**myo-Inositol and sucrose concentrations affect the accumulation of raffinose family oligosaccharides in seeds**

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Abstract

Raffinose family oligosaccharides (RFOs) fulfil multiple functions in plants. In seeds, they possibly protect cellular structures during desiccation and constitute carbon reserves for early germination. Their biosynthesis proceeds by the transfer of galactose units from galactinol to sucrose. Galactinol synthase (GolS), which mediates the synthesis of galactinol from myo-inositol and UDP-galactose, has been proposed to be the key enzyme of the pathway. However, no significant relationship was detected between the extractable GolS activity and the amount of RFOs in seeds from seven pea (*Pisum sativum* L.) genotypes selected for high variation in RFO content. Instead, a highly significant correlation was found between the levels of myo-inositol and RFOs. Moderately strong relationships were also found between sucrose and RFO content as well as between myo-inositol and galactinol. Further evidence for a key role of myo-inositol for the synthesis of galactinol was obtained by feeding exogenous myo-inositol to intact pea seeds and by the analysis of four barley (*Hordeum vulgare* L.) low phytic acid mutants. In seeds of three of these mutants, the reduced demand for myo-inositol for the synthesis of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate) was associated with an increased level in myo-inositol. The mutants seeds also contained more galactinol than wild-type seeds. The results suggest that the extent of RFO accumulation is controlled by the levels of the initial substrates, myo-inositol and sucrose, rather than by GolS activity alone.

Key words: Galactinol, *Hordeum*, myo-inositol, *Pisum*, phytic acid, raffinose, seeds, sucrose.

Introduction

Raffinose family oligosaccharides (RFOs) have multiple functions in plants. They serve as transport carbohydrates in the phloem (Ayre et al., 2003), as storage reserves and cryoprotectants in frost-hardy plant organs (Sprenger and Keller, 2000; Pennycooke et al., 2003), and they accumulate in maturing seeds, where they may play a role in the acquisition of desiccation tolerance and storability (Horbowicz and Obendorf, 1994). However, RFOs also have antinutritional properties (they cause flatulence) and so research efforts have been aimed at modulating the RFO content of crop seeds (Wang et al., 2003).

The biosynthesis of RFOs proceeds by the reversible addition of galactose units from galactinol (*O*-α-D-galactopyranosyl-(1→1)-L-myoinositol) to sucrose. Chain elongation is catalysed by the consecutive action of raffinose synthase and stachyose synthase (Peterbauer and Richter, 2001). Some stachyose synthases are able to add two galactose units to raffinose, yielding the pentasaccharide verbascose (Peterbauer et al., 2003). However, the key enzyme of the pathway is believed to be galactinol synthase (GolS, EC 2.4.1.123), which reversibly synthesizes galactinol from UDP-D-galactose and myo-inositol (Keller and Pharr, 1996). This model, which was originally based on a correlation of GolS activity with the RFO content of a range of plant species (Handley et al., 1983), implies that the concentration of galactinol is the critical factor, because...
Materials and methods

addition, a range of pea cultivars, selected for high variation in myo-inositol (Raboy et al., 2002). Consequently, recent work focused on GolS expression in seeds and in vegetative tissues exposed to cold or drought stress (Cunningham et al., 2003; Downie et al., 2003; Volk et al., 2003; Zhao et al., 2003). A tight relationship between GolS and RFO content, however, was not found in all cases. For example, there were no significant differences in GolS expression and enzyme activity in developing seeds of two pea cultivars with contrasting RFO content (Peterbauer et al., 2001). GolS mRNA levels were also poorly correlated with the presence of raffinose in developing and germinating tomato (Lycopersicon esculentum) seeds (Downie et al., 2003). These results suggest that other factors modulate RFO accumulation in seeds.

