https://doi.org/10.17221/96/2019-CJGPB

Grain hardness in triticale: a physical and molecular evaluation

Veronika Kselíková[1], Tomáš Vyhnánek[2], Pavel Hanáček[2], Petr Martinek[3]*

[1]Department of Experimental Biology, Faculty of Science, Masaryk University, Brno, Czech Republic
[2]Department of Plant Biology, Faculty of AgriSciences, Mendel University in Brno, Brno, Czech Republic
[3]Agrotest fyto, Ltd., Kroměříž, Czech Republic

**Corresponding author: kselikova.veronika@gmail.com*

Citation: Kselíková V., Vyhnánek T., Hanáček P., Martinek P. (2020): Grain hardness in triticale: a physical and molecular evaluation. Czech J. Genet. Plant Breed., 56: 102–110.

Abstract: Grain hardness is an important quality trait of cereals. In the present work, the particle size index (PSI) was used to assess the variability in grain hardness of thirteen triticale genotypes, three wheat genotypes and one rye genotype grown in two subsequent seasons. The PSI values of the triticale samples ranged between 7.7 to 19.2%, representing a medium hard to a very hard grain. Moreover, the sequencing of genes in the *Hardness* (*Ha*) locus revealed a limited genetic variability in the grain hardness genes, namely secaloindoline-a (*Sina*) and secaloindoline-b (*Sinb*). Two possible new allelic variants were identified, one for each of the secaloindoline genes. The existing variability in the grain hardness as well as polymorphisms in its candidate genes are a good starting point for efforts in breeding new varieties of triticale with improved grain hardness.

Keywords: friabilin; *Ha* locus; particle size index; puroindoline; secaloindoline

Triticale (× *Triticosecale* Wittmack), an inter-genus hybrid of wheat and rye, is traditionally considered good feed for livestock (Barneveld & Cooper 2002), as it is of high nutritional quality and rich in essential amino acids (Boros 2002). In the baking industry, triticale flour can be an alternative to wheat-rye flour mixtures (Peña 2004). Besides the low gluten level and its poor quality, which were considered major problems of triticale flour, but have recently been improved by creating cytogenetically modified lines carrying parts of the wheat chromosome 1D (Lukaszewski 2000, 2006), grain hardness is yet another trait awaiting to be improved.

Grain hardness is an economically important trait as it determines the potential end-use of the crop. It is known to have a direct influence on the milling yield, water absorption, damaged starch content and fermentation capacity of flours (Simeone & Lafiandra 2005). The molecular mechanisms determining grain hardness were first described in wheat, when Greenwell and Schofield (1986) discovered an inverse proportion between the grain hardness and the amount of friabilin, a protein complex of 15 kDa, found on the surface of starch granules. Giroux and Morris (1997) then proved friabilin to be coded by the genes of the *Ha* locus, namely puroindoline-a (*Pina*) and puroindoline-b (*Pinb*). The grain softness protein gene (*Gsp*-1), another gene of the *Ha* locus, was not proven to have any relationship with the grain hardness (Tranquilli et al. 2002) although it is highly conserved in the genus *Aegilops* and *Triticum* (Massa et al. 2004; Gollan et al. 2007) implying some evolutionary important function, supposedly, its involvement in plant defence mechanisms (Gollan et al. 2007). Orthologous genes to puroindolines were found in oat (Tanchak et al. 1998; Gazza et al. 2015); barley (Darlington et al. 2001; Beecher et al. 2002) and rye (Gautier et al. 2000; Simeone & Lafi-

Supported by the Ministry of Agriculture of the Czech Republic, Project No. RO1118.

https://doi.org/10.17221/96/2019-CJGPB

andra 2005); their expression products were denoted as tryptophanins, hordoindolines and secaloindolines, respectively. In triticale, carrying genomes A and B from wheat and genome R from rye, the grain hardness genes of genomes A and B were lost in evolution, probably as a result of low-copy DNA sequences elimination following polyploidisation events, therefore, triticale carries the *Ha* locus on chromosomes 5R originating from rye (Gautier et al. 2000). Therefore, puroindoline orthologues in triticale are denoted as secaloindoline-a (*Sina*) and secaloindoline-b (*Sinb*) as is the case in rye.

The aim of this work was to study the variability of grain hardness and find the potential polymorphisms in the candidate genes responsible for the grain hardness in the selected triticale genotypes.

MATERIAL AND METHODS

Plant material. Experiments were conducted with thirteen samples of hexaploid triticale, three wheat samples and one rye sample serving as a reference to its parental species (Table 1). All triticale samples are part of the ongoing breeding programme at Agrotest fyto, Ltd. (Kroměříž, Czech Republic). Moreover, all the samples are winter varieties grown at the same location to minimise the effect of the environment on the tested traits. The allelic status of the secaloindoline/puroindoline genes was unknown in all of the tested samples.

