Variability in the Oligosaccharide Concentration in Seeds of the Mapping Population of Pea (*Pisum sativum* L.)

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Abstract

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Anti-nutritional compounds are among the obstacles to the use of pea seeds as a protein source in both feed and food. These compounds are poorly digested by both monogastric animals and humans. There are three main oligosaccharides in pea: raffinose, stachyose and verbascose (raffinose family oligosaccharides – RFOs). The concentration of oligosaccharides in dry seeds, the oligosaccharide percent to the total content of soluble sugars and quantitative trait loci (QTLs) were analysed in the mapping population Wt10245 × Wt11238. The composition and concentration of soluble carbohydrates in seeds harvested from two field experiments (2002 and 2004) were analysed by the high resolution gas chromatography method. The Wt10245 × Wt11238 population was chosen because of the greater difference in the concentration of RFOs in seeds between parental lines (56.48 mg/g seed in Wt10245 and 99.1 mg/g seed in Wt11238). The average levels of oligosaccharides (mg/g seed) from both field experiments in the mapping population were: *myo*-inositol 1.5, sucrose 33.3, galactinol 0.8, raffinose 9.6, stachyose 30.1, verbascose 37.1. The total oligosaccharide concentration was 76.8 mg/g seed. This comprised anaverage of 68% soluble sugars, with the range from 59% to 75%. There was no interaction between lines and years of experiments (significance of lines × year interaction, *F* statistic > 0.01). One main quantitative trait locus was found for both experiments in LG VA (the *tl-r* interval) and three additional: in LG I (five traits 2002 and 2004 near *afp1k*), LG II (two traits 2002 near *afp15h*) and LG IIIB (five traits 2004 and 2002 near *afp4i* and *M16*). The main QTL was responsible for the level of RFOs and the total soluble sugar concentration in seeds. The results are in agreement with the knowledge of RFO biosynthesis. This makes selection for changes in the proportion of the particular oligosaccharides difficult, like in *Phaseolus*. However, it is possible to decrease the RFO content in pea seeds. The linkage between QTL and the gene *r* is interesting. The *rugosus* (*r*) locus changes the morphology and distribution of starch grains, decreases the total starch accumulation, produces a higher ratio of amylose to amylopectin and higher sugar and water content during development along with changes in cell size and lipid content.

Keywords: genetic map; molecular markers; QTL; *rugosus* (*r*) locus; seed oligosaccharide

Pea seeds are an important source of protein. However, their value as ingredients in food and feed is limited by a considerable amount of anti-nutritional factors, among them sucrose α-galactosides (raffinose, stachyose and verbascose, named the raffinose family of oligosaccharides, RFOs). Because of the lack of α-galactosidase in the human digestive system, RFOs appear unchanged in the large intestine, where they are degraded and fermented by microflora, causing cramps and flatulence (Coon *et al.* 1990). In effect, the true metabolizable energy and digestibility can be decreased. However, the presence of RFOs in the digestive tract stimulates development of some probiotic bacteria. The nutritional importance of

