinol synthase activity. At these low levels of enzyme activity and sugar concentration, the limits of detection may be confounding.

Table 2. Different species ranked according to galactinol synthase activity on a gfw of leaf basis and their respective concentrations of sucrose and raffinose saccharides as a percentage of the leaf soluble carbohydrate. Soluble carbohydrate was estimated by anthrone analysis and individual sugars by HPLC (See text).

Accessions	Galactinol synthase (munits enzyme activity/g fresh wt)	Raffinose saccharides (% of soluble carbohydrate)	Sucrose (% of soluble carbohydrate)
Luffa cylindrica	2501	33	45
Lagnearia siceraria	2322	26	23
Cucuribita pepo var. melopepo	1758	33	26
Cucumis melo	1636	45	29
Cucumis myriocarpus	1479	43	20
Cucuribita peop var. *ovifera*	1300	46	31
Citrullus lanatua	1152	30	48
Cucumis sativus 'Little Leaf'	912	49	20
Cucumis sativus 'Calypso'	667	22	42
Cucumis anguria	568	34	24
Cucumis sativus var. *hardwickii*	529	45	36
Lamium maculatum	320	18	35
Coleus blumei	133	15	29
Stachys byzantina	73	27	43
Vigna unguiculata	35	3	71
Zea mays	15	1	80
Capsicum annuum var. annuum	11	4	59
Triticum aestivum	4	3	73
Ipomoea setosa	3	8	38
Glycine max	2	ND	64
Phaseolus vulgaris	2	4	63
Nicotinan tabacum	1	ND	60
Lycopersicon esculentum 'Manapal'	ND[z]	1	36
Lycopersicon esculentum 'Floramerica'	ND	1	32
r values for correlation with munits activity/g fresh wt	---	+0.84**	−0.73**

[z]ND = not detected.
**Significant at 1% level.

Galactinol synthase activity was first detected in maturing soybean seeds 40 days after the beginning of flowering (Fig. 2). This rise in activity was followed by the appearance of raffinose and stachyose. Stachyose was the predominant raffinose-type sugar stored. This pattern of carbohydrate accumulation in cotyledons follows closely that previously reported for soybean (33) and other legumes such as *Phaseolus vulgaris* (30, 32), *Phaseolus lunatus* (16), and *Pisum sativum* (27). Accumulation occurs in the sequence: sucrose → raffinose → stachyose. Galactinol synthase appeared just prior to the appearance of raffinose saccharides. As the seeds reached the very late stages of maturity and began to dry, galactinol synthase activity dropped off as the level of carbohydrate reached a maximum.

Mature cucumber seeds contained appreciable amounts of raffinose and stachyose (Fig. 3). Raffinose was the dominant storage sugar in cucumber seeds. During germination, the levels of raffinose and stachyose dropped rapidly, whereas sucrose levels first increased and then decreased. Galactinol synthase activity appeared after 36 hr. Raffinose and stachyose in the cotyledon did not disappear completely during germination but reached a steady state. Conversely, it has been reported that raffinose saccharides decrease rapidly and within 4–6 days disappear completely during germination of cotton seeds (28), soybean (1, 15, 21), *Brassica*, and peanut (6) based on paper chromatographic methods. At least 3 of these studies (1, 15, 28) should have been able to detect the low levels of raffinose and stachyose in seedlings as detected here by HPLC, but did not. Therefore, in cucumber, unlike other species, raffinose-sugars do not disappear completely during germination. Cotton and soybean have not been reported to contain raffinose saccharides in leaf and stem tissues of the mature plant. Unlike cucurbits, these plants are not known to transport raffinose saccharides. Cucumber, however, does contain and transport raffinose-type sugars (23, 34). Reserve raffinose and stachyose are rapidly utilized during germination, but within 3 days galactinol synthase has appeared (Fig. 3). Thus unlike these other species, a steady-state level of raffinose and stachyose is reached in the cucumber cotyledon during germination.

Discussion

Results from our survey of species (Table 2) indicate a positive correlation between galactinol synthase activity and the fraction of the total soluble carbohydrate comprised of raffinose saccharides in leaves. A negative correlation was found between activity of this enzyme and percentage as sucrose. Because galactinol synthase is the first enzyme to commit carbon to raffinose saccharide biosynthesis (Fig. 1), it may play a role in regulating the partitioning of carbon between these sugars and sucrose. Carbon may be shuttled to the raffinose saccharides at the expense of sucrose. This could be importance to plant breeders who would like to reduce or eliminate these sugars from the seeds of legumes since they are only 20% as sweet as sucrose