Assessing grain hardness. The grain hardness was assessed in grains from the 2015 and 2016 harvests via the particle size index (PSI) method according to the AACC 55-30 methodology as described in Faměra et al. (2004). The relative moisture of the grain samples was measured on an near-infrared (NIR) Inframatic 9100 grain analyser (Perten Instruments, Hägersten, Sweden) and ranged within 11–13% as required for the PSI method. Prior to the PSI measurement, 25 g of each sample was processed in a KT-3303 laboratory mill (Perten Instruments, Hägersten, Sweden) equipped with a No. 2 milling head. The PSI was measured twice for each sample, using 10 g of meal for every run. The meal was sifted through stainless steel sieves with 75 µm holes using a Swing 200 laboratory sifter (Mezos, Hradec Králové, Czech Republic) set at 180 rpm for 10 min. After sifting, the weight of the material that passed through the sieves was assessed and the PSI was determined using the formula: PSI % = [weight of the meal that passed through the sieve (g)/sample weight (g)] × 100. Based on the determined PSI, the relative grain hardness was assessed

Table 1. The samples of the winter hexaploid triticale (1–14) wheat (15–16) and rye (17) used in the present study

No.	Designation	Ancestry
1	1m^2-397-15	603-07(= Presto RM1B/*2 Krakowiak)/Valentin
2	1m^2-408-15	519-07(= Presto FC1/*1 BOH504)/Valentin
3	1m^2-411-15	509-07(= Presto FC1/*2 Krakowiak//BOH504/3/Krakowiak)/Valentin
4	1m^2-439-15	Inpetto/UCRL DH 445
5	V1-283-15	BOH504/Mungis//BOH505
6	V1-287-15	BOH504/Mungis//BOH505
7	V1-342-15	629-07 (= RM2/*1 BOH504)/Pawo
8	V1-380-15	BOH504/Mungis//BOH505
9	V3-9a-15	UCR DH 424
10	V1-188-15	Selection from 48M
11	V1-190-15	Selection from 48M
12	KM 686-14	Kitaro/2117-06-A//Avangard
13	KM 690-14	Inpetto/UCRL DH 445
14	wheat Vanessa	Rapsodia/SG–S 2040-97 (GRIN: 01C0107410)
15	wheat Matchball	Carenius/Boomer (GRIN: 01C0107409)
16	wheat Bohemia	(540i/U6192)/(540i/Kontrast) (GRIN: 01C0106924)
17	rye "Doupovské"	Gene bank CRI (GRIN: 03C0300435)

Origin of the parental material: UCR – University of California; BOH – Hodowla Roslin Strzelce, breeding institute Borowo, Poland; 48M – received from Hodowla Roslin Strzelce, true origin unknown; 2117-06-A – received from Selgen, JSC breeding institute in Úhřetice; GRIN – Germplasm Resources Information Network; CRI – Crop Research Institute

https://doi.org/10.17221/96/2019-CJGPB

Table 2. The primers used in this study to amplify the puroindoline and secaloindoline genes

Target gene	Primer name	Primer sequence	Product size (bp)	Reference
Pina/Sina	Pina-D1F	5'-GGTGTGGCCTCATCTCATCT-3'	515[a]	Massa et al. (2004)
	Pina-D1R	5'-AAATGGAAGCTACATCACCAGT-3'		
Pinb/Sinb	Pinb-D1F	5'-AATAAAGGGGAGCCTCAACC-3'	525[a]	Massa et al. (2004)
	Pinb-D1R	5'-CGAATAGAGGCTATATCATCACCA-3'		
	Sinb-F	5'-GAGCCTCAACCCATCTATTCATC-3'	548[b]	Li et al. (2006)
	Sinb-R	5'-CAAGGGTGATTTTATTCATAG-3'		

[a]expected size of the product in *Triticum aestivum*; [b]expected size of the product in × *Triticosecale* Wittmack

using the criteria described in a study by Hrušková and Švec (2009). The PSI values were statistically analysed using a two-way ANOVA (analysis of variance) in the MS Excel 2016 add-in Solver to determine the effect of the genotype and cultivation year.

Molecular analysis of grain hardness genes. The grain samples were sprouted on wet filter paper in Petri dishes. After approximately 7 days, 100 mg of each sample was harvested for DNA isolation using a DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The concentration and purity of the isolated DNA was measured using a Picopet 1.0 (Picodrop, Cambridge, United Kingdom). The candidate genes for the grain hardness were analysed via PCR (polymerase chain reaction). The primers Pina-D1F/Pina-D1R and Pinb-D1F/Pinb-D1R by Massa et al. (2004) and the primers Sinb-F/Sinb-R by Li et al. (2006) were used in this study to obtain the full-length sequences of the grain hardness candidate genes (Table 2). The PCR reactions in the volume of 25 µl containing 0.5 U *Taq* polymerase (Promega, Madison, USA), a 1× aliquot buffer, 0.1 mmol/L of each deoxynucleotide (Promega), 0.3 mol/L of each primer and 20 ng of the template DNA were conducted in a T3 thermocycler (Biometra, Göttingen, Germany). The cycling conditions for the Pina-D1F/Pina-D1R and Pinb-D1F/Pinb-D1R primers were 94 °C for 4 min followed by 35 cycles of 94 °C for 1 min, 58 °C for 90 s, and 72 °C for 2 min with an extension for 10 min at 72 °C. The cycling conditions for the Sinb-F/Sinb-R primers were 94 °C for 3 min followed by 36 cycles of 94 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min, with an extension for 10 min at 72 °C. The PCR products were run on a 1.5 % agarose gel in a 1× standard Tris Acetate-EDTA buffer.

