EVALUATION OF SLOVAK WINTER WHEAT QUALITY IN TERMS OF PUROINDOLINE GENES

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The grain hardness of 100 current and 24 old superior Slovak winter wheat cultivars was studied at molecular level. Using polymerase chain reactions (PCRs), normal and null alleles of both puroindoline *Pina* and *Pinb* genes were identified. Three different genotypes were found: 1) normal allele of both genes (dominant wild type with soft endosperm) – *Pina*-D1a/*Pinb*-D1a; 2) normal allele of the *Pina* gene and null allele of the *Pinb* gene – *Pina*-D1a/*Pinb*-D1b; and 3) null allele of the *Pina* gene and normal allele of the *Pinb* gene *Pina*-D1b/*Pinb*-D1a. No Slovak current as well as old wheat cultivar had together null allele of both puroindoline genes. The frequencies of wild-type *Pinb*-D1a and null *Pinb*-D1b allele in current cultivars were 62.0% and 38.0%, respectively, whilst in

old cultivars, 8.3% and 91.7%, respectively. Regarding null allele *Pina*-D1b of puroindoline *Pina* gene, only in Rheia current cultivar, one was found. All other cultivars had wild-type *Pina*-D1a allele. Alacris, Alana, Axis, Balada, Blava, Bona Dea, Bruta, Charger, Hana, Ilona, IS Karpatia, Ludwig and Sulamit current cultivars were selected as donors of the null *Pinb*-D1b allele for molecular breeding in order to improve the grain hardness as important wheat quality trait. Statistically significant correlations between null *Pinb*-D1b allele and grain size as well as colour were found. In comparison with wild type, cultivars with this null allele have paler and longer grain with higher length-to-width ratio and lighter grain colour.

Key words: current and old Slovak cultivars, grain size and colour, null allele, polymerase chain reaction, puroindoline genes, winter wheat

The variation in grain hardness (hard or soft endosperm texture) is one of the most important traits that determine the utilisation and marketing of hexaploid wheat. Wheat grain texture is determined by the degree of grain hardness or softness. Hardness is defined as ‘difficulty to penetrate or reduce to smaller fragments’. This characteristic strongly influences the functionality of wheat and affects a range of parameters including the milling (tempering, milling yield, flour particle size, shape and density of flour particles), baking and end-use properties (Giroux & Morris 1998; Morris 2002). An important functional

difference between hard and soft wheats is in their water absorption. Hard wheat varieties are typically higher in protein content (12–15%) and stronger gluten-forming proteins than soft wheat ones (5–10%). Grain hardness was negatively correlated with break flour yield, flour yield and mixing score and positively correlated with flour ash (Martin *et al.* 2001). Grain hardness was not correlated with loaf volume or crumb grain score (Hogg *et al.* 2005).

Wheat hardness (the degree of adhesion between the starch granules and the protein matrix) is regulated by the friabilin. The discovery of this 15-kDa

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protein provided a biochemical way to distinguish between hard and soft wheats. It is present in larger amounts in soft wheats compared to hard ones and consists of three major polypeptides (Gautier *et al.* 1994; Rahman *et al.* 1994; Giroux & Morris 1997): puroindoline-a (*Pina*), puroindoline-b (*Pinb*) and grain softness protein 1 (*Gsp-1*). A single locus *Hardness* (*Ha*) for the grain endosperm texture was identified at the short arm of chromosome 5D (Turnbull *et al.* 2003). They designated the gene *Hardness*, with the soft allele *Ha* and the hard allele *ha*. Softness is a dominant trait.

Previous studies suggested that the grain hardness is correlated with the soft type *Pina* and *Pinb*, not total puroindoline (Swan *et al.* 2006). Igrejas *et al.* (2001) found *Pinb* to be more closely correlated with grain hardness than *Pina*. The soft grain texture in wheat is the result of both puroindoline genes occurrence in the wild-type form. When one of the puroindolines is either absent or altered by various mutations, it results in a hard texture. Gene sequence variation and mutations of both puroindoline genes account for the majority of variation in the wheat grain texture.