Interestingly, a reduction in the myo-inositol level has been shown to reduce galactalin and RFO levels drastically in mutant soybean (Glycine max) seeds (Hitz et al., 2002) and transgenic potato (Solanum tuberosum) tubers (Keller et al., 1998). The objective of this study was, therefore, to determine the effect of different myo-inositol levels on RFO accumulation. Use was made of low phytic acid mutants, because these seeds require less myo-inositol for the biosynthesis of phytic acid (myo-inositol 1,2,3,4,5,6-hexakisphosphate), the major seed storage reserve for phosphorus (Raboy et al., 2001), and may, therefore, contain elevated levels of myo-inositol (Shi et al., 2003). In addition, a range of pea cultivars, selected for high variation in RFOs (Jones et al., 1999), was analysed. Evidence is provided that an increase in myo-inositol combined with a sufficient availability of sucrose promotes RFO accumulation in seeds.

Plant material and chemicals

Seeds of barley (Hordeum vulgare L. cv. Harrington) wild type and low phytic acid homozygous mutant sibling lines (lpa1-1, M635, M955, and lpa2-1) have been described by Raboy and co-workers (Raboy et al., 2001; Dorsch et al., 2003). Seeds of pea (Pisum sativum L. cv. Kelvedon Wonder) were obtained from a local supplier (Austrosaat, Vienna, Austria). Seeds of the pea genotypes SD1, SD3, SD5, SD7, SD9, and the round-seeded pea genotype BC3 have been described by Jones et al. (1999). Raffinose was purchased from Merck (Vienna, Austria), myo-inositol and sodium phytate from Sigma (Vienna, Austria), stachyose and galactinol from Fluka (Vienna, Austria), and verbascose from Megazyme (Wicklow, Ireland).

Extraction and analysis of carbohydrates

Mature seeds were ground to a fine powder in a sample mill. About 50 mg of the powder was suspended in 0.5 ml of a mixture of methanol, chloroform, and water (12:5:3, by vol.) containing 0.6 mg phenyl β-D-glucopyranoside as an internal standard and heated for 30 min at 60 °C. After cooling, 0.5 ml water was added and phases were separated by centrifugation. Aliquots of the aqueous phase were taken to dryness. Trimethylsilyl derivatives of soluble carbohydrates were separated by capillary gas chromatography as described (Peterbauer et al., 1998).

Extraction of soluble protein and assay of activity of GolS

Seeds were frozen in liquid nitrogen and powdered using a mortar and pestle. About 250 mg of the frozen powder was suspended in 1.25 ml ice-cold extraction buffer consisting of 50 mM HEPES-NaOH (pH 7.0) and 1 mM dithiothreitol, homogenized with a Polytron tissue homogenizer (3×5 s), and centrifuged (20 000 g, 20 min, 4 °C). The supernatant was desalted by centrifugal gel filtration and concentrated by ultrafiltration as previously described (Peterbauer et al., 2001). GolS activity was assayed in reaction mixtures (20 μl) containing 50 mM HEPES-NaOH (pH 7.0), 1 mM dithiothreitol, 5 mM MnCl2, 20 mM myo-inositol, 5 mM UDP-D-galactose, and 10 μl of the desalted extracts. Reaction mixtures were incubated at 30 °C for 15 min and stopped by boiling at 100 °C for 5 min. The amount of galactinol formed was determined by HPLC with pulsed amperometric detection (Peterbauer et al., 2002a).

Extraction and determination of phytic acid

Seed powder (50 mg) was mixed with 1 ml 0.5 M HCl and shaken for 3 h at room temperature. Extracts were frozen, thawed, and centrifuged. Phytic acid was quantified by anion-exchange chromatography using a modification of the method of Rounds and Nielsen (Rounds and Nielsen, 1993). Samples of 50 μl were loaded onto an IonPac AG11 column (50×4 mm, Dionex), which had been equilibrated with 10 mM HNO3. Phytic acid was eluted at 1 ml min−1 with a 10 min linear gradient from 0 to 0.9 M KNO3 in 10 mM HNO3. Effluent from the column was mixed with a colorimetric reagent (0.015% FeCl3, 0.15% sulphasalicylic acid) at a flow rate of 1 ml min−1 and passed through a 3 m reaction coil (0.5 mm i.d.) prior to absorbance detection at 525 nm. Standard curves were constructed from peak areas obtained by injecting different amounts of sodium phytate standard (5–50 μg).

myo-Inositol feeding experiment

Pods were harvested 25–30 d after flowering from pea plants (cv. Kelvedon Wonder) grown in a greenhouse. Seeds with a fresh mass of 450–650 mg seed−1 were excised and divided into two groups. Single seeds from one group were placed in tubes containing a sterile-filtered solution (1 ml) of 50 mM myo-inositol and 200 mM mannitol. Seeds from the other group were placed in tubes containing 250 mM mannitol (control). The tubes were capped with aluminium foil and incubated for 2 d at 22 °C with gentle agitation. After this period, the seeds were washed with water, killed in a microwave oven (3×30 s) and dried at 60 °C. Soluble carbohydrates were extracted and assayed as described above.

