Down-regulation of galactinol synthesis in oilseed Brassica napus leads to significant reduction of antinutritional oligosaccharides

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Down-regulation of galactinol synthesis in oilseed *Brassica napus* leads to significant reduction of antinutritional oligosaccharides[1]

Cheryl Bock, Heather Ray, and Fawzy Georges

Abstract: The utility of defatted seed meal from many crops such as canola (*Brassica napus* L.) is limited by the presence of antinutritional factors, including sucrose galactosides, raffinose, and stachyose. Anaerobic breakdown of these sugars in the digestive tract of livestock is a major source of production of farm gases. In this report, the gene encoding galactinol synthase was isolated from *B. napus* and reintroduced into the same species in an antisense orientation to limit the production of galactinol, an important intermediate in the biosynthesis of raffinose and stachyose. This approach substantially reduced the accumulation of galactinol and stachyose in mature transgenic canola seed. Substantial changes in the mRNA levels of galactinol synthase and several sugar-related genes were also observed.

Key words: antisense galactinol synthase, *Brassica napus*, canola, inositol, galactinol, raffinose, stachyose.

Résumé : La présence de facteurs non nutritifs, incluant les galactosides de saccharose, le raffinose et la stachyose, limite l'utilité des farines de grains dégraissées comme le canola (*Brassica napus* L.). La décomposition anaérobie de ces sucres dans le tractus digestif des animaux constitue une source majeure de production de gaz sur la ferme. Les auteurs ont isolé le gène codant pour la synthase du galactinol à partir du *B. napus* et l'ont réintroduit chez cette même espèce en utilisant une orientation antisense afin de limiter la production de galactinol, un important intermédiaire dans la biosynthèse du raffinose et du stachyose. Cette approche réduit substantiellement l'accumulation de galactinol et de stachyose chez les graines transgéniques mûres du canola. On observe également des modifications importantes dans les teneurs en mARN de la synthase du galactinol et de plusieurs gènes reliés aux sucres.

Mots-clés : synthase du galactinol antisense, *Brassica napus*, canola, inositol, galactinol, raffinose, stachyose.

[Traduit par la Rédaction]

Introduction

Oligosaccharides of the raffinose family (RFOs) are complex carbohydrates that are found at variable levels in seeds of many crops. They are also known as sucrose α-galactosides. Raffinose, stachyose, and in some cases verbascose and larger oligosaccharides in the RFO series accumulate to high levels during seed development, where they are believed to contribute to seed viability by allowing dehydration without loss of function (Obendorf 1997). In some species they also appear to contribute cold-, drought-, heat-, or oxidative-stress tolerance (Liu et al. 1998; Taji et al. 2002; Panikulangara et al. 2004; Nishizawa et al. 2008). During seed germination they are broken down by the activity of α-galactosidases and the sugars used as an energy source (Downie et al. 2003). However, it has recently been reported that RFOs are not required for proper germination in some plants, such as soybean (Dierking and Bilyeu 2009).

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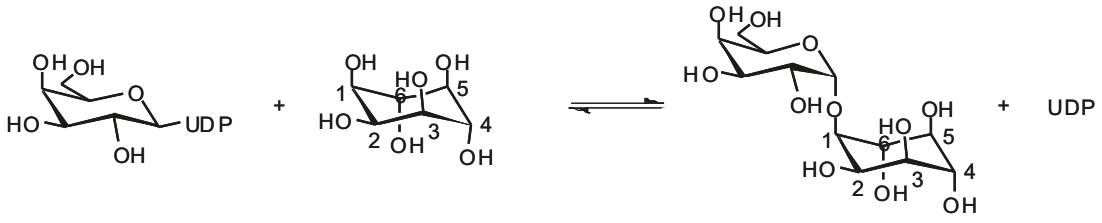
Galactinol (*myo*-inositol-α-D-galactoside) is the first intermediate substrate in the biosynthesis of RFOs (Fig. 1). It functions as a galactosyl-residue donor in subsequent steps of sucrose α-galactosides formation. Galactinol synthase (GolS) [UDP-D-Gal:*myo*-inositol (1-α-D) galactosyl transferase (EC 2.4.1.123)] catalyses a key step in the production of RFOs. The enzyme utilizes UDP-galactose for the transfer of a galactosyl residue to *myo*-inositol and the production of galactinol.