To date, in winter wheat, 16 and 24 recessive null alleles were identified at the *Pina* and *Pinb* loci, respectively (Bhave & Morris 2008). However, still – from time to time – new mutations of both puroindoline genes are discovered (Chen *et al.* 2009). Most of mutate alleles occur only isolated in some localities (Lilemo & Morris 2000; Chen *et al.* 2005, 2006), for instance, in Northern Europe (*Pinb-D1c* allele), Sweden and Netherlands (*Pinb-D1d*), a few of Chinese landraces (*Pina-D1l*, *Pina-D1n*, *Pinb-D1p*), Jiangsu province (*Pina-D1m*) and Guizhou province (*Pinb-D1t*).

Some of the alleles result in particular single nucleotide change or deletion at various positions in the coding region, another result in the ‘stop’ codon TGA (tryptophan). The dominant wild-type alleles (*Pina-D1a* and *Pinb-D1a*), as well as recessive *Pinb-D1b* null allele, occur most frequently. The *Pina-D1a* allele (wild type) is present in all soft hexaploid and possibly all hard hexaploid wheats carrying the hardness mutation in puroindoline-b. The *Pinb-D1a* allele (wild type) occurs in all soft hexaploid and possibly in all hard hexaploid wheats carrying the mutation in puroindoline-a. The *Pinb-*

D1b is a ‘loss of function’ mutation. It is prevalent amongst a wide set of both recent and historical cultivars (Giroux & Morris 1997).

Grain hardness is largely controlled by genetic factors; however, it can be also partly affected by the environmental and other factors allowing N management, tillage system, fertiliser as well as fallow management, pest infestations, moisture, gliadin composition, lipid, starch and pentosan content (Huebner & Gaines 1992; Peterson *et al.* 1992; Lyon & Shelton 1999; Konopka *et al.* 2005b; Oury *et al.* 2015).

Owing to the lack of information about hardness of Slovak winter wheat cultivars at molecular level, the specific objective of this study was to discover allelic variation of both puroindoline genes in current and old Slovak cultivars. Another aim was to recognize the relation of glycine → serine point mutation to the grain size and colour as well as to determine suitable cultivars as donors of the *Pinb-D1b* null allele for marker-assisted selection (MAS) in order to manipulate the grain hardness.

MATERIAL AND METHODS

Plant materials

Seed samples (200 grains of each cultivar) of 100 current (registered in the Slovak National List of Varieties) and 24 old superior Slovak winter wheat cultivars were obtained from The Gene Bank of Slovak Republic in Piešťany (kindly provided by Dr. Pavol Hauptvogel). Following the published data (McIntosh *et al.* 2003), Bolero cultivar (Italy) was used as standard for the detection of normal allele of both puroindoline genes. In addition, Amidon cultivar (USA), which has null allele (*Pina-D1b*) of the *Pina* gene, as well as Brasilia (Italy) and Pascal (France) cultivars possessing null allele (*Pinb-D1b*) of the *Pinb* gene were used.

Genomic DNA preparation

DNA was extracted from 100 mg of young wheat leaf tissue using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions.

Primers and PCR conditions

Amplification of the *Pina* and *Pinb* puroindoline genes was performed using sequence-specific primers Pina-D1-F, Pina-D1-R, Pinb-D1-F, Pinb-D1-R, Pinb-glyR and Pinb-serR (Gautier *et al.* 1994; Giroux & Morris 1997; Tranquilli *et al.* 1999). The sequences for forward (F) and reverse (R) primers were as follows:

Pina-D1-F: 5'-CCC TGT AGA GAC AAA GCT AA-3'

Pina-D1-R: 5'-TCA CCA GTA ATA GCC AAT AGT G-3'

Pinb-D1-F: 5'-ATG AAG ACC TTA TTC CTC CTA-3'

Pinb-D1-R: 5'-TCA CCA GTA ATA GCC ACT AGG GAA-3'

Pinb-glyR: 5'-CTC ATG CTC ACA GCC GCC-3'

Pinb-serR: 5'-CTC ATG CTC ACA GCC GCT-3'

The polymerase chain reactions (PCRs) were performed in a final reaction volume of 15 µl using 25 ng of genomic DNA, 1 × PCR buffer, 1.5 mM MgCl₂, 0.2 mM dNTP, 0.2 µM of each forward and reverse primers and 0.8 U of *Taq* DNA polymerase (Invitrogen).