Results

Analysis of pea lines for soluble carbohydrates and GolS activity

The RFO content of mature seeds from the pea selected genotypes varied considerably, ranging from 60.9 μmol g−1 dry mass in the cultivar SD9 to 166.5 μmol g−1 in Kelvedon Wonder (Fig. 1). Stachyose was the major oligosaccharide in SD1, SD3, SD5, and Kelvedon Wonder, whereas verbascose was predominant in the other cultivars. Raffinose was a minor constituent, representing 9.5% of the total amount of RFOs (in SD9) to 19.0% (in SD1; data not shown). A large variation was also detected for the levels of myo-inositol. SD9 exhibited the lowest myo-inositol content.
(2.3 µmol g⁻¹). All the pea genotypes analysed showed a high sucrose content, ranging from 65.1 µmol g⁻¹ dry mass in SD9 to 196.1 µmol g⁻¹ in SD7. RFO levels were highly correlated with those of myo-inositol ($r^2=0.92$, $P<0.0001$; Fig. 2A) and to a lower extent with those of sucrose ($r^2=0.49$, $P<0.0001$; Fig. 2B) and galactinol ($r^2=0.52$, $P<0.001$, data not shown).

Extractable GolS activity ranged from 1.8 nkat g⁻¹ dry mass in BC3 to 15.2 nkat g⁻¹ in SD1. There was no correlation between GolS activity and the total amount of RFOs when expressed in µmol g⁻¹ dry mass ($r^2=0.09$, n.s., data not shown). However, raffinose, stachyose, and verbascose contain one, two, and three galactose units, respectively, which are all derived from GolS. Therefore, the relationship between GolS activity and the total amount of galactose units in RFOs were also analysed, but again no significant correlation was found ($r^2=0.05$, n.s., data not shown). A moderately strong correlation was detected between GolS activity and galactinol content ($r^2=0.31$, $P<0.01$; Fig. 3A), but a better relationship was observed for myo-inositol and galactinol ($r^2=0.59$, $P<0.0001$; Fig. 3B).

**Analysis of barley low phytic acid mutants**

Barley lpa1-1, M635, M955, and lpa2-1 mutants and the respective wild-type sibling lines were analysed. In each of the four mutants, phytic acid levels were significantly lower than in the respective wild types (Fig. 4). Seeds homozygous for the M635 and the M955 mutation had reductions in seed phytic acid of about 75% and 95%, respectively, while lpa1-1 and lpa2-1 showed reductions of about 65%, in agreement with previous studies (Raboy et al., 2001; Dorsch et al., 2003). The levels of myo-inositol, galactinol, sucrose, and raffinose in lpa1-1 mutants were similar to the wild-type sibling (Fig. 4). M635 mutants showed an increase in both myo-inositol and galactinol (by about 87% and 89%, respectively), whereas the levels of sucrose and raffinose were similar to the wild type (Fig. 4). In M955 and lpa2-1 mutants, myo-inositol, galactinol, and raffinose were significantly increased compared with the corresponding wild types. Raffinose content increased by about 91% and 65%, respectively (Fig. 4). M955 and lpa2-1 also had increased sucrose levels (about 54% and 33% more than found in the corresponding wild types), but these differences were statistically not significant. In all lines, traces of stachyose (<0.5 µmol g⁻¹ dry mass) were present. No verbascose was detected.

