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Improving Grain Quality in Pulses: Strategies to Reduce Raffinose Family Oligosaccharides in Seeds

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ABSTRACT

In human diet, pulses are an excellent source of carbohydrates, proteins, dietary fibers, vitamins, minerals and other bioactive compounds. However, the presence of high concentration of raffinose family oligosaccharides (RFO) limits their consumption and acceptance worldwide especially in developed countries. Humans and mono-gastric animals cannot digest RFO but are fermented by large intestinal microflora that produces carbon dioxide, hydrogen and methane causing flatulence and stomach discomfort. Hence, it is imperative to develop strategies to reduce RFO concentration in pulses to promote their consumption in human diet around the world.RFO are sucrosyl galactosidessynthesized during the later stages of seed development. RFO accumulation in seeds is affected by crop species, genotype and growing environment. Genetic strategies have been used to reduce the accumulation of RFO in pulses. Several post-harvest processing methods have also been used to reduce RFO concentration in pulses used for human consumption.

Keywords: oligosaccharides,pulses, raffinose, RFO, stachyose and verbascose

Introduction

Pulse crops, members of the family Fabaceae, are defined by the presence of unusual flowers, podded fruits and their ability to fix nitrogen in their root nodules (de Faria *et al*.1989). The family Fabaceae is further divided into three subfamilies: Papilionoideae, Caesalpinioideae and Mimosoideae (Andrews and Andrews 2017). The three subfamilies show distinct flower characteristics: Papilionoideae has two partially fused petals, two wing petals and a banner like petal; Caesalpinioideae has irregular flowers with no distinct petals; and Mimisoideae is characterized by the presence of spikes. Most pulse crops belong to the subfamily Papilionoideae.

Major pulse crops cultivated for human and animal consumption include field pea (*Pisum sativum* L.), common bean (*Phaseolus vulgaris* L.), chickpea (*Cicer arietinum* L.), broad bean (*Vicia faba* L.), pigeon pea (*Cajanus cajan* L.), cowpea (*Vigna unguiculata* L. Walp.), and lentil (*Lens culinaris* Medik.) (Chibbar *et al*., 2010). Two other legume crops grown primarily for oil production include soybean (*Glycine max* L.) and peanut (*Arachis hypogaea* L.). Pulse crops in general have gained a great significance in crop rotation, due to their nitrogen fixing capability that enriches soil with nitrogen.

Production of pulses has constantly increased during the past decades in Canada that contributed about 35% and 30% to total world production of lentil and beans from 2011-2013, respectively (Table 1). Canada is the largest exporter of lentil/ pea and third largest exporter of beans in the world during 2013 (FAOStAT 2016). Nutritionally,pulses have higher amount of protein (20.6-32.2 g/100 g dry weight) compared to cereal grains (10-12

g/100g dry weight), but are also enriched in the essential amino acid lysine that is deficient in cereal grains (Chibbar *et al*., 2010; Shewry and Halford 2002). The major constituents in pulse seeds are carbohydrates contributing 49-68% to total seed weight (Chibbar *et al*., 2010). Carbohydrates can be classified as monosaccharides, disaccharides, oligosaccharides and polysaccharides based on their polymeric structure (Chibbar *et al*., 2010). The total soluble sugars concentration in pulse seeds range from 3 -13 g/100g (Oomah *et al*., 2011). Total soluble sugars in the pulses include monosaccharides (ribose, fructose and glucose), disaccharides (sucrose, maltose, melibiose) and oligosaccharides (raffinose, stachyose, verbascose, ajugose and ciceritol). Among the soluble sugars, concentration of galacto-oligosaccharides or raffinose family oligosaccharidesishighranging from 2.7 to 5.9 g/100g in seeds (Sosulski *et al*., 1982).

RFO or α-galactosides are sucrosyl derivatives characterized by the presence of α(1→6) linkage between the galactose residue and the C-6 of the glucose moiety of sucrose (Gangola *et al*., 2014a).A major limitation to increase human consumption of pulses is the presence ofhigh seed RFO concentration (Gangola *et al*., 2012).Human and monogastric animals lack alpha-galactosidase required to hydrolyze α(1→6)glycosidic linkages, therefore RFO remain undigested in the upper gastrointestinal tract (Gangola *et al*., 2014b). The undigested oligosaccharides are fermented in the lower gut by anaerobic bacteria producing carbon dioxide, hydrogen and methane (Reddy *et al*., 1984). The higher production of these gases causes flatulence that can lead to stomach discomfort, abdominal rumblings, cramps, pain, and diarrhea. RFO in animal diets have also been associated with a reduction in net dietary energy. Adult roosters fed with diets containing 5.3% RFO showed a 20% reduction in net metabolizable energy compared to a diet containing 1% RFO (Coon *et al*., 1990). Diets with high RFO content caused osmotic imbalance (before fermentation by microbial flora) resulting in reduced nutrient absorption and protein utilization (Wiggins 1984; Van Barneveld 1999). In humans, RFO when consumed in low concentrationsmay have potential beneficial effects as prebiotics promoting the growth of beneficial bacteria like bifidobacterium in the large intestine (Guillon and Champ 2002; Martínez-Villaluenga *et al*., 2008b; Roberfroid 1999; Roberfroid *et al*., 1998). In rats, diets rich in RFO showed an increase in bifidobacterial growth and increased immune response (Gulewicz *et al*., 2002). In humans, consumption of soybean α-galactosides increased bifidobacterial and eubacterial growth in the large intestine (Hayakawa *et al*., 1990; Wada *et al*., 1991). RFO also play an important role in plants and participate in several metabolic processes (Obendorf and Górecki 2012; Sengupta *et al*., 2015) such as phloem transport, and defense responsesduring abiotic (Hannah *et al*., 2006; Nishizawa *et al*. 2008) and biotic stresses (Gil *et al*., 2012). RFO are synthesized during later stages of seed development and are postulated to confer desiccation tolerance (Martínez-Villaluenga *et al*., 2008a).

In pulses such as lentil (Tahir *et al*., 2012) and chickpea (Gangola *et al*., 2013), high RFO concentration has been attributed as one of the reasons for reduced consumption of pulses by humans (Gangola *et al*., 2014b).Hence, reduction of RFO concentration might help to promote human consumption of pulses.However, a major consideration is that RFO concentration needs to be reduced to an optimal amount that reduces stomach discomfort while still maintaining adequate amounts required for seed germination and plant growth.