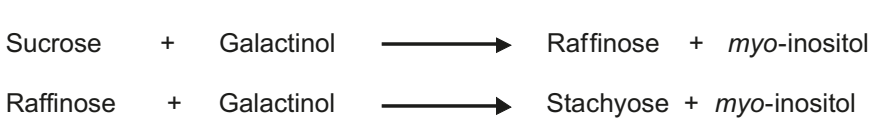
Since RFOs in seeds or seed meal fed to monogastic livestock can only be digested through anaerobic fermentation in the small intestine, they lead to deleterious side effects, such as increased production of methane gas (Minorsky 2003). Similar side effects occur in humans when RFO-rich legume products are consumed (Naczk et al. 1997; Dierking and Bilyeu 2009).

In the animal feed industry, a number of processes have been suggested for the removal of RFOs (Hansen 1977). However, these processes increase the cost of feed meal production considerably, and in the food industry, such processes are mostly inapplicable; hence, the problem with such crops persists. Therefore, the ability to reduce the production of these undesirable glycosides during seed development to levels closer to the minimum required for seed viability would not only improve the value of the crop, but would also eliminate the associated costs of post harvest processing.

We hypothesized that a reduction in available galactinol, the key intermediate in the biosynthesis of RFOs, should re-

Fig. 1. The gyratory role of *myo*-inositol in the biosynthesis of galactinol and sucrose galactosides. The synthesis of each galactinol molecule requires a molecule of *myo*-inositol, which is later released through the action of a galactosyltransferase.





duce their production. Herein, an antisense strategy was designed against *Brassica napus* L. (canola) galactinol synthase (*GolS*) and tested in the same species as a model system for other crops.

Materials and methods

Preparation and cloning of galactinol synthase gene of *Brassica napus*

Data from Liu et al. (1998) were used to assist in the design of forward and reverse primers to isolate a galactinol synthase gene from *B. napus*. Primers were designed to amplify a 1028 bp region of *B. napus golS*, with restriction sites incorporated into the primers for directional cloning. Total RNA was extracted from *B. napus* and cDNA generated using a first strand cDNA Synthesis Kit for reverse-transcription polymerase chain reaction (RT-PCR; Boehringer-Mannheim, Amsterdam, Netherlands). PCR was then carried out to amplify the gene, which was cloned and sequenced according to standard methods (NCBI accession No. AF106954). The gene was subcloned in antisense orientation into the *Agrobacterium* vector pBI121 (Clontech) which was inserted into competent *Agrobacterium tumefaciens* EHA105 cells by electroporation (IBI Gene Zapper™; 21 μF, 400 Ω, 2500 V, Eastman-Kodak, New Haven, Conn.).

Brassica napus cultivar 'Westar' was transformed with the *Agrobacterium* vector following the method of Moloney et al. (1989).

Quantitative reverse-transcription polymerase chain reaction

For quantitative reverse transcription PCR (qRT-PCR), transformed and control plants were grown under standard growth chamber conditions of 16 h light at 300 μE and 20 °C and 8 h darkness at 15 °C. Tissues from the third true leaf of the T_3 generation and seed of the T_4 generation harvested 25 days after pollination (dap) were collected and placed in liquid nitrogen. Tissue was finely ground in a mortar in liquid nitrogen. RNA was prepared using RNeasy plant RNA columns (QIAGEN, Venlo, Netherlands). RNA quality was assessed on agarose gels, then 1–2 μg RNA was treated with 1 U DNase for 10 min at 25 °C, the reaction was stopped with EDTA to 2.5 mmol·L^{-1} and 10 min 65 °C, and the RNA ethanol-precipitated for 16 h. Alternatively, on-column DNase digestion was incorporated into the RNA isolation procedure. Reverse transcription was carried out on 1–2 μg of the resuspended RNA, using Superscript III (Invitrogen, Carlsbad, Calif.) for 60 min at 50 °C. The reaction was stopped by 15 min at 70 °C.