Sequence analysis. The selected samples were further analysed by sequencing. The selection was based on the PSI values and samples from all the grain hardness categories were included to see if there is a difference in the allelic status of their *Ha* locus. Based on these criteria, triticale samples 4, 5 and 10, as well as wheat sample 14 and rye sample 17 were selected. Prior to the sequencing, the PCR products were purified using an Invisorb Fragment CleanUp kit (Stratec Molecular, Berlin, Germany). The purified products were sequenced (Macrogen, Amsterdam, Netherlands).

The amplification product of the Pina-D1F/Pina-D1R primers proved to be a mixture of at least two PCR products very close in size. The PCR products of this marker were cloned to separate them from each other using a pGEM-T Vector System (Promega) following the protocol described by Trojan et al. (2014). Products of length 160 bp (plasmids without insert) + the length of the original PCR product (515 bp) were identified as the recombinant products and sequenced (Macrogen). In total, fourteen recombinant products were sequenced.

The obtained sequences were analysed using the BLAST (Basic Local Alignment Search Tool) algorithms (blast.ncbi.nlm.nih.gov/Blast.cgi) and The National Center for Biotechnology Information (NCBI) database GenBank.

RESULTS

Assessing grain hardness. The grain hardness was measured in two subsequent harvests (2015 and 2016) using the PSI method. The medium hard, hard and very hard samples have been identified. The average PSI in the 2015 and 2016 harvests was 13.3% and 12.9%, respectively. The highest PSI was in sample No. 5 in both observed harvests, while the lowest PSI was identified in sample No. 4 in the 2015 harvest and sample No. 17 in the 2016 harvest

https://doi.org/10.17221/96/2019-CJGPB

Table 3. The grain hardness of the tested samples from the 2015 and 2016 harvests measured as a particle size index (PSI)

No.	Designation	PSI (%)		
		2015	2016	average
1	1m^2-397-15	15.0^b	13.8^b	14.4^b
2	1m^2-408-15	16.0^b	11.8^c	13.9^b
3	1m^2-411-15	13.3^b	12.9^b	13.1^b
4	1m^2-439-15	19.2^a	17.4^a	18.3^a
5	V1-283-15	9.9^c	7.7^c	8.8^c
6	V1-287-15	11.1^c	8.3^c	9.7^c
7	V1-342-15	11.2^c	12.3^c	11.7^c
8	V1-380-15	11.1^c	13.8^b	12.5^b
9	V3-9a-15	13.8^b	13.7^b	13.7^b
10	V1-188-15	13.4^b	10.3^c	11.8^c
11	V1-190-15	11.0^c	9.6^c	10.3^c
12	KM 686-14	13.6^b	15.1^b	14.3^b
13	KM 690-14	14.5^b	13.2^b	13.8^b
14	wheat Vanessa	15.0^b	16.6^a	15.8^b
15	wheat Matchball	10.5^c	10.4^c	10.4^c
16	wheat Bohemia	9.9^c	12.8^b	11.3^c
17	rye "Doupovské"	17.2^a	20.0^a	18.6^a

The superscript letters represent the grain hardness categories based on the PSI values: ^{a}medium hard, ^{b}hard, ^{c}very hard following the criteria by Hrušková and Švec (2009)

(Table 3). The influence of both the harvest year and the sample (genotype) were found to be statistically significant at a 5% level of significance (Table 4).

Molecular analysis of selected markers. The PCR primers Pina-D1F/Pina-D1R and Pinb-D1F/Pinb-D1R were used to obtain the full-length *Pina/Sina* and *Pinb/Sinb* genes, respectively. All the samples showed an amplification product of an expected size about 520 bp for both the *Pina/Sina* (Figure 1) and *Pinb/Sinb* genes (Figure 2). Moreover, all the tested wheat samples showed extra amplification products of a size about 650 bp when using the Pinb-D1F/Pinb-D1R primers. The identity of these extra products was not further analysed. Similar results were obtained for the amplicons of the Pina-D1F/Pina-D1R primers. When carefully separated on the agarose gel, each of the amplicons was found to consist of two very close and yet separate bands corresponding to two amplicons differing in size by approximately 50 bp (Figure 3). The use of the Sinb-F/Sinb-R PCR primers designed to specifically detect the *Sinb* gene by Li et al. (2006) led to the amplification of a fragment of an expected size about 550 bp in all the samples tested, although for all the wheat samples, the signal was weaker accompanied by an extra amplification product of a size about 350 bp (Figure 4).

Sequence analysis. The cloned sequences of the Pina-D1F/Pina-D1R amplicons were compared with

Table 4. The effect of the harvest year, sample (genotype) and their interaction on the grain hardness measured as a particle size index (PSI) – the statistical evaluation using a two-way ANOVA

Source of variability	DF	SS	MS	*F*	*P* value	Critical *F* (α = 0.05)
Harvest year	1	2.28	2.28	56.11	1.08E-08***	4.13
Sample (genotype)	16	485.74	30.36	746.12	1.73E-38***	1.95
Interaction	16	76.31	4.77	117.22	5.58E-25***	1.95
Error (residual)	34	1.38	0.04			
Total	67	565.72				

SS – sum of squares; DF – degrees of freedom; MS – mean square; *F* – test criteria value; α – level of significance, ***$P \leq 0.001$