Structures of raffinose family oligosaccharides

RFO are ubiquitous in plant seeds (Blöchl *et al*., 2007).Raffinose is the first member of this oligosaccharides family followed by stachyose, verbascose and ajugose (Figure 1). The first RFO was found and purified in chickpea hence named as "Ciceritol" (Quemener and Brillouet 1983). The RFO nomenclature is as follows:

Raffinose [*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D- fructofuranoside]

Stachyose [*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D- fructofuranoside]

Verbascose [*α*-D-galactopyranosyl-(1→6) -*α*-D-galactopyranosyl-(1→6)-*α* - D- galactopyranosyl-(1→6)-*α*-D-glucopyranosyl-(1→2)-*β*-D-fructofuranoside]

Ajugose [*α*-D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)-*α*-D- galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→6)*α*-D-glucopyranosyl-(1→2)-*β*-D- fructofuranoside]

Ciceritol [*α*- D-galactopyranosyl-(1→6)-*α*-D-galactopyranosyl-(1→2)-1D-4-O-methyl-chiro-inositol]

Biosynthetic pathway of raffinose family oligosaccharide

Raffinose family oligosaccharides are formed by α-(1→6) galactoside linkages between the linear chain galactosyl residues and the glucose moiety of sucrose (Avigad and Dey 1997) (Figure 1). The three galactosyl donors involved in RFO biosynthesis are: UDP-D-galactose, galactinol and RFO. The biosynthesis of RFO is initiated by galactinol synthase (EC 2.4.1.123) which catalyzes the transfer of a galactosyl residue from UDP-D-galactose to *myo*-inositol to synthesize galactinol (Figure2). Raffinose synthase (EC 2.4.1.82) catalyzes the synthesis of raffinose by the transfer of a galactosyl residue from galactinol to sucrose. Stachyose synthase (EC 2.4.1.67) catalyzes the synthesis of stachyose by the transfer of a galactosyl residue from galactinol to raffinose. The enzyme verbascose synthase (VS) catalyzes the synthesis of verbascose by addition of a galactosyl residue from galactinol to stachyose. The main enzymes involved in the RFO pathway, galactinol synthase (GS or*GolS*), raffinose synthase (RS) and stachyose synthase (StS) have been isolated from some plants and the gene sequences coding for these enzymes have been submitted in NCBI or patented (Allan and Hitz 2000; Oosumi *et al*., 1998). RFO are also synthesized by a galactinol independent biosynthetic pathway. In *Ajuga reptans* the enzyme galactan:galactan galactosyl transferase (GGT), that catalyzes chain elongation by galactosyl transfer between two RFO molecules, was reported (Bachmann *et al*., 1994).

In *Ajuga reptans* two different RFO pools were reported: (i) storage pool - RFO synthesized in mesophyll cells, and (ii) transport pool - RFO synthesized in intermediary cells involved in phloem transport (Bachmann *et al*., 1994). Further compartmentalization studies by purification of vacuoles from mesophyll cells indicated that GGT, stachyose and higher RFO (verbascose) were vacuolar; and GS, StS, *myo*-inositol, galactinol, sucrose and fructose were extra-vacuolar. Raffinose was reported to be distributed in both the vacuole and the cytoplasm (Bachmann and Keller 1995). Stachyose synthesized in the cytoplasm was proposed to be transferred to the vacuole through a stachyose transporter in the tonoplast (Bachmann and Keller 1995). Two allelic variants of *GolS* were isolated in *Ajuga reptans* (Sprenger and Keller 2000). Gene expression, RFO accumulation and GS activity suggested functional differences among the two isoforms. *ArGolS1* was predominantly present in the storage RFO pool in mesophyll cells and *ArGolS2* was predominant in transport RFO pools in intermediary cells (Sprenger and Keller 2000).

A. Galactinol synthase

In Cucurbitaceae leaves, GS was a monomeric polypeptide of 38-43 kDa with 318-348 amino acid residues. GS enzyme activity hadpH optima between 5.6 and 7.5, and it requiredMn^{2+} as a cofactor. The K_m values for UDP-D-galactose and *myo*-inositol ranged from 0.16 – 0.53 mM and 4.0 – 6.5 mM, respectively (Keller and Pharr 1996; Peterbauer *et al*., 2001b). Most of the studies on GS suggest that RFO accumulation is controlled by the concentrations of initial substrates, *myo*-inositol and sucrose, rather than only by galactinol synthase activity (Karner *et al*., 2004).RFO concentration in chickpea seeds also showed a significant positive correlation to initial substrates concentrations (Gangola *et al*., 2013).

The presence of more than a single isoform of *GolS* showing differential expression during abiotic stresses has been reported in several plant species. Three genes coding for GS were characterized in *Arabidopsis thaliana* as, *AtGolS1*, *AtGolS2* and *AtGolS3*. All three recombinant AtGolS1, AtGolS2 and AtGolS3 proteins expressed in *E. coli* showed GS activity. Differential expressions of GS genes were obtained during abiotic stresses, where, *AtGolS1* and *AtGolS2* were induced during drought and salinity stress, but not by cold stress. The third isoform, *AtGolS3* was induced by cold stress but not by drought and salinity stress (Taji *et al*., 2002).

In *Zea mays*, three *GolS* isoforms were isolated, but they showed differential genes expressions. *ZmGolS1* expression was not observed during seed development. Transcript accumulation of *ZmGolS2* was observed towards later stages of seed development and rapid decrease of transcripts was observed at imbibition during seed germination. *ZmGolS3* transcripts were only detected when seed germination was interrupted by desiccation (Zhao *et al*., 2004). In *Coffea arabica* three allelic variants, *CaGolS1*, *CaGolS2* and *CaGolS3* encoding polypeptides with 388, 334, 344 amino acids, also showed differential transcript accumulation under drought, salinity and heat stress conditions. *CaGolS1* showed high expression during stressed and non-stressed conditions, *CaGolS2* was expressed only during severe water deficit and *CaGolS3* was expressed during all experimental stresses but at reduced level than *CaGolS1* (dos Santos *et al*., 2011). In tomato (*Lycopersicon esculentum*), *LeGolS1* transcripts weredetected 35 days after anthesis until seed maturity (60 days after anthesis) (Downie *et al*., 2003).