The Primer3 program (Untergasser et al. 2007) was used to design primers with Tm 59–60 °C, amplifying sequences 170–190 bp long. All primer pairs were previously tested for efficiency and single product formation by amplification of *B. napus* DNA. Primers were designed for *B. napus* galactinol synthase (AF106954), *B. napus myo*-inositol phosphate synthase (*MIPS*) (U66307), phosphatidylinositol 3-kinase (*PI3K*) (AY142114), phospholipase C2 (*PLC2*) (AF108123) (Das et al. 2005), and several additional sugar-related genes (see Discussion).

Quantitative PCR was carried out on an I-Cycler (Bio-Rad, Hercules, Calif.) with fluorescent substrate Platinum SybR Green qPCR Supermix UDG (Invitrogen), 0.4 μL of each primer, and 0.05 μL of cDNA in a 25 μL reaction. PCR cycling comprised 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 57 °C, and 60 s at 72 °C, followed by a standard dissociation curve. Expression was quantified relative to expression of actin in the same sample. Duplicate samples were analysed and the data averaged.

Sugar analysis

The T_4 generation of canola plants transgenic for antisense galactinol synthase gene (AGS) and 'Westar' controls were grown. Flowers were tagged on the day of opening and developing seeds were dissected at 25 dap, frozen in liquid nitrogen, and dried overnight under vacuum without heating. Mature seed samples were stored at room temperature. Mature seed samples were prepared and run in triplicate. Developing seed samples were prepared and run once for multiple sublines of the independent transgenic lines 1 and 28.

Dichloromethane-rinsed glassware was used throughout lipid extraction. Mass of seed and leaf tissue were recorded to ±0.0001 g. Leaf tissue aliquots were kept frozen until dichloromethane was added.

Tissue was ground with a PRO200 homogenizer (PRO-Scientific, Oxford, Conn.), triple-rinsed with dichloromethane between samples. Centrifugation at 7500*g* for 5 min aided clarification, and the dichloromethane-lipid layer was transferred to a fresh tube. Tissues were extracted twice more. All tubes were dried under gaseous nitrogen, and mass of the contents determined.

Sugars were extracted from defatted tissue in 2 mL 80:20 *v*/*v* ethanol–water with agitation at 70 °C for 30 min, followed by centrifugation at 7500*g* for 5 min. One millilitre of each sample was evaporated to dryness under vacuum, reconstituted in 1 mL of 18-MΩ water, diluted 10-fold, and filtered through a 0.45 μm nylon filters for HPLC analysis.

High performance anion exchange-pulsed amperometric detection (HPAE-PAD) of sugars was accomplished with a system from Dionex Corp. (Sunnyvale, Calif.), which consisted of an AS50 autosampler equipped with a 25 μL injection loop, an AS50 thermal compartment at 30 °C, a GP50 gradient pump, an ED_{50} electrochemical detector with an Ag/AgCl pH electrode, and a gold electrode for ion detection. Glucose, galactose, fructose, sucrose, stachyose, and raffinose were separated with 25 mmol·L^{-1} isocratic NaOH at 0.25 mL·min^{-1} on a CarboPac™ PA10 analytical column (2 mm × 250 mm), preceded immediately by a CarboPac™ PA10 guard column (2 mm × 50 mm) and then an AminoTrap™ guard column (2 mm × 50 mm) (Dionex Corp.) Galactinol and *myo*-inositol were eluted with 500 mmol·L^{-1} isocratic NaOH at 0.40 mL·min^{-1} on a CarboPac™ MA1 column (4 mm × 250 mm), preceded by a CarboPac™MA1 guard column (4 mm × 50 mm).

Phytate analysis of mature seed meal

Eighteen mega-Ohm deionized water was used throughout. Meal samples of 0.2 g were weighed to ±0.0001 g. Two hundred microlitres of 2.4% HCl was added with boiling for 10 min, then water was added to a total volume of 1400 μL. Centrifugation settled the meal. Filtration through a 0.45 mm GHP Acrodisk filter (Pall Gellman Laboratory, Ann Arbor, Mich.) clarified the supernatant, which was diluted four-fold with water for analysis.