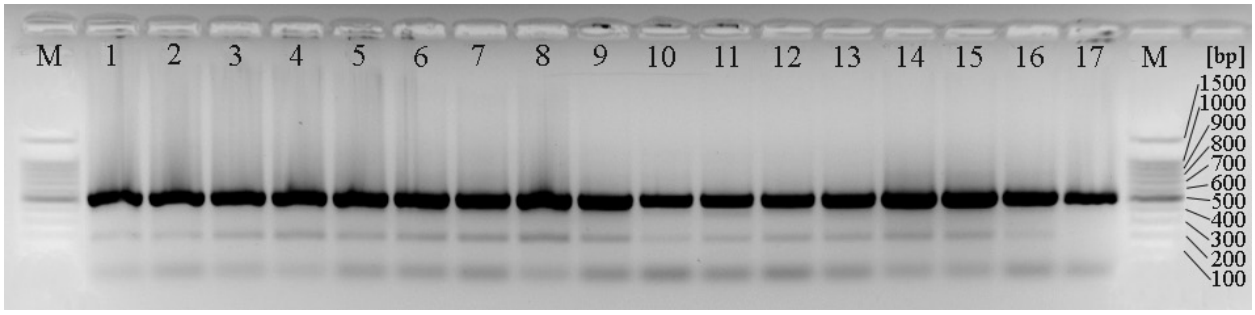

Figure 1. *Pina* and *Sina* detection using primers Pina-D1F/Pina-D1R – product approximately 550 bp in size
M – size marker, the numbers correspond to the sample numbers from Table 1

https://doi.org/10.17221/96/2019-CJGPB

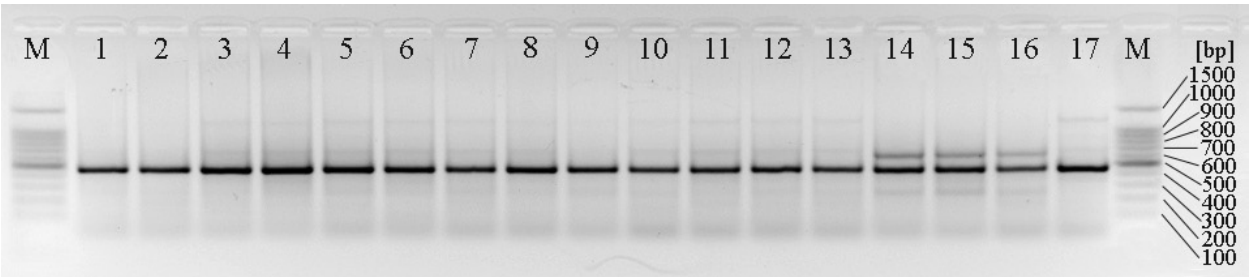

Figure 2. *Pinb* and *Sinb* detection using primers Pinb-D1F/Pinb-D1R – product approximately 500 bp in size
M – size marker, the numbers correspond to the sample numbers from Table 1

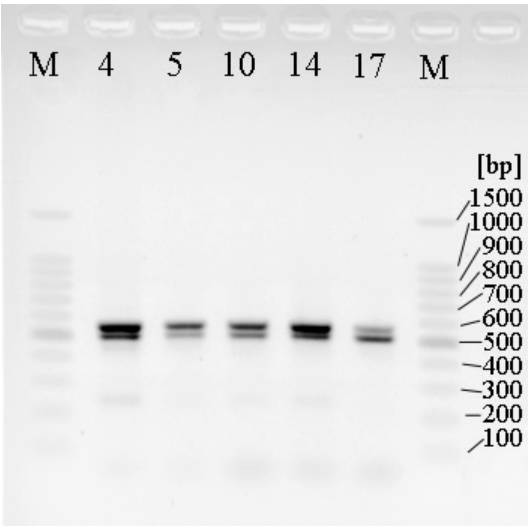

Figure 3. The detailed electrophoretic separation of the *Pina/Sina* amplification product in the selected samples showing two separate amplification products around 500 and 550 bp
M – size marker, the numbers correspond to the sample numbers from Table 1

the Genbank database and the sequences with the best hits by means of the percent identity are summarised in Table 5. From fourteen analysed sequences, five of them gave the best alignment with the *Sina* gene (triticale and rye) or the *Pina* gene (wheat). In the case of the cloned sequence 4-2 (MN457918) there is a 99% match to the KC784350 sequence (the *Sina* gene obtained from the triticale described by Gasparis et al. (2013)) with two mismatches in their alignment. The translated MN457918 was aligned with the respective *Sina* protein sequence (AGO65289) to reveal that one of the observed single nucleotide substitutions leads to a conservative substitution (i.e., substitution to an amino acid with similar biochemical properties) of L to I (i.e., leucine to isoleucine) at position 9 of the AGO65289 sequence. The second observed single nucleotide substitution leads to the substitution of W to R (i.e., tryptophan to arginine) corresponding to position 68 in the amino acid sequence of AGO65289. In the case of the cloned sequence 4-3 (MN457919) there is again a 99% match to the KC784350 sequence. The only mismatch in their alignment is a synonymous substitution without any effect on the amino acid sequence of the respective protein. The same *Sina* sequence was aligned to the cloned sequence 5-1 (MN457923) with a 100% match in the aligned region. Moreover, nine of the analysed sequences gave the best match with some variant of the *Gsp-1* gene. These findings point to a high sequence identity with the analysed DNA sequences.