Two GS isoforms in *Ajuga reptans*, *ArGolS1* and *ArGolS2*were characterized. *ArGolS1*was source-leaf specific and *ArGolS2* participated in RFO transport. Gene expression studies showed that *ArGolS1*

transcripts were found in mesophyll and *ArGolS2* in intermediary cells explaining its role in phloem transport (Sprenger and Keller 2000). Recently, two galactinol synthase isoforms, *LcGolS1* and *LcGolS2*, were reported in lentil (*Lens culinaris* Medik.; Kannan *et al*. 2016). Both the isoforms showed expression during lentil seed development however, *LcGolS2* showed maximum expression at 24 days after flowering (DAF) whereas, *LcGolS1* transcripts accumulated mainly during 26-32 DAF.

B. Raffinose synthase

The purification of raffinose synthase (RS) was first reported by Lehle and Tanner (1973) from *Vicia faba*. The purified RS had a molecular mass of 90 kDa and exhibited a pH optimum between 6.5 and 7.0. In a subsequent study pea (*Pisum sativum*) RS was partially purified, which showed apH optimum of 7.0, and K_m values of 7.3 mM and 22.9 mM for galactinol and sucrose, respectively (Peterbauer *et al*. 2002a). Expression of a *RS* cDNA clone in *Spodoptera frugiperda* Sf21 insect cells, produced recombinant RS with kinetic properties like those of the purified RS (Peterbauer *et al*., 2002a). A *RS* cDNA clone isolated from rice (*Oryza sativa*) was expressed in *E. coli* to produce recombinant RS. The rice recombinant RS also showed maximum activity at pH 7.0 at 45°C (Li *et al*., 2007).

In *Arabidopsis*, five putative RS genes (*AtRS1-5*) or seed imbibition proteins (SIP) were described (Nishizawa *et al*., 2008). Among the five *AtRS* genes described, *AtRS5* showed high sequence similarity to the RS characterized in *Pisum sativum*. Heterologous expression of recombinant *AtRS5* showed RS activity (Egbert *et al*., 2013). Further, raffinose concentration was reduced in seeds of Arabidopsis *AtRS5* mutant. No *AtRS5* expression or activity was detected in leaves in mutant plants under unstressed or stressed conditions (Egbert *et al*., 2013). *RS2*/*ATSIP2*showed sequence similarity to α-galactosidase genes. Recombinant protein of *ATSIP2* expressed in Sf9 insect system showed raffinose specific α-galactosidase activity (Peters *et al*., 2010). RS has been reported as the most unstable enzyme in the RFO biosynthetic pathway (Castillo *et al*., 1990; Peterbauer *et al*., 2002a). Low RFO line (*stc*1 mutant) identified in *Glycine max* was associated with RS2 allele showing low raffinose synthase activity and higher accumulation of galactosyl cyclitols (Dierking and Bilyeu 2008; Hitz *et al*., 2002; Obendorf and Górecki 2012; Sebastian *et al*. 2000). The low RFO soybean genotypes showed good field emergence and yield like wild type.

Stachyose synthase

Stachyose synthase (StS) has been purified from adzuki bean, kidney bean, lentil and pea (Hoch *et al*., 1999; Peterbauer and Richter 1998; Peterbauer *et al*., 2002b; Tanner and Kandler 1968). StS purified from mature lentil seeds had a specific activity of 9.09 pkat/mg protein, a molecular mass of 88.6 kDa and an isoelectric point of 4.8 (Hoch *et al*., 1999). The amino acid sequence of StS (853-868 amino acids) was first obtained from *Vigna angularis* (Peterbauer *et al*., 1999). The molecular weight of StS was 85 to 95 kDa, with a pH optima of 6.5 - 7.0 (Richter *et al*., 2000). StS shows a broad range of substrate specificity which includes inositols and inositol O-methyl ethers. StS from adzuki bean showed no conversion of pinitols, whereas lentil StS catalyzed the synthesis of galactopinitol A and ciceritol, in addition to stachyose synthesis (Hoch *et al*., 1999; Peterbauer *et al*., 2001a). StS purified from adzuki bean and lentil showed no synthesis of verbascose (Hoch *et al*., 1999; Peterbauer and Richter 1998). StS from *Pisum sativum* synthesized both stachyose and verbascose (Peterbauer *et al*., 2002b and 2003)

In *Ajuga reptans,* RFO biosynthesis also occurred through a non-galactinol independent enzyme galactan:galactan galactosyl transferase (GGT) present in leaf vacuoles (Bachmann and Keller 1995; Haab and Keller 2002). GGT amino acid sequence showed high similarity to α-galactosidases and a non-sequence specific vacuolar sorting determinant at the C-terminal (Haab and Keller 2002; Tapernoux-Luthi *et al*., 2007). The presence of GGT activity (neutral pH) was reported in pea seeds with a high verbascose concentration and undetectable activities in a low verbascose pea line (Obendorf and Górecki 2012; Peterbauer *et al*., 2002b and 2003;). Since previous reports suggest that galactinol is exclusively cytoplasmic and stachyose is exclusively vacuolar, higher RFO might be produced by the galactinol independent pathway (Peterbauer *et al*., 2001a).

Interestingly, RFO biosynthetic enzymes have shown a wide range of substrate specificity. Galactinol synthase also catalyzed the synthesis of fagopyritol B1 where D-*chiro*-inositol was the galactosyl acceptor (Lahuta *et al*., 2005; Obendorf and Górecki 2012; Ueda *et al*., 2005). Raffinose synthase synthesized galactosyl ononitol and galactopinitol A from D-ononitol, D-pinitol and O-methyl cyclitols not naturally present in pea (Obendorf and Górecki 2012; Peterbauer *et al*., 2002a). Stachyose synthase from Lentil also synthesized fagopyritol B1 from D-chiroinositol and galactinol. Synthesis of galactopinitol B was also catalyzed by lentil stachyose synthase at a lower rate (Hoch *et al*., 1999).