Phytates were quantified by electrochemical detection using Dionex system described above. An OmniPac PAX-100 analytical column (4 mm × 250 mm) preceded an OmniPac PAX-100 guard (4 mm × 50 mm) and the GM-3 Gradient Mixer was replaced by an IonPac ATC-3 Trap Column (9 mm × 24 mm) (all from Dionex Corp.), which was regenerated daily with 2 mol·L^{-1} NaOH. Before reaching the detector, the eluant passed through an ASRS Ultra II 4 mm self-regenerating suppressor at 297 mA with regenerating water at 5 mL·min^{-1}. Eluants were water (A), 200 mmol·L^{-1} NaOH (B), and 50:50 *v*/*v* isopropanol–water (C), with a flow rate of 1.0 mL·min^{-1}. The gradient was: 0–13 min, B = 6% and C = 12%; 13.1–30 min, B = 30% and C = 2%; 30.1–43 min, B = 56% and C = 2%; 55–65 min, B = 61% and C = 8%; 65.1–70 min, B = 6% and C = 12%.

Results

A strategy was developed using a galactinol synthase gene from *B. napus* in antisense orientation under the control of the 35S promoter, transformed into *B. napus*, with the expectation of finding plants with reduced seed RFOs and improved digestability. The majority of the transformed plants was of normal phenotype. Southern blot analysis showed that lines 1-1 and 28-1 carried a single copy of the transgene (see supplementary data,[3] Fig. S1), and these lines were selected for more detailed examination in the T_4 generation. Expression profiles of selected genes, and seed sugar and phytate profiles were examined.

Expression of *GolS* and functionally related genes

The expression of several sugar pathway genes in transgenic and control leaf and developing seed was examined. When quantified by qRT-PCR, expression of the antisense *GolS* substantially increased the relative expression of *GolS* in both tissues. A number of other genes, selected on the basis of connection to the metabolism of RFOs, galactinol, *myo*-inositol, or phytates, were also profiled. Some of these genes (an additional form of *MIPS*, sucrose phosphate synthase (*SPS*), two forms of sucrose synthase (*SuSy*), inositol polyphosphate kinase 1 and 2 (*IPK1*, and *IPK2*) and two raffinose synthase (*RS*)-like genes) had not previously been isolated from *B. napus*, so we used GenBank data to identify strong candidate sequences. While the degree of similarity to *Arabidopsis thaliana* (L.) Heynh. sequences is high (about 85% base pair identity), the attributed functions for some of these genes have not been ascertained.

In leaf material, antisense *GolS* was strongly expressed in the selected transgenic lines, at 16- to 60-fold higher than wild type expression (Fig. 2). The expression of *MIPS* (U66307) increased about two-fold, as did a second form of *MIPS*, while *SPS* was unchanged and *SuSy* and *RS* increased moderately. Line 1-1 showed increased expression of one form of RS and one form of SuSy, together with higher AGS expression. Expression of other genes in leaf material was not tested.

The expression of selected genes in 25 dap developing seed was also examined. In *B. napus*, expression of the CaMV 35S promoter has been frequently observed to be weaker in developing seed than in leaf (unpublished results). Expression of antisense *GolS* increased about 10-fold above the wild type levels of native *GolS*, while expression of phospholipase C2 (*PLC2*) increased up to 7-fold (Fig. 3). Additional changes included smaller increases in *SPS* and *PI3K* (results not shown). Expression of *MIPS*, which increased in leaf, did not do so in developing seed. Expression of phosphatidylinositol synthase (*PIS*), *IPK1* and *IPK2* did not change noticeably, nor did that of two *RS*-like genes or *SuSy* (results not shown).

Sugars

The accumulation of sugars in mature seed, particularly of the products and precursors of *GolS*, was examined. Data

[3] Supplementary data for this article are available on the journal Web site (http://botany.nrc.ca) or may be purchased from the Depository of Unpublished Data, Document Delivery, CISTI, National Research Council Canada, Building M-55, 1200 Montreal Road, Ottawa, ON K1A 0R6, Canada. DUD 3944. For more information on obtaining material refer to http://cisti-icist.nrc-cnrc.gc.ca/eng/ibp/cisti/collection/unpublished-data.html.