RFOs and other α-D-galactosides – galactosyl cyclitols – present in legume seeds was reviewed in detail by Martinez-Villaluenga *et al.* (2008). The anti-nutritional effect of RFOs can be decreased by seed germination, soaking, cooking, fermentation, enzyme addition and genetic manipulation (Martinez-Villaluenga *et al.* 2008). The last approach needs the discovery in detail of the RFO biosynthetic pathway and its genetic background. The RFO biosynthesis pathway is initiated by producing galactinol from UDP-galactose and *myo*-inositol by galactinol synthase. Galactinol serves as the main galactosyl donor for the galactosylation of sucrose by raffinose synthase and for the galactosylation of raffinose by stachyose synthase (Peterbauer & Richter 2001; Obendorf & Górecki 2012). According to Peterbauer and Richter (2001) three enzymes participate in the RFO biosynthesis pathway: galactinol synthase (GolS; EC 2.1.4.123), raffinose synthase (RS; EC 2.4.1.82), and multifunctional stachyose synthase (STS; EC 2.4.1.67), which is able to synthetize both oligosaccharides – stachyose and verbascose (Peterbauer *et al.* 2003). GolS catalyzes the first step in the biosynthesis of RSOs. RS and STS control subsequent steps in the biosynthesis of raffinose saccharides by adding a galactose unit to sucrose (RS) or raffinose and stachyose (STS), respectively. Some experiments indicate that an additional enzyme – verbascose synthase – can be responsible for the synthesis of verbascose (Lahuta *et al.* 2010). However, verbascose synthase, the enzyme exclusively responsible for the galactosylation of stachyose, has not yet been identified (Obendorf & Górecki 2012). Mutations in genes encoding enzymes engaged in the RFO biosynthetic pathway can affect RFO accumulation during seed development and maturation. In the soybean genome appears one (out of two identified) raffinose synthase gene, completely associated with the low raffinose and low stachyose phenotype (Dierking & Bilyeu 2008). A single mutation in a soybean seed expressing the *myo*-inositol 1-phosphate synthase gene leads to a dramatic reduction of enzyme activity and decreases the accumulation of RFOs (Hitz *et al.* 2002). The reduction of RFO level can be achieved also by genetic manipulation. Downregulation of galactinol synthase significantly reduces the accumulation of RFOs in canola (*Brassica napus*) seeds (Bock *et al.* 2009). Recent advances in the development of improved molecular marker technology enabled the genetic dissection and characterization of many quantitatively inherited seed quality traits. Maughan *et al.* (2000) identified genomic regions significantly associated with quantitative trait loci (QTL) controlling sucrose content in soybean. In soybean four QTL for oligosaccharide content were identified (Kim *et al.* 2006). In *Medicago truncatula* QTL mapping revealed one QTL that explained variation in the stachyose/verbascose ratio co-located with a stachyose synthase gene, determining the oligosaccharide composition (Vandecasteele *et al.* 2011). For QTL analyses saturated genetic maps are necessary. Irzykowska and Wolko (2004) constructed and used a linkage map of pea for the mapping of QTLs controlling pea yield parameters. Krajewski *et al.* (2012), using pea lines derived from the crosses Wt3557 × Wt11238 and Wt10245 × Wt11238, identified new loci with alleles coming from the protein-rich Wt11238 line. The new markers were recently placed by Święcicki *et al.* (2012) on the Wt10245 × Wt11238 map. In the present study a QTL approach was used on pea seeds to investigate the possibility of genetic determination of seed oligosaccharide concentration.

MATERIAL AND METHODS

The plant material consists of the pea mapping population (Wt10245 (round seeded) × Wt11238 (wrinkled seeded) 101 lines). This population of F_2-derived lines was described by Krajewski *et al.* (2012). The linkage map was constructed in JoinMap ver. 3.0 on the basis of 219 markers (AFLP, RAPD, STS, CAPS, SSR, SNP markers) using the maximum likelihood method (Van Ooijen & Voorrips 2001), as described by Krajewski *et al.* (2012); Święcicki *et al.* (2012); Knopkiewicz *et al.* (2013). The seeds, harvested from two field experiments (2002 (F_5) and 2004 (F_7)), were homogenized in a mixer mill (MM200, Retsch, Verder Group, Vleuten, The Netherlands) at a 22Hz frequency for 2 min. Soluble carbohydrates were extracted from 40 to 45 mg of meal with 800 µl of ethanol and water mixture (1:1, v/v, at 90°C for 30 min), containing 100 µg of xylitol (internal standard), centrifuged, deionized and dried in a speed vacuum rotary evaporator to dryness. Dry residues were derivatized with 200 µl of TMSI and pyridine mixture (Sigma, St. Louis, USA) at 70°C for 45 min. The TMS-derivatives of soluble carbohydrates were analysed by high resolution gas chromatography on a ZEBRON ZB-1 capillary column (Phenomenex, Torrance, USA) according to the method described previously (Lahuta 2006). The data were subjected to analysis of variance according to the model including effects of lines, years and of line × year interactions

(Payne *et al*. 2013). Mixed-model based composite interval mapping (MCIM) (Zhu 2000) was applied for QTL mapping in the QTL-Network 2.0 software (Yang *et al.* 2005). Statistical significance *F* was declared at 0.05. The likelihood-ratio statistic (LR) threshold of $P = 0.005$ (equivalent to logarithm of the odd (LOD) = 2.79 for df = 3) used in simulations is considered as the typical one for most QTL mapping studies, but it gave a consistent high power in detecting QTLs of moderate additive/epistatic effects (Wang *et al.* 1999).