Variation in RFO concentration among different legume crops

RFO concentration varies widely among different legume crops (Table 2). The concentration and composition of RFO depend on type of crop, growing environment and the genotype (Andersen *et al*., 2005; Gangola *et al*., 2013; Martín-Cabrejas *et al*., 2008; Reddy and Salunkhe 1980; Tahir *et al*., 2011). Reddy and Salunkhe (1980) reported verbascose (34.4 g/kg) as predominant RFO followed by stachyose (8.9 g/kg) and raffinose (trace) in black gram (*Vigna mungo* L. Hepper). Faba bean was reported to contain higher amount of verbascose (27.0 g/kg) while field pea was found to have higher amount of stachyose (27.0 g/kg). Sosulski *et al*. (1982) studied the variation in RFO concentration in eleven legumes and reported stachyose as the major RFO component in chickpea (Gangola *et al*., 2014b) and lentil flours. They also reported verbascose as the predominant RFO in mung bean and fababean. Quemener and Brillouet (1983) detected ciceritol in chickpea (28.0 g/kg dehulled seed), lentil (16.0 g/kg), white lupin (6.5 g/kg), soybean (0.8 g/kg), and bean (in traces). Saini and Knights (1984) studied the variation for total oligosaccharides in desi and kabuli chickpeas (seven varieties of each). They concluded that on average kabuli chickpeas (14.7, 53.0 and 1.2 g/kg of raffinose, stachyose and verbascose, respectively) contained 3.2% higher levels of total oligosaccharides compared to desi types (14.8, 50.6 and 1.5 g/kgof raffinose, stachyose and verbascose, respectively). Gangola *et al*., (2013) also reported a relatively higher concentration of total RFO in kabuli type (21.1 - 53.8 mmol/kg) compared to desi type (15.8 - 53.1 mmol/kg) chickpea genotypes.

In cowpea (*Vigna unguiculata*L. Walp.) and soybean, RFO concentration contributed more than 50% to total soluble sugars (Martín-Cabrejas *et al*., 2008). Cicek *et al*., (2006) studied various soybean seed characteristics including RFO variation using recombinant inbred lines among which stachyose (30 - 60 g/kgseed) was found as the major RFO constituent followed by raffinose (2 - 9 g/kgseed). Comparable results were reported by Kumar *et al.,* (2010) in soybean seeds showing range of 6.4 - 25.3 and 20.9 - 71.0 mmol/kgfor raffinose and stachyose concentrations, respectively.

Andersen *et al*., (2005) studied the compositional variations of α-galactosides in barley and various species of Leguminosae and Brassicaceae. The highest concentration of total RFO was reported in Lupin (91.0 ± 26.0 g/kg seeds), while *Brassica* species contained 14.0 ± 5.0 g RFO/kg of seeds (only raffinose and stachyose). Barley (*Hordeum vulgare* L. cv. Vega) contained 5.0 g raffinose/kg of seeds, which was the sole RFO component. However, Lupin was reported to have 3.0 - 19.0, 23.0 - 86.0 and up to 35.0 g/kg of raffinose, stachyose and verbascose, respectively (Martinez-Villaluenga *et al*., 2008a). Among studied species of Leguminosae and Brassicaceae, ajugose was present exclusively in lupin seeds. *L. albus* and *L. mutabilis* contained the lowest ajugose concentration (2.0 - 5.0 and 2.0 g/kg, respectively) followed by *L. angustifolius* (17.0 - 26.0 g/kg) and, *L. luteus* (6.0 - 46.0 g/kg; Andersen *et al*. 2005; Martinez-Villaluenga *et al*. 2008a).

Vidal-Valverde *et al.,* (1998) observed higher amount of verbascose [22.9 g/kg dry matter (DM)] followed by stachyose (11.0 g/kg DM) and raffinose (2.8 g/kg DM) in fababean. Total α-galactosides concentration of 18 pea varieties varied from 22.6 to 63.4 g/kgDM. Stachyose (10.7 - 26.7 g/kgDM) was found in higher amount than raffinose (4.1 - 10.3 g/kgDM), while verbascose was present in fifteen varieties ranging from 1.7 - 26.7 g/kgDM (Vidal-Valverde *et al*., 2003). Tahir *et al.,* (2011) analyzed eleven lentil cultivars, grown in two different environments, varying for stachyose, raffinose and verbascose concentrations that ranged 22.0 - 25.5, 19.5 - 22.2 and 11.5 - 13.3 g/kgof lentil seed meal, respectively. In another study, Lentil seeds RFO concentrations ranged from 9.22 to 19.68 g/kgfor verbascose and from 23.19 to 27.93 g/kg for raffinose+stachyose (Johnson *et al*., 2013).

The significant impacts of genotype, environment and their interaction on seed RFO concentrationshave been reported in some of the legume crops like soybean (*Glycine max* L. Merr.; Cicek *et al*. 2006; Kumar *et al*., 2010; Jaureguy *et al*., 2011), lentil (*Lens culinaris* Medikus subsp. Culinaris; Tahir *et al*., 2011) and chickpea (*Cicer arietinum* L.; Gangola *et al*., 2013). Consequently, broad sense heritability of RFO traits in legumes has been reported from low to high (0.25 - 0.85) depending on the crop, genotype and environment (Cicek *et al*., 2006; Gangola *et al*., 2013; Tahir *et al*., 2011). The environment influenced variation in RFO concentration suggest their role as antioxidants and phloem-mobile signaling compounds during diverse types of stresses. Therefore, environmental conditions like temperature, rainfall and light intensity influence RFO concentration, *i.e.* more adverse condition would result in higher RFO concentration (ElSayed *et al*., 2014).

Strategies to reduce RFO in seeds

Two main strategies have been employed to reduce RFO concentration in the seeds: (i) Post-harvest processing methods, and (ii) Molecular approaches

A. Procession methods

(i) De-hulling

Dehulling of cowpea (*Vigna unguiculata* L. Walp) seeds caused a significant reduction in RFO

concentration (Onyenekwe *et al*. 2000). However, in *Lens culinaris* varieties, dehulling decreased raffinose but increasedstachyose and verbascose concentrations (Wang *et al*., 2009). This shows that interaction of variety and processing method can led to different results in different crop species.

(ii) Germination

Germination has been found to remove RFOs quite effectively in legumes (Chilomer *et al*., 2010; Gulewicz *et al*., 2014; Khalil and Mansour 1995; Martín-Cabrejas *et al*., 2008; Mubarak 2005; Urbano *et al*., 1995; Vidal-Valverde *et al*., 1998). The decrease in RFO concentration during germination has been attributed to increased activity α-galactosidase which hydrolyses the α(1,6)- linkages, thus increasedthe total soluble sugar content and decreased RFO concentration (Martin-Cabrejas *et al*., 2008).