Fig. 2. Relative expression of various genes in the third to fourth true leaf of 'Westar' and selected T_3 transgenic plants expressing antisense galactinol synthase (*GolS*). *GolS*; raffinose synthase 1(*RS1*) and 2(*RS2*), RS gene fragments 1 and 2; sucrose synthase 2(*Susy2*), sucrose phosphate synthase (*SPS*); *myo*-inositol phosphate synthase 2 (*MIPS2*); *myo*-inositol phosphate synthase (*MIPS*) (U66307). W indicates 'Westar'. For almost all data points, standard error on technical replicates was less than 3% of expression level, rising to less than 10% of expression level in one sample.

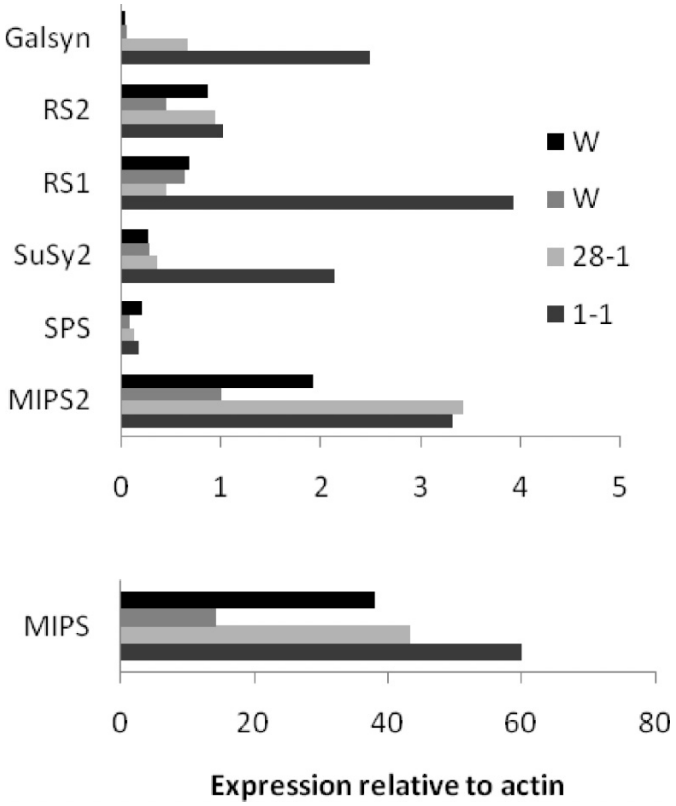
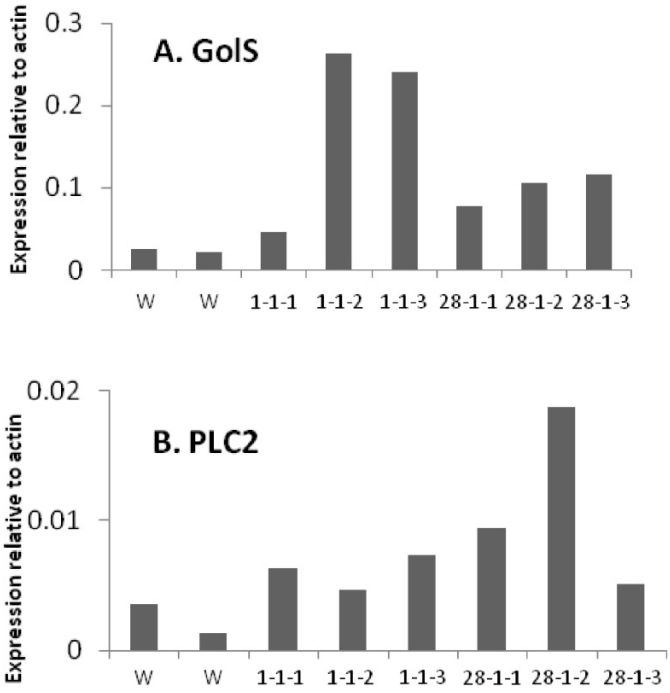


Fig. 3. Relative expression in 25 dap developing seed of T_4 generation, relative to actin, of galactinol synthase and phospholipase C2, for 'Westar' and three sublines each of transgenic lines 1 and 28. (A) galactinol synthase; (B) phospholipase C2. W indicates 'Westar'. For almost all data points, standard error on technical replicates was less than 3% of expression level, rising to 6% of expression level in one sample.



from mature seed of the T_1 and T_2 generations had identified lines 1-1 and 28-1 as having reduced total RFOs (results not shown). Data from T_3 and T_4 mature seed showed decreased concentrations of stachyose in the transgenic lines versus the 'Westar' control, with the lowest-stachyose subline, 1-1-1, having 36% of control levels (Figs. 4A and 4B), while sucrose was slightly elevated (Fig. 4C). *Myo*-inositol levels remained negligible (0.02–0.03 $mg \cdot g^{-1}$ seed) while galactinol levels were decreased in the transgenics by 19%–39%, with subline 1-1-1 having 50% of control levels. These data confirm the prediction that sucrose levels should rise and galactinol and stachyose levels should decrease. Raffinose did not decrease, contrary to this prediction.