**Feeding of myo-inositol to intact seeds**

To analyse the role of myo-inositol for RFO accumulation further, a feeding experiment was conducted with immature seeds from the pea cultivar Kelvedon Wonder. Excised seeds were incubated with either 50 mM myo-inositol and 200 mM mannitol, or with 250 mM mannitol alone (control). After 2 d of incubation the levels of carbohydrates were determined. Seed myo-inositol content was almost (4.9 µmol g⁻¹ dry mass), while the highest level was found in Kelvedon Wonder (23.7 µmol g⁻¹). Kelvedon Wonder was also highest in galactinol (10.4 µmol g⁻¹ dry mass), whereas the lowest galactinol content was found in SD3.
3-fold higher in samples incubated with myo-inositol compared with those incubated without myo-inositol (Fig. 5). The higher myo-inositol level was associated with an increased galactinol level. Galactinol content in the myo-inositol treated seeds was almost doubled compared with the control seeds. No significant differences in sucrose and RFO levels were observed (Fig. 5).

Discussion

Expression of GolS is certainly a prerequisite for RFO biosynthesis, because its product galactinol is the only known galactosyl donor to RFOs. Since galactinol has apparently no other function in plant metabolism, it is reasonable to expect some kind of correlation between the presence of GolS and RFOs. However, in contrast to other researchers (Handley et al., 1983; Saravitz et al., 1987), a significant relationship between GolS activity and the amount of RFOs was not found. Further, GolS activities correlated only moderately with the levels of galactinol (Fig. 3A). It has previously been shown that the extractable GolS activity in developing seeds of two pea cultivars is 50–150-fold higher than that required to explain the observed RFO accumulation (Peterbauer et al., 2001). Thus, even fairly high variations in the amount of GolS may have little effect on the rate of galactinol formation.

Axis tissue has frequently been found to contain higher RFO and GolS levels than cotyledon tissue on a dry matter basis (Kuo et al., 1997; Obendorf et al., 1998). However, the mass of axes is very small compared with that of cotelydons, analysis of entire seeds as performed in this study could have blurred a relationship between enzyme activities and substrate or product concentrations. Hence, it was surprising to find a highly significant correlation between myo-inositol and the RFO contents of the pea lines (Fig. 2A). Strong effects of myo-inositol on the pathway have already been reported in work on 1L-myo-inositol 1-phosphate synthase (MIPS, EC 5.5.1.4). MIPS converts D-glucose 6-phosphate into myo-inositol 1-phosphate, which is dephosphorylated by a specific phosphatase.
A single point mutation in soybean (Glycine max) MIPS results in a low seed myo-inositol phenotype, which is associated with strongly reduced RFO levels (Hitz et al., 2002). Similarly, a reduction in myo-inositol levels (to 7% of the wild type) by antisense suppression of MIPS activity gave reduced galactinol and raffinose content in potato (Solanum tuberosum) leaves (to 5% and 12%, respectively) (Keller et al., 1998).

To demonstrate the opposite effect, i.e. an increase in galactinol in response to elevated myo-inositol, the content of myo-inositol was raised in intact, excised pea seeds by applying exogenous myo-inositol (Fig. 5). Myo-Inositol is a common constituent of phloem saps and is taken up into the seed from the apoplast, since maturing seeds are not symplastically connected to the maternal tissue (Sasaki and Loewus, 1980). Therefore, this approach to feed myo-inositol to excised pea seeds mimics uptake from the apoplastic fluid. Indeed, when exogenous myo-inositol was fed to pea seeds, galactinol levels were increased compared with control seeds, demonstrating that sufficient GolS activity was present to convert the additional myo-inositol into galactinol. To see whether this effect is operative in planta, barley mutants low in phytic acid were analysed (Fig. 4). The isolation of these mutants was based on a high

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**Fig. 4.** Content of phytic acid, myo-inositol, galactinol, raffinose, and sucrose in mature seeds of the barley lpa lines lpa1-1, M635, M955, and lpa2-1 (open bars) and the respective wild-type sibling lines (closed bars). Data represent means ±SE of three replications. An asterisk indicates a significant difference to the respective wild-type line (P<0.05).