(iii) Aqueous or alcoholic extraction

Soaking has been commonly used during legume processing which decreased RFO concentration in severalpulses (Aguilera *et al*., 2009; Han and Baik 2006; Martín-Cabrejas *et al*., 2004 and 2006; Onyenekwe *et al*., 2000). Reduction due to hydration depends on differential solubility of individual oligosaccharides and their diffusion rates (Aguilera *et al*., 2009; Shimelis and Rakshit 2007; Upadhyay and Garcia 1988) but activation of enzymes like α-galactosidases upon hydration may also be responsible for reduced RFO concentrations (Aranda *et al*., 2001; Onyenekwe *et al*., 2000; Wang *et al*.,2003). Autolysis during soaking and extraction in the soak water and the cook water also decreased oligosaccharide concentrations in seeds (Wang *et al*., 2009). Ethanol extraction of RFO also increased amino acid usage and availability in soybean meal and resulted in more protein and energy dense product (Glencross *et al*., 2003; Leske and Coon 1999; Leske *et al*., 1995). Though it may be an effective method, ethanol extraction is not economically viable for the large-scale production of low RFO concentration seeds (Hagely 2013).

(iv) Changes in temperature or humidity or pressure treatment

Heat treatment (e.g. boiling, autoclaving, microwave cooking and extrusion at elevated temperature) decreased anti-nutritional RFOs (Alajaji and El-Adawy 2006; Devindra *et al*., 2011;El-Adawy 2002; Frias *et al*., 2011; Jenkins *et al*., 1982; Khalil and Mansour 1995; Vijayakumari *et al*., 2007; Wang *et al*., 2008; Wang *et al*., 2010). It was proposed that the decrease in raffinose and stachyose during cooking was due to thermal hydrolysis and formation of disaccharides and monosaccharides or other compounds from the RFOs (Onigbinde and Akinyele 1983; Wang *et al*., 2008). Industrial process of dehydration also affected the α-galactoside content by inducing changes in the carbohydrate fraction including hydrolysis of α-galactosides (Aguilera *et al*., 2009). However, long cooking time can also cause loss in proteins which could be attributed to partial removal of certain amino acids on heating (Aguilera *et al*., 2009; Rehman and Shah 2005; Wang *et al*., 2010; Youssef *et al*., 1986). Cooking after soaking treatment showed more noticeable decrease in RFO content than soaking alone and depended on the crop type (Aguilera *et al*., 2009; Martín-Cabrejas *et al*., 2006; Sánchez-Mata *et al*., 1999). Blanching has also been tried to reduce RFO concentration in pulses (Wang *et al*., 1997). Autoclaving has been suggested as a better method of processing to reduce RFO concentration in seeds (Vijayakumari *et al*., 2007).

(v) Treatment with microbial or plant α-galactosidase

Alpha-galactosidase is present in plants, microorganisms and animals (Dey and Campillo 1984; Kim *et al*., 2002). Endogenous synthesis of α-galactosidases increases during seed germination and results in lower concentrations of RFO in germinating seeds (McCleary and Matheson 1974). This is the biochemical principle for using germination and fermentation to reduce RFO concentration (Devindra *et al*. 2011; Glencross *et al*., 2003; Granito *et al*., 2002; Ibrahim *et al*., 2002; Torres *et al*., 2006; Vidal-Valverde *et al*., 1998;Yamaguishi *et al*., 2009). These procedures take advantage of the natural role of plant and microbial α-galactosidases. However, the potential microbial contamination of germinated grain legume seeds decreased their shelf life, making them unsuitable for food and feed use (Kadlec *et al*., 2006). While many studies showed positive effects for the enzymatic removal of RFOs (Anisha and Prema 2008; Cao *et al*., 2010; Girigowda *et al*., 2005; Leblanc *et al*., 2004; Veldman *et al*., 1993; Yamaguishi *et al*., 2009), still there are reports where diets supplemented with α-galactosidase showed no improvements in protein digestibility (Brasil *et al*., 2010; Irish *et al*., 1995; Smiricky *et al*., 2002). The potential of this approach is restricted due to poor stability of enzymes or their origin from microbes without generally recognized as safe (GRAS) status (Gote *et al*., 2004; King *et al*., 2002; Viana *et al*., 2007). Use of isolated enzymes is another option but it greatly increases processing costs. Since α-galactosidase is sensitive to pH and heat, and loses its activity rapidly during storage at

room temperature, novel coating treatments have been suggested such as encapsulation of α-galactosidase in chitosannanoparticles that could be developed into a pH-sensitive feed enzyme-releasing system (Liu *et al*., 2011). Though microbial enzymes are more efficient (Falkoski *et al*., 2006) and provide convenience of easy growth and isolation, soybean α-galactosidase may be a better choice because it is more suited for high protein and buffered environment of soybean (Viana *et al*., 2005). Alpha-galactosidase from coconut kernel immobilized to sepharose-4B gel also reduced total flatulence by 53-73%. This was advantageous as no clogging occurred when soy milk was passed through glass columns with enzyme containing gels (Dharamsena and Mathew 2002).

(vi) Irradiation

Irradiation is commonly used to control insect infestation and extend the shelflife of pulses (Machaiah *et al*., 1999), but it can also lower RFO levels by their rapid degradation (Al-Kaisey 2003; Machaiah *et al*., 1999). Gamma radiation along with germination showed distinct legume-specific quantitative changes in RFO concentration without altering their positive sensory attributes (Machaiah and Pednekar 2002; Rao and Vakil 1983). However,such treatment increase the cost as well as energy required for pulses production.

B. Molecular approaches to reduce RFO in seeds

(i) Up-regulation of α-galactosidase and galactosyl cyclitols synthesis

Alpha-galactosidase is a well-known enzyme for RFO break down by hydrolyzing α(1→6) linkage. Overexpression of α-galactosidase from coffee (*Coffea arabica* L.) was used to reduce RFO concentration in peas (Polowick *et al*., 2009). The transgenic pea lines showed up to 40% reduction in raffinose and stachyose concentrations without affecting seed germination rate (96%). Zuo *et al*., (1996) had showed that although much lower oligosaccharide concentrations were present in genetically altered soybean meal; no differences were noted between conventional and low oligosaccharide soybean meal in any of the digestion responses in ileally-cannulated dogs.

Further reductions in the endogenous RFOs could be obtained by use of improved vectors, RNAi or antisense technology and development of homozygous lines (Polowick *et al*., 2009). It would be a better strategy if α–galactosidase could be activated after harvesting to degrade RFO after harvesting. This can be based on the transfer of α-galactosidase from a thermophilic bacterium into grain legumes which can be activated during canning (Griga *et al.,* 2001; Wang *et al.,* 2003).