In 25 dap T_4 developing seed, galactinol was substantially decreased, while stachyose was relatively little affected, but raffinose was detectable only in one subline of the six sublines of two independent lines that were tested (Fig. 5). The quantity of *myo*-inositol was negligible under all tested conditions (Fig. 5).

Inositol phosphates

In mature seed, phytate and total inositol phosphates were lower in both transgenic lines than in the 'Westar' control (Fig. 6).

Discussion

In leaf and developing seed of several transgenic lines, substantial increases in antisense *GolS* expression were observed. Reductions of raffinose and (or) stachyose and galactinol in mature seed were also found in several transgenic lines, to different degrees. Two lines, 1-1 and 28-1, with single copies of the transgenic sequence, were selected for more detailed analysis. Line 1-1 had a large increase in expression of antisense *GolS*, and was therefore anticipated to have strongly reduced GolS activity and substantial reductions in enzyme products galactinol, raffinose and stachyose, while line 28-1 had a more moderate increase in expression of antisense *GolS* and was therefore anticipated to have smaller changes in GolS activity and enzyme products.

In mature seed, galactinol decreased in both transgenic lines in the T_3 and T_4 generations, while stachyose decreased substantially only in line 1, although some decrease was seen in the T_4 generation of line 28 (Figs. 4A and 4B). Raffinose did not decrease and in several sublines increased slightly. In developing seed of the T_4 generation, the amount of galactinol was lower in both transgenic lines (Fig. 5), while little reduction in stachyose relative to the wild type, but an entire absence of raffinose, was found in the majority of sublines of both lines. The difference from 'Westar' was larger in line 1-1, which had higher antisense *GolS* expres-

Fig. 4. Sugar accumulation in mature seed of the T_3 and T_4 generation. (A) Oligosaccharides and galactinol in 'Westar' and transgenic lines 1 and 28 of the T_3 generation; (B) oligosaccharides and galactinol in 'Westar' and three sublines each of transgenic lines 1 and 28, in the T_4 generation; (C) sucrose in 'Westar' and lines 1 and 28 of the T_3 generation (left) and 'Westar' and three sublines each of lines 1 and 28 in the T_4 generation. Error bars indicate standard error on three samples each.