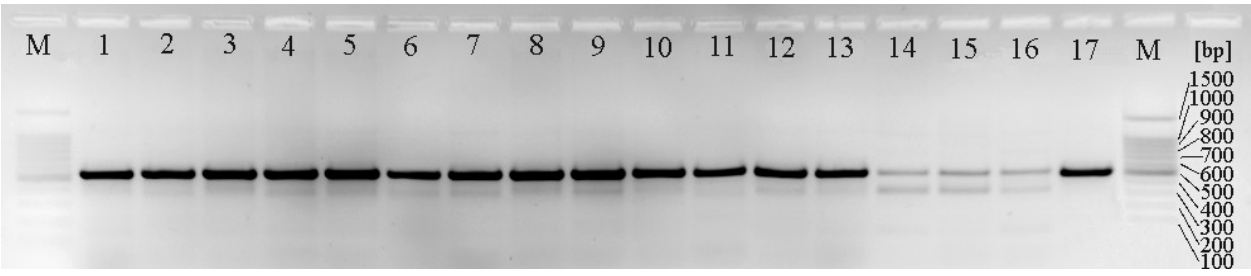

Figure 4. *Pinb*/*Sinb* amplification using primers Sinb-F/Sinb-R – product 550 bp in size
M – size marker, the numbers correspond to the sample numbers from Table 1

https://doi.org/10.17221/96/2019-CJGPB

Table 5. The alignment of the Pina-D1F/Pina-D1R amplicon sequences with the nucleotide database GenBank

Sequences of *Pina/Sina*		Best match in the GenBank database (based on percent identity)				
Sample-clone No.	GenBank accession No.	GenBank accession No.	score (bit)	match	sequence description	reference
4-2	MN457918	KC784350.1	809	442/444 (99%)	× *Triticosecale* Wittmack *Sina* gene	Gasparis et al. (2013)
4-3	MN457919	EU835980.1	1031	558/558 (100%)	*Triticum aestivum Gsp-A1* gene	Ragupathy and Cloutier (2008)
4-4	MN457920	KC784350.1	815	443/444 (99%)	× *Triticosecale* Wittmack *Sina* gene	Gasparis et al. (2013)
4-6	MN457921	EU835980.1	1014	555/558 (99%)	*Triticum aestivum Gsp-A1* gene	Ragupathy and Cloutier (2008)
4-8	MN457922	KF570248.1	935	508/509 (99%)	*Secale africanum Gsp-1* gene	Li et al. (2016)
5-1	MN457923	KC784350.1	821	444/444 (100%)	× *Triticosecale* Wittmack *Sina* gene	Gasparis et al. (2013)
5-3	MN457924	EU307526.1	1031	558/558 (100%)	*Triticum turgidum* subsp. *dicoccoides Gsp-A1* gene	unpublished
5-5	MN457925	EU307526.1	1026	557/558 (99%)	*Triticum turgidum* subsp. *dicoccoides Gsp-A1* gene	unpublished
10-4	MN457926	EU835980.1	1026	557/558 (99%)	*Triticum aestivum Gsp-A1* gene	Ragupathy and Cloutier (2008)
10-7	MN457927	EU835980.1	1020	556/558 (99%)	*Triticum aestivum Gsp-A1* gene	Ragupathy and Cloutier (2008)
14-3	MN457928	CT009735.1	1018	557/560 (99%)	*Triticum aestivum Gsp-1* gene	Chantret et al. (2008)
14-4	MN457929	AJ302091.1	953	516/516 (100%)	*Triticum aestivum Pina* gene	Lillemo et al. (2002)
14-8	MN457930	EU307526.1	1014	555/558 (99%)	*Triticum turgidum* subsp. *dicoccoides Gsp-A1* gene	unpublished
17-4	MN457931	DQ269850.1	815	443/444 (99%)	*Secale cereale Sina* gene	Massa and Morris (2006)

Sample numbers correspond to sample specifications in Table 1

Table 6. The alignment of the Pinb-D1F/Pinb-D1R amplicon sequences with the nucleotide database GenBank

Sequences of *Pinb/Sinb*		Best match in GenBank database (based on percent identity)				
Sample No.	GenBank accession No.	GenBank accession No.	score (bit)	match	sequence description	reference
4	MN457932	KC784351.1	763	415/416 (99%)	× *Triticosecale* Wittmack *Sinb* gene	Gasparis et al. (2013)
5	ND	KC784351.1	769	416/416 (100%) %)	× *Triticosecale* Wittmack *Sinb* gene	Gasparis et al. (2013)
10	MN457933	KC784351.1	756	414/416 (99%)	× *Triticosecale* Wittmack *Sinb* gene	Gasparis et al. (2013)
14	MN457934	CT009735.1	859	471/474 (99%)	*Triticum aestivum Pinb* gene	Chantret et al. (2008)
17	ND	DQ269886.1	761	414/416 (99%)	*Secale cereale Sinb* gene	Massa and Morris (2006)