Frias *et al*., (1999) suggested an alternative strategy to reduce RFO concentration byincreasing the synthesis of related compounds such as the galactosyl cyclitols. This would maintain the protective nature of these compounds but decrease their flatus potential, as the ciceritol was more slowly hydrolyzed by α–galactosidase than the RFO. Ciceritol is present in chickpea and lentil but has not been detected in pea. The key to introduce galactinol cyclitols into pea with an accompanied reduction in the RFO content appears to lie with stachyose synthase, which has a vital role in the synthesis of the galactinol cyclitols and in the synthesis of stachyose (Peterbauer and Richter 2001b). It represents a link between the RFO and galactinol cyclitol pathways (Wang *et al*., 2003).The ratio of D-pinitol and *myo*-inositol influenced the RFO concentration in developing tiny vetch [*Vicia hirsute* (L.) S. F. Gray] seeds (Lahuta *et al*., 2005). Galactosyl pinitols can replace RFOs as reserve carbohydrates for seed germination in *Vicia villosa* (Lahuta and Goszczyńska 2009). It has also been reported that free cyclitols inhibit StS and/or VS activity in developing seeds of *Vicia*species (Lahuta *et al*., 2010). Since accumulation of d-chiro-inositol strongly reduced accumulation of verbascose, the main RFO in pea seeds, transformation of pea with genes encoding d-chiro-inositol synthesizing enzymes has been suggested as a strategy to reduce the accumulation of RFO by inhibiting the synthesis of verbascose (Lahuta and Dzik 2011).

(ii) Down-regulation of key biosynthetic enzyme

Galactinol synthase (GS) is considered as the first committed and key regulating step of RFO biosynthesis influencing carbon partitioning between sucrose and RFO (Nishizawa *et al*., 2008). There has already been a patent regarding genetic manipulation of RFO levels by inhibiting galactinol synthase activity (Kerr *et al*., 1993). Bock *et al*., (2009) used an antisense approach to down-regulate the expression of galactinol synthase in canola (*Brassica napus* L.). Consequently, a decrease in galactinol and stachyose concentrationswas observed in transgenic canola seeds. Out of four main targets (*myo*-inositol concentration, sucrose concentration, galactinol synthase and other biosynthetic enzymes) to regulate RFO biosynthesis,galactinol synthase has been suggested as a potential target to reduce RFO concentration in chickpea seeds (Gangola *et al*., 2016)

(iii) Mapping and Breeding

Genetic manipulation of RFO content by plant breeding can be an effective tool to prevent flatulence

caused by legumes. It is known that there is considerable variation in the raffinose and stachyose content among different varieties of legumes. This variation can be either natural or created through mutagenesis. Transgenics require high energy and time input. Further different regulations make it difficult to release varieties especially for food and feed purposes. In such cases, plant breeding can be a good approach, as used in case of soybean. Methods of germplasm screening as well as chemical mutagenesis have been used to select soybean strains with low RFO or high sucrose (Clarke and Wiseman 2000) and in addition, it also helped in determining the genetic basis of some of the available low RFO traits (Hagely *et al*., 2013). These soybean lines with variant alleles for low RFO not only provided soybean meal that was nutritionally superior to conventional soybean meal (Parsons *et al*., 2000), but also a resource to introgress the low RFO phenotypes into other genetic backgrounds including elite cultivars (Hagely 2013).

The breeding program for soybeanutilized mutants characterized by Hitz *et al*., (2002)[including LR33 having low raffinose, stachyose, *myo*-inositol, and phytic acid (with mutation in MIPS1 gene)] and a soybean plant introduction line, PI 200508, (with lower raffinose and stachyose and increased sucrose) identified by Kerr and Sebastian (1998). This linealso showed decreased raffinose synthase enzyme activity in maturing seeds (Hitz *et al*., 2002). Further studies showed that the line had mutant allele for *RS2* gene (Dierking and Bilyeu 2008). Another independent mutant allele of the *RS2* gene was further identified (Dierking and Bilyeu 2009) and, recurrent selection and plant breeding have led to the development of new soybean lines containing more distinct alterations in sucrose, raffinose, and stachyose contents. Carbohydrate profiles of lines representing a range of characterized RS2 genotypes grown together in one location showed that these profiles were heritable in general and RS2 genotype appeared to be the single largest determinant of carbohydrate profile (Hagley *et al*., 2013). In another recent study, though altered carbohydrate soybeans could produce low RFO phenotype across distinct locations, the carbohydrate profile was found to be affected by the environment (Bilyeu and Wiebold 2016)

In pea also, variant stachyose synthase gene resulted in reduced verbascose content in genotype SD1 (Peterbauer *et al*., 2003). Another recent report from soybean showed the inheritance of high sucrose and low raffinose/stachyose contents in V99-5089 soybean seeds. Due to the strong correlations between sucrose and raffinose ($r = -0.88$), and between sucrose and stachyose ($r = -0.96$), V99-5089- can be a good genotype for use as parent in soybean food-grade improvement programs (Mozzoni *et al*., 2013). Another study identified a 33-bp deletion mutant in the putative *StS* gene (Glyma19g40550) of PI 603176A responsible for ultra-low stachyose content (0.5%) therefore, an indel marker associated with low stachyose content was developed (Qiu *et al*., 2015). Identification of variation both natural and through mutation is necessary for successful pulses improvement programs to reduce RFO concentration in seeds. Anti-nutritional factors in legumes can be efficiently and economically reduced through molecular breeding. Marker-assisted selection has proven a rapid and reliable method for selecting desirable lines for seed quality traits. Recent breakthroughs in genomic sequencing of legumes (Das and Parida 2014), molecular breeding becomes more attractive strategy for RFO reduction in pulses (Hagely 2013). Molecular markers correlating with raffinose family oligosaccharides in soybean are already available, and the availability of markers in other species will also increase with increasing genomic information. Although several quantitative trait loci (QTLs) and associated markers have been identified for sugar content in soybean, it is still necessary to validate these QTLsand confirm associated molecular markers in several genetic backgrounds (Mozzoni *et al.,* 2013).

Concluding remarks

Pulses are environmentally friendly due to their nitrogen fixing capability, which reduces the input costs and enriching the soil with nutrition. Pulses are also very nutritionally diverse grains, and rich source of proteins rich in essential amino acid lysine that is deficient in cereal grains. To completely utilize the nutritional benefits of pulses, more emphasis should be placed on pulse seed quality improvement. Genetic and molecular biological techniques have been used to reduce RFO concentration in some pulses. However, similar strategies can be used to improve the pulse seed quality to increase the protein concentration, improve the amino acid composition and above all enhance protein digestibility so that complete benefit can be realized from pulses consumption in human diet. Pulse carbohydrates also have very good health benefits as pulse starch has higher amylose concentration that cereal grain starch. Research to improve starch concentration and composition in pulses will add to the human health benefits of pulses (Chibbar *et al.,* 2010). In conclusion, pulse improvement should focus both to increase yield as well as improve pulse seed quality to realize the complete benefits of these environmentally friendly grains that have the potential to assure global food and nutritional security.