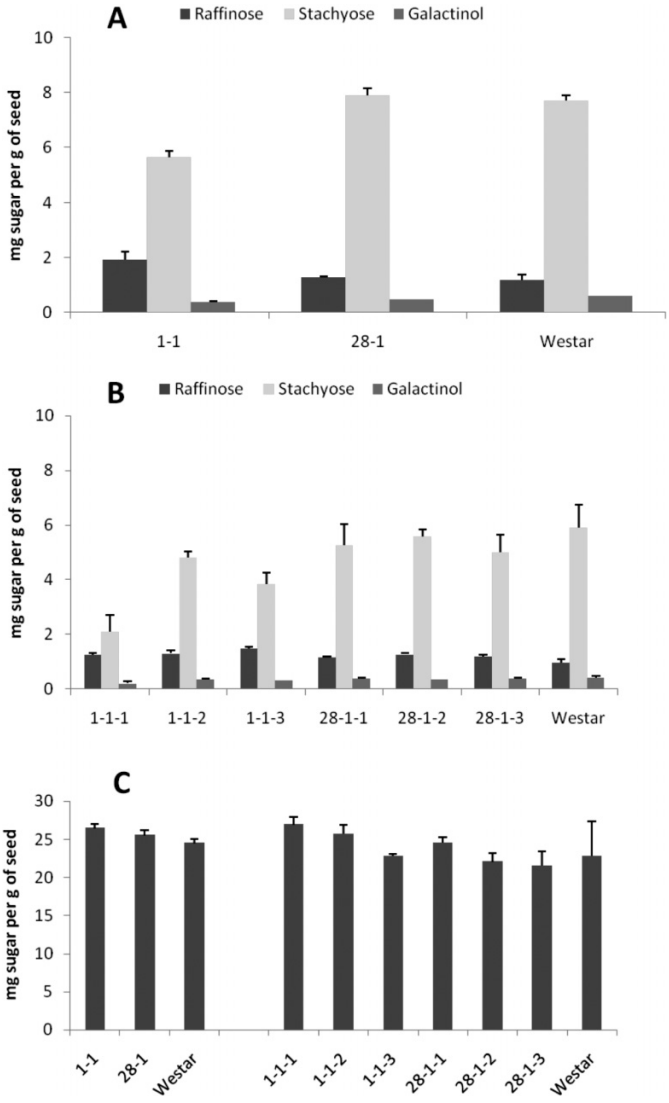


Fig. 5. Sugar accumulation in 25 dap developing seed of the T_4 generation, for raffinose, stachyose, and galactinol, in 'Westar' and three sublines each of transgenic lines 1 and 28.

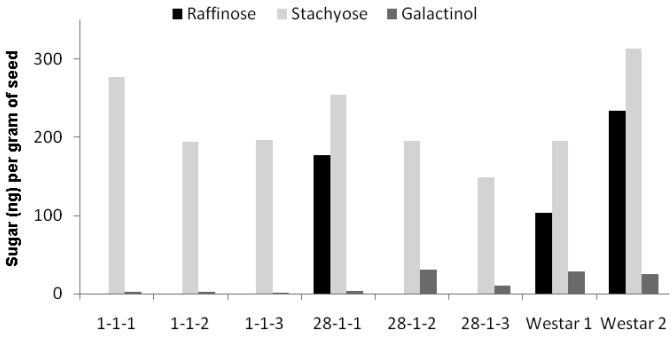
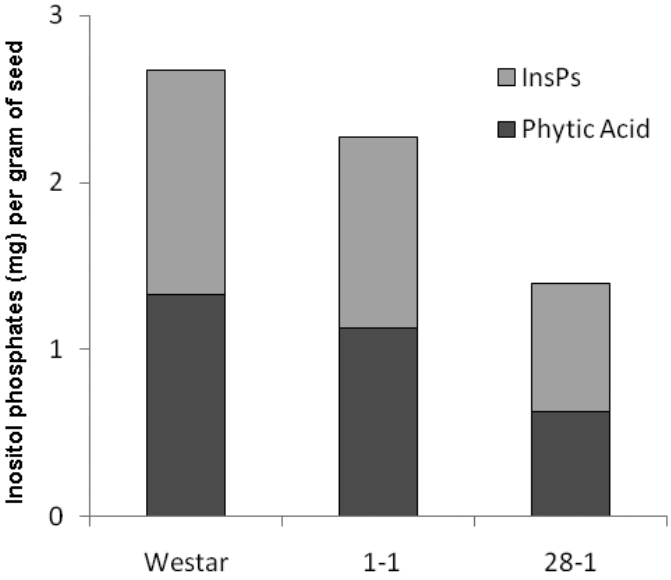


Fig. 6. Phytic acid and total inositol phosphates in mature seed of 'Westar' and transgenic T_3 lines, 1 and 28. The HPLC method does not distinguish completely the several 4-phosphate and 5-phosphate phosphorylated derivatives of *myo*-inositol and precursors to phytic acid (inositol 6-phosphate), which are pooled as "InsPs".



sion. Verbascose was not detected in these tissues, nor have we previously detected verbascose in *B. napus* (C. Bock, unpublished results).

The expression of raffinose synthase-like genes did not change in developing seed according to qRT-PCR (results not shown). It is possible that raffinose synthase transcription is not induced unless galactinol or stachyose has accumulated to a certain level. The gene encoding stachyose synthase of *B. napus* has not been isolated, but the pattern seen in developing seed suggests that it is not regulated by the concentration of its precursor.