The amplification conditions for *Pina* included initial denaturation at 94°C for 3 min, followed by 37 cycles at 94°C for 90 s, at 55°C for 90 s, at 72°C for 2 min and final extension at 72°C for 10 min. To amplify *Pinb*, annealing temperature was raised up to 58°C. PCRs were conducted using PTC-200 thermal cycler (MJ Research Inc., Waltham).

Amplified PCR products were analysed on 1.4% agarose gels, stained with ethidium bromide. Amplification is observed only for the wild-type *Pina-D1a*, yielding 330 bp fragment; null allele *Pina-D1b* amplifies no band.

After amplification of a 447-bp segment of the *Pinb-D1* gene using primers designed by Gautier *et al.* (1994), the PCR product was digested by the restriction endonuclease BsrBI. The enzyme recognises the sequence with the glycine → serine mutation but not the sequence without the mutation. After digestion, fragments of 320 bp are expected for genotypes lacking the mutation (normal allele), whilst fragments of 200 bp are expected for genotypes carrying the null allele for hard texture (Tranquilli *et al.* 1999). For differen-

tiation of normal *Pinb-D1a* from null *Pinb-D1b* allele, primers Pinb-glyR/Pinb-serR and Pinb-D1-F were used (Gautier *et al.* 1994).

Grain parameters assessment

In triplicate, 36 grains of two genetically related pairs of old wheat cultivars Slovenská B and Slovenská 777 (Slovenská B line II and Slovenská B line III as well as Slovenská 777 line I and Slovenská 777 line V) were examined for grain dimensions and 24 grains for colour. Digital image analysis (images were obtained with scanner HP Scanjet 4370, Hewlett Packard) was used to measure grain length and width (Tahir *et al.* 2007) as well as red, green and blue (RGB) colour components. The methods of measurement were similar as described by Tańska *et al.* (2005). Dimension parameters were measured in the Microsoft photo editor 3. To determine the grain colour histograms of RGB component, the Assess Image Analysis Software for Plant Disease Quantification were used (Lamari 2002).

Statistical methods

Standard statistical testing including analysis of variance (ANOVA), general linear model, Bonferroni test and Tukey's HSD test (statistical software package SPSS® 11.5) was used for data evaluation. The value $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

All 124 evaluated wheat cultivars belong to following alleles of the *Pina* and *Pinb* genes (Table 1): normal *Pina-D1a* and *Pinb-D1a* and null *Pina-D1b* and *Pinb-D1b* alleles. Concerning the *Pina* gene, null allele *Pina-D1b*, which is associated with harder texture than *Pinb-D1b* mutation (Morris & Massa 2003), was found only in Rheia current cultivar. Other current cultivars, as well as old cultivars, have wild-type *Pina-D1a* allele. Regarding the *Pinb* gene, frequencies of normal (wild-type) *Pinb-D1a* and null *Pinb-D1b* allele were 62.0% and 38.0%, respectively, in current cultivars, whilst 8.3% and 91.7%, respectively, in historical ones. Recessive null *Pinb-D1b* allele was found mostly in cultivars with good bread-making quality (Alacris, Alana, Axis, Balada,

Blava, Bona Dea, Bruta, Charger, Hana, Ilona, IS Karpatia, Ludwig, and Sulamit). Surprisingly, almost all (22 from 24) old superior cultivars were homozygous for recessive null *Pinb-D1b* allele. The null *Pinb-D1b* allele was detected in Slovenská 777 (I–IV), Stupická Bastard (I–V), Kaštická bezosinátá (I–III), Radošinská Dorada (I–V), Slovenská 200 I, Slovenská B (I and II) and Bučianska červenoklasá (I and II) cultivars. Because all varieties had normal (*Pina-D1a* and *Pinb-D1a*) or the most frequent null allele (*Pina-D1b* and *Pinb-D1b*) of both puroindoline genes, we did not look for other sporadically occurring null alleles.