Table 1. Production of pulses in the world and Canada

Legumes	Years	World		Canada		
		Area harvested	Total production	Area harvested	Total production	Export value
Chickpea (*Cicer arietinum*)	1961-1970	11122839	6671439	NA	NA	NA
	1971-1980	10217041	6511618	NA	NA	NA
	1981-1990	9862896	6674667	NA	NA	NA
	1991-2000	10824941	8050048	52343	72269	7183
	2001-2010	10656554	8750603	124410	149350	44986
	2011-2013	13052911	12155054	67267	140533	68260
Soybean (*Glycine max*)	1961-1970	26548450	34442545	108373	210716	6123
	1971-1980	41172412	66468637	204565	439081	10399
	1981-1990	53306881	95580502	421870	998160	40962
	1991-2000	64109804	135011549	854520	2243850	140805
	2001-2010	90766274	213207790	1190820	2979290	539093
	2011-2013	106664474	259829434	1680333	4843700	1445843
Lentils (*Lens culinaris*)	1961-1970	1688968	969648	NA	NA	NA
	1971-1980	2069018	1233391	10083	6806	NA
	1981-1990	2879647	2113347	104720	115630	27346
	1991-2000	3454660	2818101	373487	482190	115842
	2001-2010	3798903	3485664	714900	938620	418174
	2011-2013	4289906	4693991	985500	1650100	883659
Beans, dry (*Phaseolus vulgaris*)	1961-1970	23656624	11930983	34288	48739	3391
	1971-1980	24016286	13040812	55767	80363	20481
	1981-1990	26224268	15649445	42530	71700	34439
	1991-2000	25239779	16935482	99258	178450	71581
	2001-2010	27378366	20425247	153380	297910	184821
	2011-2013	29656745	23422508	90597	207787	214073
Peas, dry (*Pisum sativum*)	1961-1970	9524463	9824185	25240	32154	1576
	1971-1980	7657213	9096667	34036	55223	8002
	1981-1990	8839051	12620974	126360	215560	34303
	1991-2000	6869300	12350106	690110	1516870	161560
	2001-2010	6300669	10302944	1305530	2677390	457821
	2011-2013	6444392	10378984	1233433	3101900	1099015
Cow peas, dry (*Vigna unguiculata*)	1961-1970	4355361	1117818	NA	NA	NA
	1971-1980	4185682.2	1273051	NA	NA	NA
	1981-1990	4328346	1548741	NA	NA	NA
	1991-2000	8336560	2967803	NA	NA	NA
	2001-2010	10587941	4965438	NA	NA	NA
	2011-2013	11061517	5472601	NA	NA	NA

Units for area harvested, total production and export value are hectare, tonnes and ×1000 US$, respectively.

Table 2. Variation in RFO (including Ciceritol) concentrations among different legume crops

Legume crops	Concentration (g/kg dry matter)*					
	Raffinose	**Stachyose**	**Verbascose**	**Ajugose**	**Ciceritol**	**References**
Chickpea (*Cicer arietinum*)	4.5 – 21.0	17.2 – 61.5	ND - 45.0	ND	~28.0	1, 3, 4, 5, 6, 7, 12, 13, 14, 15
Soybean (*Glycine max*)	6.7 - 11.5	27.5 - 28.5	ND - 3.0	ND	0.5 - 0.8	1, 2, 3, 6, 7
Lupin (*Lupinus albus*, L. *luteus*, L. *angustifolius* and L. *mutabilis*)	3.0 - 19.0	23.0 - 86.0	ND - 35.0	02.0 - 46.0	6.5	1, 3, 6, 7
Cow pea (*Vigna unguiculata*)	4.1	32.2 - 44.4	4.8	ND	0.4	1, 2, 3
Lentil (*Lens culinaris*)	3.1 - 10.0	14.7 - 31.0	4.7 - 31.0	ND	16.0	1, 3, 4, 6, 7
Field pea (*Pisum sativum*)	6.0 - 14.0	17.1 - 27.0	23.0	ND	ND	1, 3, 6, 7, 9, 10
Mung bean (*Vigna radiata*)	2.3	9.5	18.3	ND	ND	1
Fababean (*Vicia faba*)	1.0 - 3.0	6.7 - 15.0	14.5 - 31.0	ND	ND	1, 3, 4, 6, 7, 9, 11
Black gram (*Vigna mungo*)	Traces	8.9	34.4	ND	ND	8
Bean (*Phaseolus vulgaris*)	<0.5 - 25.0	2.0 - 42.0	0.6 - 40.0	ND	Traces	3, 4, 5, 6, 7

[1] Sosulski *et al*., 1982; [2] Martín-Cabrejas *et al*., 2008; [3] Quemener and Brillouet 1983; [4] Dilis and Trichopoulou 2009; [5] Wang *et al*., 2010; [6] Andersen *et al*., 2005; [7] Martinez-Villaluenga *et al*., 2008a; [8] Reddy and Salunkhe 1980; [9] Huynh *et al*., 2008; [10] Vidal-Valverde *et al*., 2003; [11] Vidal-Valverde *et al*., 1998; [12] Saini and Knights 1984; [13] Alajaji and El-Adawy 2006; [14] Frias *et al*., 2000; [15] Jukanti *et al*., 2012
*ND = not detected

Figure 1. Chemical structure of *myo*-inositol, UDP- Galactose, galactinol, raffinose, stachyose, verbascose, ajugose and ciceritol