A slight increase in sucrose and total sugars, was observed in mature seed of antisense *GolS* lines (Fig. 4C). Expression of *SuSy*, the gene for the sucrose degradation enzyme, did not change substantially in immature seed, but expression of *SPS*, which leads to its synthesis, increased slightly, according to qRT-PCR (results not shown). This might produce an alteration in the balance of sucrose synthesis and catabolism, which together with the decreased synthesis of RFOs, might drive final sucrose accumulation to higher levels.

Inositol phosphate analysis of mature seed showed a slight decrease in the antisense *golS* lines, for both phytate and other inositol phosphate compounds, which are based on *myo*-inositol but not on RFOs (Fig. 6). The level of free *myo*-inositol was negligible in both control and transgenic lines. A multiplicity of functions for *myo*-inositol in plants means that any fluctuation caused by the alteration in RFO metabolism may have been absorbed by other pathways. Although we cannot speculate on the mechanism of reduction in seed phytate levels, the increase in sucrose and the concurrent decrease in seed stachyose and phytate is nutritionally interesting.

An inverse relationship was found between phospholipase Dα (PLDα) activity and raffinose accumulation in *Arabidop-*

sis (Zhou 2002), which may be attributed to the second messenger, phosphatidic acid. The latter may also result from the action of PLC coupled with a diacylglycerol kinase activity (Ruelland et al. 2002). The substantial increase in *PLC2* transcripts in our transgenic lines may be indicative of similar stress-related signals that could be triggered in response to low galactinol levels. If such is the case, the higher expression levels of *PLC2* in transgenic lines may represent a secondary mechanism contributing to the observed low levels of RFOs. Overexpression of *BnPLC2* in *B. napus* was found to delay the formation of RFOs in developing transgenic seeds (unpublished results).

The effects of RFO reduction on seed development, long-term viability, resistance to oxidative stress, freezing or heat stress during seed development and germination have not been determined in *B. napus*. A modest reduction in RFOs should not affect seed viability, but this has not been empirically determined for this species. In petunia plants, reduction of α-galactosidase was accompanied by increased raffinose in tissues, and better frost tolerance (Pennycooke et al. 2003). In *Arabidopsis*, on the other hand, a *GolS*-overexpressing transgenic line had substantially increased raffinose but no more tolerance to cold than the untransformed line (Zuther et al. 2004). Among ecovars of *Arabidopsis*, substantial variation was found in seed raffinose and stachyose. Mature seed of *Arabidopsis* had sucrose concentrations of 1.3%–2.3%, raffinose concentrations of 0.15%–0.55%, and stachyose concentrations of 0.1%–0.6%, with more stachyose than raffinose in almost every line (Bentsink et al. 2000); *Brassica napus* had similar levels of seed RFOs (Fig. 4), which are much lower than those found in many plant species (Kuo et al. 1988) and RFOs in *B. napus* leaf are almost undetectable (results not shown). Together, these data suggest that *B. napus* has minimal dependence on RFOs as a mechanism of cold tolerance. Nishizawa et al. (2008), however, showed that oxidative stress tolerance and salt tolerance were enhanced in *Arabidopsis* which overexpressed *GolS*. The effects of these stresses on the antisense *B. napus* have yet to be determined.

In pea and maize, galactinol synthase activity appeared to be far in excess of requirements for the concentrations of RFOs detected, and therefore was considered an unlikely limiting factor in the synthesis of RFOs (Peterbauer et al. 2001; Zhao et al. 2004), while Nishizawa et al. (2008) found that in *Arabidopsis*, knockout or knockdown of individual *GolS* genes had no effect on the total GolS enzyme activity, that is, there was ample *GolS* redundancy. However, our observations of *B. napus* show that in spite of this redundancy, an antisense strategy can still effectively reduce RFOs.

Alternative approaches to the reduction of RFOs might include removal of the RFOs, for example, via α-galactosidase. The use of α-galactosidase for this purpose has been tested in pea (Polowick et al. 2009) and by our laboratory in *B. napus*. In both instances coffee (*Caffea arabica* L.) α-galactosidase under the control of the 35S promoter was incorporated into transgenic plants. This approach led to moderate reductions in RFOs in *B. napus* (results not shown), and in pea the approach led to up to a 48% reduction in raffinose and a 21% reduction in stachyose (Polowick et al. 2009).

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