Three different genotypes of puroindoline genes were found: 1) normal allele of both genes, *Pina-D1a/Pinb-D1a* (dominant wild type with soft grain); 2) normal allele of the *Pina* gene and null allele of the *Pinb* gene, *Pina-D1a/Pinb-D1b* (moderately hard grain); and 3) null allele of the *Pina* gene and normal allele of the *Pinb* gene, *Pina-D1b/Pinb-D1a* (very hard grain). Neither in the current nor in the old Slovak wheat cultivars we detected simultaneous presence of null alleles of *Pina* and

Pinb genes. Dominant wild type with soft grain was achieved in 61.0% of current cultivars and in only 8.3% of old wheat cultivars (Table 2). The genotype *Pinb-D1b* was detected in 38.0% of current and 91.7% of old superior cultivars. Whilst in old cultivars, the *Pina-D1a/Pinb-D1b* genotype with moderately hard grain dominated, in current cultivars, the dominant wild-type *Pina-D1a/Pinb-D1a* with soft grain associated with poorer wheat quality was more frequent. This fact points to forgetting of grain hardness importance in breeding for other wheat qualitative traits. Our results provide the possibility of Slovak wheat cultivars breeding for hardness improvement.

Obtained results from cultivars of the Slovak origin are in agreement with observations of other authors who also found the *Pinb-D1b* mutation as prevalent amongst a wide set of both recent and historical wheat cultivars of different origin (Giroux & Morris 1997). The null allele has a glycine → serine substitution at position 46 in the puroindoline *Pinb* gene (Giroux & Morris 1997). The *Pinb-D1b* allele was first characterised in the ‘Chinese Spring’

T a b l e 1

The frequency of homozygous alleles in winter wheat cultivars

Cultivars (number)	<i>Pina</i> gene		<i>Pinb</i> gene	
	Normal allele <i>Pina-D1a</i> [%]	Null allele <i>Pina-D1b</i> [%]	Normal allele <i>Pinb-D1a</i> [%]	Null allele <i>Pinb-D1b</i> [%]
Current registered (100)	99.0	1.0	62.0	38.0
Old (24)	100.0	0.0	8.3	91.7

T a b l e 2

The frequency of homozygous genotypes in winter wheat cultivars

Puroindoline genotype	Current cultivars [%]	Old cultivars [%]
<i>Pina-D1a/Pinb-D1a</i>	61.0	8.3
<i>Pina-D1b/Pinb-D1a</i>	1.0	0.0
<i>Pina-D1a/Pinb-D1b</i>	38.0	91.7
<i>Pina-D1b/Pinb-D1b</i>	0.0	0.0

Pina – Puroindoline-a gene; *Pinb* – Puroindoline-b gene

substitution line possessing the 5D chromosomes of ‘Cheyenne’. Giroux & Morris (1997) discovered that the hardness mutation is a single base change (GGC→AGC) in the codon of Gly-46, converting glycine to serine. The mutation is highly conserved and might explain the most of phenotypically hard hexaploid wheats. Lillemo and Morris (2000) showed that the prevalence of mutation might be largely related to the gene pools of interest and also the particular area of origin.

Genotypes with *Pinb-D1b* possess significantly lower flour ash content and higher milling yield than those of genotypes with *Pina-D1b*. For steamed bread, mean scores for loaf volume, crumb colour, width and structure and total score of *Pinb-D1b* genotypes were significantly higher than those of geno-

types with *Pina-D1b* and wild-type *Pinb-D1a* (Chen *et al.* 2007).