RESULTS AND DISCUSSION

The parental lines of two mapping populations, Wt10245 × Wt11238 and Wt11238 × Wt3557, were tested for their seed oligosaccharide concentration to choose an appropriate research subject. The Wt10245 × Wt11238 population was chosen because of the greater difference in terms of the RFO concentration in seeds of parental lines (56.48 mg/g seed in Wt10245 and 99.1 mg/g seed in Wt11238, Table 1). Parental lines differ also in RFO composition. Although in both lines verbascose dominated among RFOs, the concentration of verbascose and stachyose in Wt11238 was ca. twice higher than that in Wt10245. Moreover, Wt11238 contains more sucrose than Wt10238 (Table 1). In the mapping population Wt10245 × Wt11238, from both field experiments, the mean level of fructose was 0.4 mg/g seed (0.2–0.8), *myo*-inositol 1.5 mg/g seed (0.9–2.6), sucrose 33.3 mg/g seed (19.7–52.1), galactinol 0.8 mg/g seed (0.5–1.3), raffinose 9.6 mg/g seed (5.6–15.3), stachyose 30.1 mg/g seed (20.1– 53.1), and verbascose 37.1 mg/g seed (23.3–57.4). The mean concentration of RFOs was 76.8 mg/g seed (51.8–120.3). RFOs accounted on average for 68% (59–75%) of the total soluble carbohydrates (TSC), similarto lupine (Carvalho *et al.* 2005). The analysis of variance shows that differences in trait values between lines in progeny were significant ($F < 0.001$). There was no interaction between lines and years of experiments (significance of lines × year interaction, *F* statistic > 0.01). The RFO concentration in seeds of various legume species ranges, on a dry matter (DM) basis, from 2.4–3.5% in broad bean, lentil and chickpea (Górecki *et al.* 2001; Kadlec *et al.* 2008) up to 11–16% in yellow lupine (Martinez-Villaluenga *et al.* 2008). Pea has one of the highest levels of oligosaccharide content among legumes – from 2.3 to 9.6% DM (Martinez-Villaluenga *et al.* 2008). Jones *et al.* (1999) screened 70 pea lines from the John Innes Pisum germplasm collection (Norwich, UK) and selected lines which had unusual RFO composition: traces of raffinose or verbascose and a broad range of stachyose concentration. In soybean unique lines containing low raffinose or low stachyose were also found (Hitz *et al.* 2002). The QTL analysis in soybean located four QTL for oligosaccharide content. Total oligosaccharide and sucrose content in soybean has two common QTL in two linkage groups (LGs) (Kim *et al.* 2006). The total amount of RFOs in *Medicago truncatula* was similar to that in pea and appears in a rank order system between the parents of the first mapping population LR1 (71.60 versus 75.16 μg/mg) and the parents of the second population LR4 (67.06 versus 59.98 μg/mg). In contrast, the sucrose level differed between the parents of both the LR1 (5.54 versus 3.42) and LR4 (4.52 versus 6.8 μg/mg) populations (Vandecasteele *et al.* 2011).