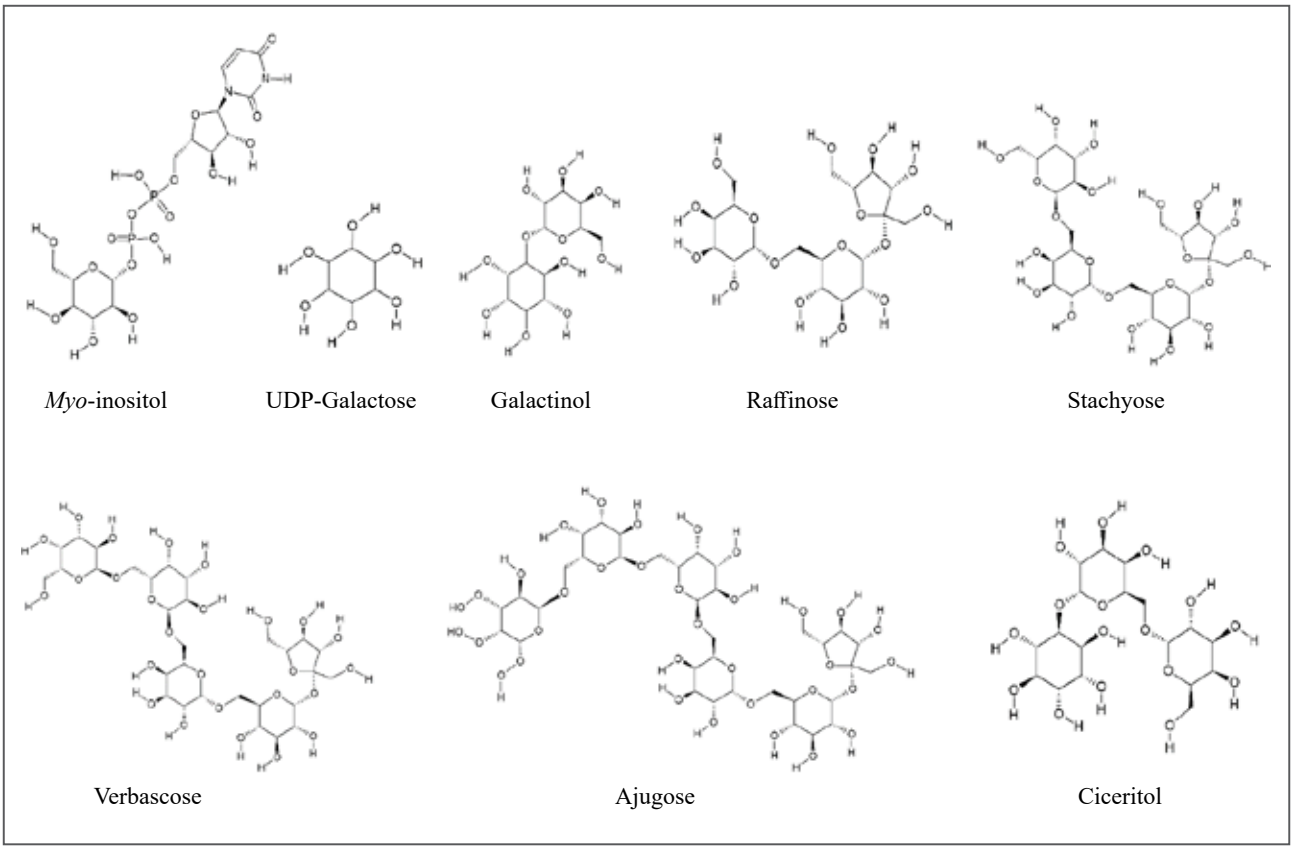
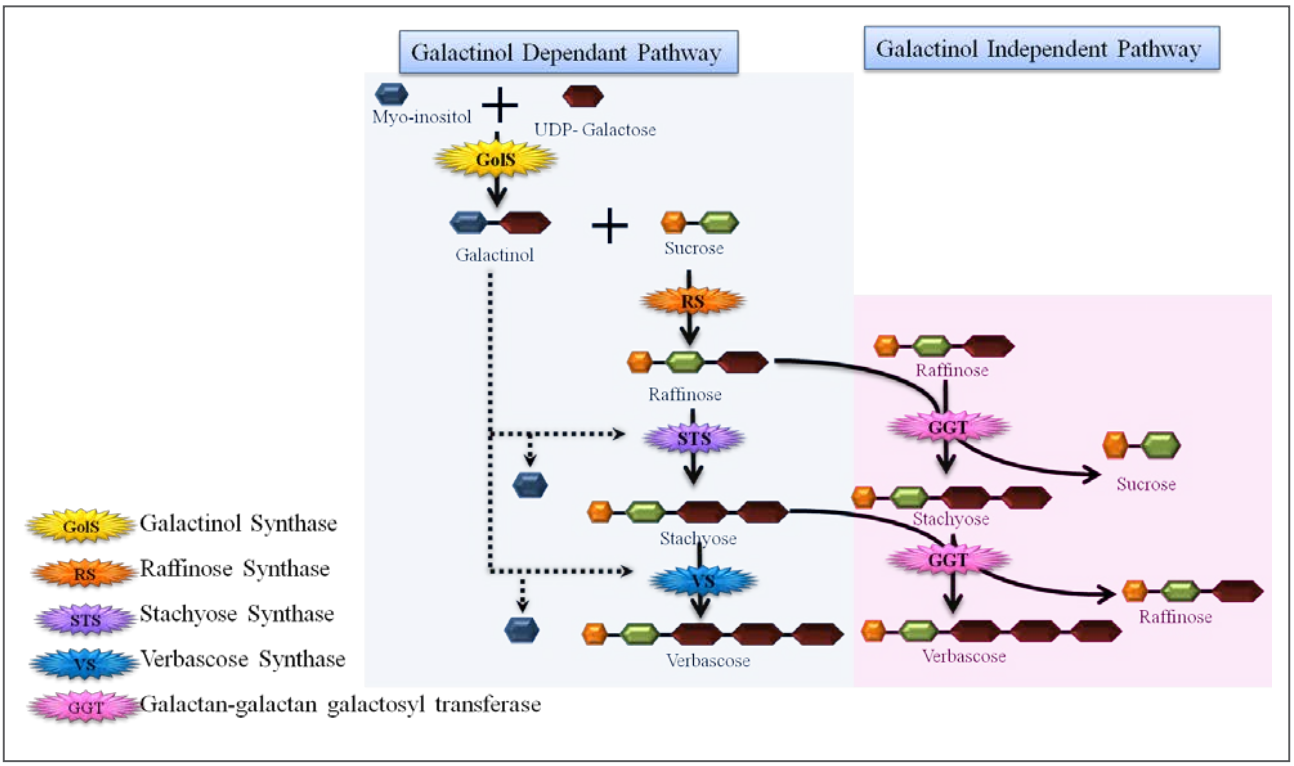


Figure 2. Schematic representation of RFO biosynthetic pathway in plants



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