**Fig. 5.** Carbohydrate content of immature pea seeds (cv. Kelvedon Wonder) incubated for 2 d with 200 mM mannitol and 50 mM myo-inositol (open bars) or with 250 mM mannitol alone (closed bars). Values represent means ±SE of seven replications. An asterisk indicates a significant difference between the two treatments (P<0.05).
inorganic phosphate phenotype, because less phosphate is required for the accumulation of phytic acid (Dorsch et al., 2003). The inositol phosphate pattern found in lpa2-1 mutants suggests that an inositol polyphosphate kinase gene may be affected (Dorsch et al., 2003). The corresponding genes of the lpa1-1, lpa2-1, M635, and M955 mutations have not yet been identified, but neither lpa1-1 and lpa2-1 (Raboy et al., 2001) nor M635 (V Roslinsky, PE Eckstein, V Raboy, BG Rossnagel, GJ Scoles, unpublished results) are MIPS mutations. There was speculation that these mutants contain increased myo-inositol levels because they convert less myo-inositol into phytic acid (as in a maize lpa2 mutant; Shi et al., 2003). If this is the case, then the mutations create a cumulatively higher in vivo steady-state pool of myo-inositol during the maturation phase (when phytic acid is synthesized alongside RFOs). The effect of the altered metabolism on RFO accumulation is clearly more informative than the analysis of metabolite levels and enzyme activities alone. With the exception of lpa1-1, which had a very similar myo-inositol content compared with the wild type, all mutants showed higher myo-inositol and galactinol levels. This was apparently not caused by the high inorganic phosphate status of the mutants: the lpa1-1 mutation had no effect on galactinol. A similar maize mutation, maize lpa1-1, was also shown to have little effect on monosaccharides and sucrose in a sweet (sugar-rich) maize genetic background (Tadmor et al., 2001).

All these results provide evidence that the biosynthesis of galactinol is controlled by the concentration of myo-inositol as the substrate for GolS rather than by the amount of the enzyme, but elevated galactinol did not in all cases promote RFO accumulation. In the feeding experiment (Fig. 5), the incubation period of not more than two days was probably too short to see an effect on the RFO content. Another explanation may be that the artificially high level of myo-inositol in these pea seeds had induced a further RFO accumulation. Since the components of the RFO pathway (galactinol, GolS, raffinose synthase, and stachyose synthase) are known to be exclusively cytoplasmic (Keller, 1992; Bachmann et al., 1994; Keller and Pharr, 1996; Braun and Keller, 2000), myo-inositol as the co-product of the raffinose synthase and stachyose synthase reactions may have favoured the reverse reactions when present in an artificially high concentration (Peterbauer et al., 2002a, b). However, there was also a lack of response on raffinose in barley M635 mutants, although these seeds had significantly more galactinol (Fig. 4). It was noted that those mutants with increased RFO levels (M955 and lpa2-1) not only had significantly more galactinol, they also tended to have higher levels of sucrose. Sucrose is the second substrate for raffinose synthase and, hence, variations in sucrose may affect the formation of raffinose as well as variations in galactinol. Evidence for a role of sucrose was already reported in work on transgenic *Vicia narbonensis* seeds inhibited for ADP-glucose pyrophosphorylase (Rolletschek et al., 2002). The transgenic seeds had increased sucrose levels (because less sucrose was converted into starch) and elevated RFO contents.

A role for sucrose in RFO accumulation is further supported by a moderately strong relationship between sucrose and RFOs in the pea cultivars (Fig. 2B). It is important to note that this simple correlation may underestimate the true effect of sucrose, because incorporation of sucrose into RFOs reduces the final sucrose pool (the same argument may apply for the sucrose data in barley seeds shown in Fig. 4). Hence, it is not easy to decide whether myo-inositol (acting via galactinol) or sucrose is finally more important for the extent of RFO accumulation. It is speculated that myo-inositol is more critical in pea than in barley. In seeds of the latter, conversion of sucrose into raffinose is the terminal step. In pea, however, raffinose is further converted into stachyose and verbascose. These steps require galactinol, but not sucrose.

In summary, these results do not support the hypothesis of the presence of a single key enzyme controlling RFO accumulation. The RFO pathway is intimately linked to primary plant metabolism via myo-inositol, sucrose, and UDP-galactose. Within this metabolic network, even changes in the distant pathway generating phytic acid can markedly affect the amount of RFOs deposited in a seed. Therefore, it is thought that a high degree of the variability in seed RFO content may be explained by differences in the concentrations of central metabolites.

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**References**