Two old Slovak wheat cultivars, Slovenská B and Slovenská 777, had different alleles of the *Pinb* gene (Slovenská B line III had normal allele and Slovenská B line II the null allele, Slovenská 777 line V had normal and Slovenská 777 line I had the null allele). Therefore, we focused on comparing the width, length and RGB colour components in relation to the presence of the null and normal allele of *Pinb-D1b* gene in these genetically related lines of cultivars Slovenská B and Slovenská 777.

Concerning *Pinb* gene, significant influence on grain length, grain length-to-width ratio, width-to-length ratio and grain colour components red, green and blue in RGB colour model was found (Table

T a b l e 3

Mean squares from ANOVA for wheat grain size and grain colour components to alleles of the *Pinb* gene

	Variability of the <i>Pinb</i> gene	df	Mean squares
Grain dimensions			
Length	Between groups	1	8,576.053 ⁺
	Within groups	46	1,257.283
	Total	47	–
Width	Between groups	1	1,313.985 ^{NS}
	Within groups	46	809.291
	Total	47	–
Length/width	Between groups	1	0.373 ⁺⁺
	Within groups	46	0.043
	Total	47	–
Width/length	Between groups	1	0.018 ⁺⁺
	Within groups	46	0.002
	Total	47	–
Grain colour components			
Red	Between groups	1	18,292.563 ⁺⁺
	Within groups	142	303.642
	Total	143	–
Green	Between groups	1	18,225.000 ⁺⁺
	Within groups	142	235.359
	Total	143	–
Blue	Between groups	1	33,580.562 ⁺⁺
	Within groups	142	282.534
	Total	143	–

⁺⁺Significant at the 0.01 level; ⁺significant at the 0.05 level; ^{NS} not significant

3). From Table 4, the rounder kernel in cultivars having null allele of the *Pinb* gene is evident. The mean values of grain length and width were 623 and 288 pixels for normal allele *Pinb-D1a* and 596 and 299 pixels for null allele *Pinb-D1b*, respectively. In comparison with normal allele *Pinb-D1a*, the cultivars with the null allele *Pinb-D1b* have significantly shorter kernel ($P < 0.05$) and lower length-to-width ratio ($P < 0.01$).

Using digital image analysis, significant differences in wheat grain size and colour were ascertained. Grain size influences milling quality of wheat (Marshall *et al.* 1986). Sizes together with density determine grain weight, which has a favourable effect on agronomic and flour yield of wheat (Dziki & Laskowski 2005). Quantitative trait loci (QTL) for grain length and width in bread wheat were found on various chromosomes (Dholakia *et al.* 2003; Breseghello & Sorrells 2007). From the low genetic correlation between grain length and

width found, Bergman *et al.* (2000) deduced their independent inheritance.

In addition, cultivars with the null allele *Pinb-D1b* have lighter grain colour. RGB colour components in cultivars with the null allele have significantly ($P < 0.01$) higher values as compared to cultivars with normal allele (Table 5). Mean squares from general linear model and coefficient of determination (R^2) for length, width, length-to-width ratio, width-to-length ratio and colour components RGB indicate that the fitted model explains significant ($P < 0.01$) great deal of variability (Table 6). Dobraszczyk *et al.* (2002) indicated that grain hardness corresponds to endosperm density. Results of Konopka *et al.* (2005a) pointed to relationship between the endosperm colour and grain hardness in wheat cultivars. However, the authors found that environment causes variability of the protein content, hardness, vitreousness, length and size of grains, and all these can affect grains colour.

T a b l e 4

Mean values of grain size in relation to the *Pinb* gene (the Bonferroni test)

Grain dimensions	Allele of the <i>Pinb</i> gene	N	Mean	Std. error	Difference
Length	Normal	24	623.089	7.027	26.733 ⁺
	Null <i>Pinb-D1b</i>	24	596.355	7.443	–
Width	Normal	24	288.473	5.426	–10.464 ^{NS}
	Null <i>Pinb-D1b</i>	24	298.937	6.330	–
Length/width	Normal	24	2.182	0.048	0.176 ⁺⁺
	Null <i>Pinb-D1b</i>	24	2.005	0.036	–
Width/length	Normal	24	0.463	0.441	–0.039 ⁺⁺
	Null <i>Pinb-D1b</i>	24	0.502	0.484	–