ND – The sequences were not submitted in the GenBank database, as they were not unique; the sample numbers correspond to the sample specifications in Table 1

https://doi.org/10.17221/96/2019-CJGPB

```
Query  1    MKTCLFLLALLALVASTTLAQYSEVGGWYNEVGGGGGAQQCPLERPKLSSCMDYVMERCF  60
            MKTCLFLLALLALVASTTLAQYSEVGGWYNEVGGGGGAQQCPLERPKLSSCMDYVMERCF
AGO65290.1  MKTCLFLLALLALVASTTLAQYSEVGGWYNEVGGGGGAQQCPLERPKLSSCMDYVMERCF  60

Query  61   TMKDFPVIWPTRWWKGGCEHEVREKCCNQLSQIAPQCRCDSIRGMIQGKFGGFFGIWRGD  120
            TMKDFPVIWPTRWWKGGCEHEVREKCCNQLSQIAPQCRCDSIRGMIQGKFGGFFGIWRGD
AGO65290.1  TMKDFPVIWPTRWWKGGCEHEVREKCCNQLSQIAPQCRCDSIRGMIQGKFGGFFGIWRGD  120

Query  121  VFKQTQRAQSLPSKCNMELKYKWVDNL    147
            VFKQTQRAQSLPSKCNM    K+
AGO65290.1  VFKQTQRAQSLPSKCNMGADCKFPGGYYW  149
```

Figure 5. The alignment of the translated nucleotide sequence MN457933 (Query) of the *Sinb* gene with the Sinb protein sequence AGO65290.1

The sequences of the Pinb-D1F/Pinb-D1R and Sinb-F/Sinb-R amplicons were aligned as they were both supposed to amplify the *Pinb* gene or its orthologue *Sinb* in the triticale. The pairwise alignment of the Pinb-D1F/Pinb-D1R and Sinb-F/Sinb-R amplicons proved their identity even though only the Pinb-D1F/Pinb-D1R primers amplified the whole gene sequence of *Pinb* or *Sinb*, hence, their amplicons were selected for further studies (data not shown). The comparison of the Pinb-D1F/Pinb-D1R amplicon sequences with the GenBank (Table 6) proved the alignment of all three triticale sequences to the triticale *Sinb* gene sequence KC784351. The alignment of KC784351 and MN457933 (sequence of triticale sample 10) revealed one single nucleotide substitution and one nucleotide deletion in the MN457933 sequence. The translated MN457933 sequence was aligned with the respective protein sequence of KC784351 (AGO65290) to analyse the effect of the observed mutations. The single nucleotide substitution is a synonymous substitution without any effect to the amino acid sequence of the resulting protein, while the single nucleotide deletion leads to a frameshift mutation in the coding region of *Sinb* gene. The frameshift causes a change in the amino acid sequence of the Sinb protein starting from residue 138 in its amino acid sequence and its premature termination (Figure 5).

DISCUSSION

Cereals used in the food industry need to fulfil numerous qualitative requirements including grain hardness which is known to have an impact on the milling efficiency, flour quality and technological properties of the resulting doughs (Dennett & Trethowan 2013). Grain hardness represents one of the limiting factors of flour use, hence, triticale flours are suitable for products in which flours of softer wheats are traditionally used, such as cookies (Leon et al. 1996) and cakes (Oliete et al. 2010), although triticale flour was even successfully used in bread-making, where flours from harder wheats are preferred (Győri et al. 2009). Introducing triticale flours into the baking industry is desirable mainly for its high nutritional value rich in essential amino acids (Doxastakis et al. 2002). Moreover, the use of triticale flours could be economically attractive as triticale exhibits high yields in less favourable growing conditions as well as the possibility to further increase this yield by conventional breeding techniques (Bassu et al. 2011).

The grain hardness assessed by the PSI method was within the range of 8.8% to 18.3% (Table 3) corresponding to a very hard, hard or medium hard grain using the criteria by Hrušková and Švec (2009). These values seem to be somewhat lower than those obtained by Ramírez et al. (2003), whose triticale samples had a PSI from 15.4% to 21.8%. A lower PSI, hence a harder grain, is a prerequisite for a high milling efficiency and the subsequent use in the milling and baking industries (Dennett & Trethowan 2013). In an older study by Williams (1986), 280 triticale samples exhibited a PSI from 7.8% to 34.6% pointing to the existing variability of grain hardness in the triticale. Our results suggest that this variability might be determined by the genotype of a variety, environmental factors as well as combination of both, even though these findings would need to be confirmed in larger data sets (Table 4). Similar results were obtained for its parental species; in wheat, both the genotype and interaction of the genotype with the environmental factors were the source of the variability in the grain hardness (Oury et al. 2015), while, for rye, only environmental factors were found to be statistically significant (Hansen et al. 2004).

On a molecular level, grain hardness in the triticale is associated with the *Ha* locus on the short arm of chromosome 5R and its genes *Sina* and *Sinb*. From ten triticale sequences analysed for the *Sina* gene, three of them gave the best alignment with the *Sina* gene (KC784350) isolated from the triticale cv.