Table 1. The concentration of soluble carbohydrates (in mg/g DM) in seed of parental pea lines of the mapping population; means ($n = 4$) ± standard deviation

Carbohydrate	Wt 10245	Wt 11238
myo-inositol	1.18 ± 0.03	1.84 ± 0.05
sucrose	27.85 ± 0.40	33.34 ± 1.91
galactinol	0.82 ± 0.02	1.40 ± 0.07
raffinose	9.25 ± 0.23	9.98 ± 0.60
stachyose	21.57 ± 0.53	38.37 ± 2.38
verbascose	25.65 ± 0.67	50.75 ± 3.00
Total RFOs	56.48 ± 1.40	99.10 ± 5.98
TSC	86.32 ± 1.82	135.68 ± 7.99

RFOs – raffinose family oligosaccharides; TSC – total soluble carbohydrates

In our study one main quantitative trait locus was found in the LG VA (the *tl-r* interval) and three additional ones: in LG I (*myo*-inositol, raffinose, stachyose concentration from both years, concentration of RFOs and total soluble carbohydrates from 2004 near *afp1k*), LG II (stachyose concentration and concentration of RFOs from 2002 near *afp15h*) and LG IIIB (*myo*-inositol, stachyose, verbascose and RFO concentration from 2004 near *afp4i*, concentration of raffinose and RFOs from 2002 near *Mtic16*) (Table 2). Alleles derived from the Wt10245 parent decreased the seed oligosaccharide concentration and the total seed soluble sugar concentration. The main locus was responsible for the total sugar concentration in pea seeds. The results are in agreement with the knowledge of the RFO biosynthesis. Also, one major QTL for the amounts of raffinose, stachyose and ver-

Table. 2. QTLs detected for oligosaccharide concentration in the Wt10245 × Wt11238 mapping population

QTLs	2011					2012				
	LG	interval	position	A	heritability	LG	interval	position	A	heritability
Myo-inositol	I	Afp1k–Afp1f	91.7	0.13	0.11	I	Afp1k–Afp1f	91.7	0.17	0.06
	IIIB	Afp4i–Afp4e	56.6	0.10	0.06					
	VA	Afp12b–*r*	12.0	–0.27	0.25	VA	Afp12b–*r*	12.0	–0.47	0.27
Sucrose	II	*k–wb*	137.6	–2.40	0.07					
	VA	*r–tl*	16.1	–6.82	0.33	VA	Afp12b–*r*	12.0	–6.41	0.26
	VB	OPG9B–Afp5b	34.9	3.3	0.1					
Galactinol		no QTL				VA	*tl*–Afp9h	20.6	–0.09	0.09
						VIA	Afp14f–Afp10g	22.2	0.11	0.13
Raffinose	I	Afp1k–Afp1f	91.7	0.95	0.12	I	Afp1k–Afp1f	91.7	0.59	0.09
						IIIB	M16–L109	45.9	–0.74	0.09
	VA	Afp12b–*r*	12.0	–1.6	0.19	VA	*r–tl*	16.1	–1.77	0.22
Stachyose	I	Afp1k–Afp1f	91.7	2.4	0.12	I	Afp1k–Afp1f	91.7	2.25	0.08
	II	Afp15h–Afp14d	75.9	2.89	0.1	II	Afp15h–Afp14d	39.0	2.75	0.09
	IIIB	Afp4i–Afp4e	55.6	1.8	0.05					
	VA	Afp12b–*r*	11.0	–3.3	0.27	VA	*tl–r*	16.1	–6.59	0.32
	VA	Afp9H–P108	26.1	–2.9	0.24					
Verbascose	IIIB	Afp4i–Afp4e	55.6	2.5	0.05					
	IV	B827B–Afp11G	46.0	3.04	0.04					
	VA	*r–tl*	16.1	–7.4	0.29	VA	*tl–r*	16.1	–6.62	0.33
Total RFO	I	Afp1k–Afp1f	91.7	8.51	0.10					
	IIIB	Afp4i–Afp4e	55.6	5.6	0.05	IIIB	M16–L109	45.9	–6.85	0.04
	VA	Afp12b–*r*	11.0	–7.7	0.29	VA	*tl–r*	16.1	–14.34	0.36
	VA	Afp9H–P108	27.1	–11.3	0.24					
Total soluble carbohydrates, TSC	I	Afp1k–Afp1f	91.7	9.6	0.11					
	VA	Afp12b–*r*	11.0	–24.7	0.32	VA	*tl–r*	16.1	–21.86	0.38
%RFO in TSC						II	Afp15h–Afp14d	39.0	0.01	0.09