⁺⁺Significant at the 0.01 level; ⁺significant at the 0.05 level; ^{NS} not significant

T a b l e 5

The effect of alleles on RGB colour components in relation to the *Pinb* gene (Tukey's HSD test)

Cultivar	Allele of <i>Pinb</i> gene	N	Red (R)	Green (G)	Blue (B)
Slovenská B III	Normal	36	187.86 ^a	139.58 ^a	50.53 ^a
Slovenská 777 V	Normal	36	200.50 ^a	148.89 ^a	52.39 ^a
Slovenská 777 I	Null <i>Pinb-D1b</i>	36	215.42 ^b	166.39 ^b	78.72 ^b
Slovenská B II	Null <i>Pinb-D1b</i>	36	218.03 ^b	167.08 ^b	85.28 ^b

Different letter (a, b) indicate statistically significant differences ($P < 0.01$)

CONCLUSIONS

Wheat marketing system established the primary classification of hexaploid wheat based on the endosperm texture. The grain hardness is one of the most important traits and affects a range of parameters including the milling, baking and end-use properties of wheat. The aim of our work was to evaluate the grain hardness of 100 current and 24 old superior

Slovak winter wheat cultivars at molecular level and determine its relation to grain size and colour. Normal (wild-type) and null alleles of both puroindoline *Pina* and *Pinb* genes were identified using PCRs. In conclusion, no one of cultivars tested had together null allele of both *Pin* genes. Only one null *Pina* allele was identified in Slovak winter wheat cultivars. Null allele *Pinb-D1b* ('loss of function' mutation resulting from the replacement of glycine by serine

T a b l e 6

Mean squares from general linear model and coefficient of determination (R^2) for length, width, length/width, width/length, and colour component red, green, blue in relation to the *Pinb* gene

	Source	df	Mean square	R^2
Grain dimensions				
Length	Model	2	8,926,552.480 ⁺⁺	0.997
	<i>Pinb</i>	2	8,926,552.480 ⁺⁺	–
	Error	46	1,257,283.000	–
	Total	48	–	–
Width	Model	2	2,070,960.041 ⁺⁺	0.991
	<i>Pinb</i>	2	2,070,960.041 ⁺⁺	–
	Error	46	809.291	–
	Total	48	–	–
Length/Width	Model	2	105.376 ⁺⁺	0.991
	<i>Pinb</i>	2	105.376 ⁺⁺	–
	Error	46	0.043	–
	Total	48	–	–
Width/Length	Model	2	5.592 ⁺⁺	0.991
	<i>Pinb</i>	2	5.592 ⁺⁺	–
	Error	46	0.002	–
	Total	48	–	–
Colour components				
Red	Model	2	3,048,285.951 ⁺⁺	0.993
	<i>Pinb</i>	2	3,048,285.951 ⁺⁺	–
	Error	142	303.642	–
	Total	144	–	–
Green	Model	2	1,749,779.514 ⁺⁺	0.991
	<i>Pinb</i>	2	1,749,779.514 ⁺⁺	–
	Error	142	235.359	–
	Total	144	–	–
Blue	Model	2	337,390.562 ⁺⁺	0.944
	<i>Pinb</i>	2	337,390.562 ⁺⁺	–
	Error	142	282.534	–
	Total	144	–	–

⁺⁺Significant at the 0.01 level

at position 46), which is prevalent amongst most of the European countries, was detected in 38.0% of current and 91.7% of old superior cultivars. Its incidence was higher in old superior in comparison with current cultivars. Statistically significant correlations between null *Pinb-D1b* allele and grain size as well as colour were found. The cultivars with the null allele *Pinb-D1b* have significantly shorter kernel, lower length-to-width ratio and lighter grain colour. We selected cultivars that would be helpful in molecular breeding programme as donors of the null allele in order to manipulate grain hardness as one of the main quality traits of winter wheat.

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