https://doi.org/10.17221/96/2019-CJGPB

Wanad (Gasparis et al. 2013). However, only MN457918 (sequence of triticale sample 4) carries a single nucleotide substitution that translates to a non-conservative substitution in the amino acid sequence of the Sina protein. The W to R substitution at position 68 of the Sina protein is located in the tryptophan-rich domain (TRD) which is highly conserved in puroindolines and its orthologues (Gautier et al. 2000). The TRD of puroindolines plays an important role in the bond of puroindolines to starch granules (Jing et al. 2003; Wall et al. 2010) as it binds to the polar lipids on the surface of starch granules (Greenblatt et al. 1995). Therefore, the observed substitution might possess an important effect on the protein function. Seven out of ten triticale sequences supposed to amplify the *Sina* gene gave the best alignment with some variant of the *Gsp*-1 gene (Table 5). The amplification of *Gsp-1* gene with primers designed for the puroindoline-like sequences can be explained by their high sequential homology. The detection of the *Gsp-A1* gene (EU835980), a copy of *Gsp-1* in the A genome, leads to the assumption that unlike the *Pina* and *Pinb* genes, the *Gsp-1* gene was conserved in the A genome of the triticale as is the case in wheat (Jolly et al. 1996; Gollan et al. 2007). In the case of the *Sinb* gene sequences, all three triticale sequences aligned with the *Sinb* sequence KC784351 described by Gasparis et al. (2013) in the triticale cv. Wanad. In the MN457934 sequence, corresponding to *Sinb* of triticale sample 10, the single nucleotide deletion leads to a frameshift mutation in the coding region of *Sinb* gene. The frameshift causes a change in the amino acid sequence of the Sinb protein starting from residue 138 in its amino acid sequence and its premature termination (Figure 5). However, the possible functional effect of this change in the amino acid sequence of Sinb is unclear as it is located on the very end of the protein.

This work shows the existing variability in the grain hardness among the tested triticale cultivars. The molecular analysis of the grain hardness genes displayed their high sequential homology to previously documented sequences revealing limited variability on a molecular level. However, two possible new allelic variants were identified, one for both the *Sina* and *Sinb* gene.

Acknowledgements. We are grateful for the technical assistance provided by Ing. O. Faměra, CSc. (Czech University of Life Sciences in Prague).

REFERENCES

Barneveld R.J. van, Cooper K.V. (2002): Nutritional quality of triticale for pigs and poultry. In: Arseniuk E. (ed.): Proc. 5th Int Triticale Symposium, Radzików, June 30–July 5, 2002: 277–282.

Bassu S., Asseng S., Richards R. (2011): Yield benefits of triticale traits for wheat under current and future climates. Field Crops Research, 124: 14–24.

Beecher B., Bowman J., Martin J., Bettge A., Morris C., Blake T., Giroux M. (2002): Hordoindolines are associated with a major endosperm-texture QTL in barley (*Hordeum vulgare*). Genome, 45: 584–591.

Boros D. (2002): Physico-chemical quality indicators suitable in selection of triticale for high nutritive value. In: Arseniuk E. (ed.): Proc. 5th Int Triticale Symposium, Radzików, June 30–July 5, 2002: 239–244.

Chantret N., Salse J., Sabot F., Bellec A., Laubin B., Dubois I., Dossat C., Sourdille P., Joudrier P., Gautier M.-F., Cattolico L., Beckert M., Aubourg S., Weissenbach J., Caboche M., Leroy P., Bernard M., Chalhoub B. (2008): Contrasted microcolinearity and gene evolution within a homoeologous region of wheat and barley species. Journal of Molecular Evolution, 66: 138–150.

Darlington H., Rouster J., Hoffmann L., Halford N., Shewry P., Simpson D. (2001): Identification and molecular characterisation of hordoindolines from barley grain. Plant Molecular Biology, 47: 785–794.

Dennett A.L., Trethowan R.M. (2013): Milling efficiency of triticale grain for commercial flour production. Journal of Cereal Science, 57: 527–530.

Doxastakis G., Zafiriadis I., Irakli M., Marlani H., Tananaki C. (2002): Lupin, soya and triticale addition to wheat flour doughs and their effect on rheological properties. Food Chemistry, 77: 219–227.

Faměra O., Hrušková M., Novotná D. (2004): Evaluation of methods for wheat grain hardness determination. Plant Soil and Environment, 50: 489–493.

Gasparis S., Orczyk W., Nadolska-Orczyk A. (2013): *Sina* and *Sinb* genes in triticale do not determine grain hardness contrary to their orthologs *Pina* and *Pinb* in wheat. BMC Plant Biology, 13: 190.

Gautier M.F., Cosson P., Guirao A., Alary R., Joudrier P. (2000): Puroindoline genes are highly conserved in diploid ancestor wheats and related species but absent in tetraploid *Triticum* species. Plant Science, 153: 81–91.

Gazza L., Taddei F., Conti S., Gazzelloni G., Muccilli V., Janni M., D'ovidio R., Alfieri M. Redaelli R., Pogna N.E. (2015): Biochemical and molecular characterization of *Avena* indolines and their role in kernel texture. Molecular Genetics and Genomics, 290: 39–54.

Giroux M.J., Morris C.F. (1997): A glycine to serine change in puroindoline b is associated with wheat grain hardness and low levels of starch-surface friabilin. Theoretical and Applied Genetics, 95: 857–864.

Gollan P., Smith K., Bhave M. (2007): *Gsp-1* genes comprise a multigene family in wheat that exhibits a unique com-

https://doi.org/10.17221/96/2019-CJGPB

bination of sequence diversity yet conservation. Journal of Cereal Science, 45: 184–198.

Greenblatt G., Bettge A., Morris C. (1995): Relationship between endosperm texture and the occurrence of friabilin and bound polar lipids on wheat starch. Cereal Chemistry, 72: 172–176.

Greenwell P., Schofield J. (1986): A starch granule protein associated with endosperm softness in wheat. Cereal Chemistry, 63: 379–380.