LG – linkage group; interval – the flanking markers of QTL, position – QTL position (cM) in relation to the first marker of the group, A – the effect of the QTL on the trait, expressed in terms of additive "weight" (alleles derived from the A parent); significant level for putative QTL detection and for QTL effects was declared for 0.05, markers named according to Gawłowska *et al.* (2005)

bascose was found by Vandecasteele *et al.* (2011) on the long arm of LG IV in *Medicago truncatula*. In the same chromosomal region, a QTL for the ratio of verbascose versus stachyose explained 80% of the variation, and the direction of the additive effect of raffinose and verbascose was opposite to that of stachyose, suggesting that the underlying mechanism is related to the conversion of these sugars. This was the only QTL detected for RFO levels for one mapping population (LR1), whereas for the second (LR4), additional QTLs (7.6–11% variation) were found for RFO levels (LG V for verbascose, LG VIII for raffinose, and both LG III and VII for stachyose) (Vandecasteele *et al.* 2011). Smýkal *et al.* (2012) concluded that pea LG V corresponds to the 7th pseudochromosome in *Medicago truncatula*. They also concluded that the 5th pseudochromosome in *Medicago truncatula* corresponds to LG I in pea (five traits 2002 and 2004 near *afp1k*), 8th pseudochromosome corresponds to LG IV (one trait from 2002) and the 3rd pseudochromosome corresponds to LG III (five traits from 2004 and 2002 near *afp4i* and *Mtic16*). A QTL related to the sucrose level was located in *M. truncatula* on LG II and LG III (Vandecasteele *et al.* 2011). Smýkal *et al.* (2012) concluded that the 2nd pseudochromosome in *Medicago truncatula* corresponds to pea LG V (sucrose level from both years). Vandecasteele *et al.* (2011) stated that the Suc and Suc/RFO QTL on LG1 (pea LG II) is co-located with two genes encoding galactinol synthase (GolS) (Medtr1g102760 and Medtr1g102770), whereas the Suc/RFO QTL on LG3 is co-located with two genes encoding raffinose synthase (RFS) (Medtr3g114540 and Medtr3g119630) and sucrose synthase (SUS) (Medtr3g086770).

Oligosaccharide genetic determination makes selection for a change in the ratio of the particular oligosaccharides difficult, like in *Phaseolus*. However, it is possible to decrease the level of the total RFO concentration in pea seeds. The linkage between QTL and the gene *r* is interesting. Wrinkled (*rr*) seeds show no isoform of starch-branching enzyme (SBEI) present in round (*RR* or *Rr*) seeds. The *rugosus* (*r*) locus changes the morphology and distribution of starch grains, decreases the total starch accumulation, produces a higher ratio of amylose to amylopectin and increased sugar and water content during development, along with changes in the cell size and lipid content (Bhattacharyya *et al.* 1990). Seeds of pea *RRRbRb* lines contain much less (ca. twofold) of RFOs than those of *rrrbrb* lines (Lahuta *et al.* 2007). Vandecasteele *et al.* (2011) stated that the majority of sugar QTLs co-located with genes involved in RFO or sucrose synthesis, indicates that they might be the regulatory loci underlying sugar variation in *M. truncatula* seeds. A major gene involved in the conversion of stachyose into verbascose (stachyose synthase) is co-located perfectly with the major QTL detected on LG IV that explained 80% of the detected variation in sugar composition. For *Arabidopsis*, Bentsink *et al.* (2000) also found a major QTL for RFO levels, and two genes encoding raffinose synthase and galactinol synthase to be closely linked to this QTL.

In conclusion our data demonstrate the main locus responsible for the level of all investigated sugar concentration in pea seeds. Also, one major QTL for the amounts of raffinose, stachyose and verbascose was found by Vandecasteele *et al.* (2011) in *Medicago truncatula*. The present study suggests that three additional loci in pea LGI, LGII and LGIIIB correspond to relevant loci and chromosomes in *Medicago truncatula* (Vandecasteele *et al.* 2011).

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