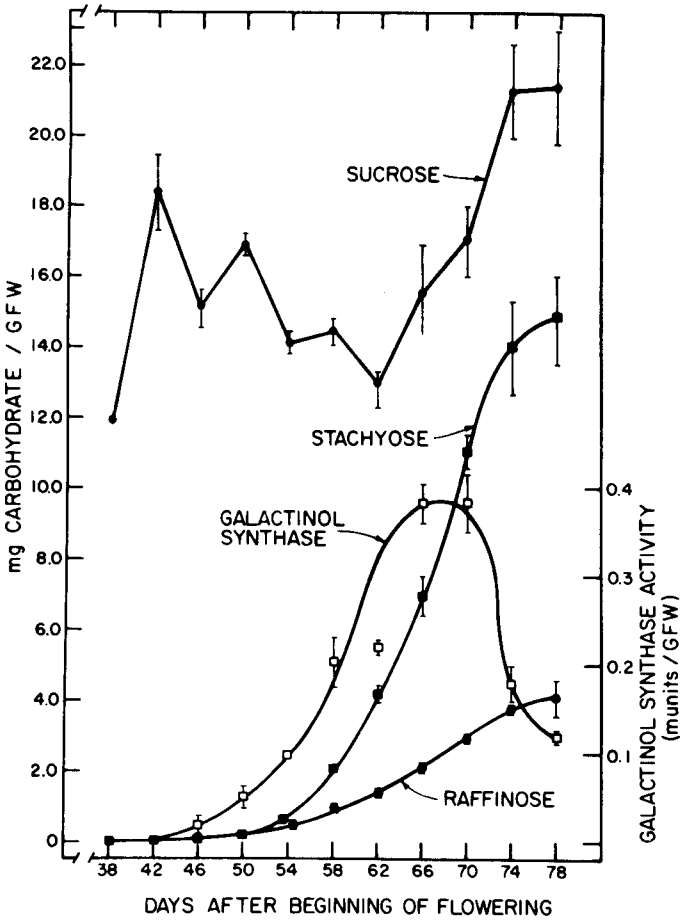

Fig. 2. Changes in the concentration of raffinose, stachyose, and sucrose, and in galactinol synthase activity in maturing soybean seeds. Data were calculated from triplicate samples and are plotted as the mean concentration/gram fresh weight ± SE.

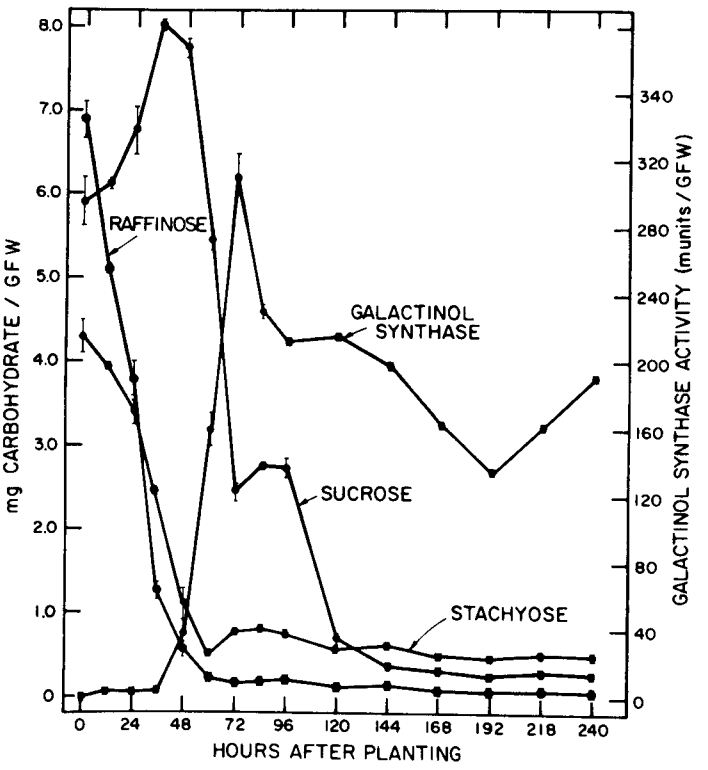

Fig. 3. Changes in the concentration of raffinose, stachyose, and sucrose, and in galactinol synthase activity in cotyledons of cucumber seeds during germination. Data were calculated from duplicate samples which are indicated by range bars.

(20, 26) and have been shown to be associated with flatulence (4). There are, however, possible advantages to having high levels of these sugars present in other crops since they have been implicated in frost resistance and cold acclimation in woody plants (14). The data reported here suggest the possibility that high or low galactinol synthase activity might have a direct effect on the proportion of soluble carbohydrate present as raffinose saccharides.

The fact that raffinose sugars are stored in mature cucumber seed raises an interesting question. Cucumber fruit tissue does not contain raffinose saccharides (23). A series of enzymatic reactions occurring in the peduncle may convert these transport sugars to sucrose (10, 29). Thus, there is either direct transport of raffinose saccharides from the vascular tissue of the stem to the developing seed through vascular traces in the fruit or the occurrence of biosynthesis of these sugars in the cucumber seed during fruit ripening.

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