Győri Z., Kruppa J., Ungai D., Mile I.G., Sipos P. (2009): Examination of technological and nutritional properties of breads made from triticale flour. In: Ugarčić-Hardi Z., Jukić M., Komlenić D.K., Planinić M., Obad L. (eds.): Proc. 5th Int. Congress Flour-Bread'09. 7th Croatian Congress of Cereal Technologists , Opatija, Oct 21–23, 2009: 503–507.

Hansen H.B., Møller B., Andersen S.B., Jørgensen J.R., Hansen Å. (2004): Grain characteristics, chemical composition, and functional properties of rye (*Secale cereale* L.) as influenced by genotype and harvest year. Journal of Agricultural and Food Chemistry, 52: 2282–2291.

Hrušková M., Švec I. (2009): Wheat hardness in relation to other quality factors. Czech Journal of Food Sciences, 27: 240–248.

Jing W., Demcoe A.R., Vogel H.J. (2003): Conformation of a bactericidal domain of puroindoline a: Structure and mechanism of action of a 13-residue antimicrobial peptide. Journal of Bacteriology, 185: 4938–4947.

Jolly C.J., Glenn G.M., Rahman S. (1996): *Gsp-1* genes are linked to the grain hardness locus (*Ha*) on wheat chromosome 5D. Proceedings of the National Academy of Sciences, 93: 2408–2413.

Leon A., Rubiolo A., Anon M. (1996): Use of triticale flours in cookies: quality factors. Cereal Chemistry, 73: 779–784.

Li G., He Z., Peña R.J., Xia X., Lillemo M., Sun Q. (2006): Identification of novel secaloindoline-a and secaloindoline-b alleles in CIMMYT hexaploid triticale lines. Journal of Cereal Science, 43: 378–386.

Li G., Gao D., La S., Wang H., Li J., He W., Yang E., Yang Z. (2016): Characterization of wheat-*Secale africanum* chromosome 5R(a) derivatives carrying *Secale* specific genes for grain hardness. Planta, 243: 1203–1212.

Lillemo M., Simeone C.M., Morris C.F. (2002): Analysis of puroindoline a and b sequences from *Triticum aestivum* cv. 'Penawawa' and related diploid taxa. Euphytica, 126: 321–331.

Lukaszewski A.J. (2000): Manipulation of the 1RS.1BL translocation in wheat by induced homoeologous recombination. Crop Science, 40: 216.

Lukaszewski A.J. (2006): Cytogenetically engineered rye chromosomes 1R to improve bread-making quality of hexaploid triticale. Crop Science, 46: 2183.

Massa A.N., Morris C.F. (2006): Molecular evolution of the puroindoline-a, puroindoline-b, and grain softness protein-1 genes in the tribe *Triticeae*. Journal of Molecular Evolution, 63: 526–536.

Massa A.N., Morris C.F., Gill B.S. (2004): Sequence diversity of puroindoline-a, puroindoline-b, and the grain softness protein genes in *Aegilops tauschii* Coss. Crop Science, 44: 1808.

Oliete B., Pérez G.T., Gómez M., Ribotta P.D., Moiraghi M., León A.E. (2010): Use of wheat, triticale and rye flours in layer cake production. International Journal of Food and Science Technology, 45: 697–706.

Oury F.X., Lasme P., Michelet C., Rousset M., Abecassis J., Lullien-Pellerin V. (2015): Relationships between wheat grain physical characteristics studied through near-isogenic lines with distinct puroindoline-b allele. Theoretical and Applied Genetics, 128: 913–929.

Peña R.J. (2004): Food uses of triticale. In: Mergoum M., Gómez-Macpherson H. (eds.): Triticale Improvement and Production. Rome, FAO: 37–48.

Ragupathy R., Cloutier S. (2008): Genome organisation and retrotransposon driven molecular evolution of the endosperm *Hardness* (*Ha*) locus in *Triticum aestivum* cv Glenlea. Molecular Genetics and Genomics, 280: 467–481.

Ramírez A., Pérez G.T., Ribotta P.D., León A.E. (2003): The occurrence of friabilins in triticale and their relationship with grain hardness and baking quality. Journal of Agricultural and Food Chemistry, 51: 7176–7181.

Simeone M.C., Lafiandra D. (2005): Isolation and characterisation of friabilin genes in rye. Journal of Cereal Science, 41: 115–122.

Tanchak M.A., Schernthaner J.P., Giband M., Altosaar I. (1998): Tryptophanins: isolation and molecular characterization of oat cDNA clones encoding proteins structurally related to puroindoline and wheat grain softness proteins. Plant Science, 137: 173–184.

Tranquilli G., Heaton J., Chicaiza O., Dubcovsky J. (2002): Substitutions and deletions of genes related to grain hardness in wheat and their effect on grain texture. Crop Science, 42: 1812–1817.

Trojan V., Musilová M., Vyhnánek T., Klejdus B., Hanáček P., Havel L. (2014): Chalcone synthase expression and pigment deposition in wheat with purple and blue colored caryopsis. Journal of Cereal Science, 59:48–55.

Wall M., Wheeler H., Huebsch M., Smith J., Figeys D., Altosaar I. (2010): The tryptophan-rich domain of puroindoline is directly associated with the starch granule surface as judged by tryptic shaving and mass spectrometry. Journal of Cereal Science, 52: 115–120.

Williams P.C. (1986): The influence of chromosome number and species on wheat hardness. Cereal Chemistry, 63: 56–58.

Received: October 7, 2019
Accepted: May 11, 2020
Published online: June 8, 2020