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STUDYING DIFFERENT MOLECULAR MARKERS FOR KERNEL TEXTURE IN BREAD WHEAT (*Triticum aestivum* L.)

Bachelor’s thesis (12 EAP)

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TABLE OF CONTENTS

TABLE OF CONTENTS 2

ABBREVIATIONS 4

PREFACE 5

1 OVERVIEW OF LITERATURE 7

1.1 The importance of the wheat quality 7

1.1 Hardness/texture of the wheat endosperm 7

1.2 Puroindolines 10

1.2.1 The discovery and nomenclature of puroindolines 10

1.2.2 Biochemical properties 11

1.2.3 The genetic background 12

1.2.4 Puroindoline b-2 14

1.2.5 Antimicrobial activities of puroindolines 15

1.3 Marker development for selection in wheat breeding 16

1.3.1 Protein markers 17

1.3.2 Antibody-based marker assays 18

1.3.3 DNA- based markers 18

1.4 High resolution melting analysis 22

2 EXPERIMENTAL WORK 23

2.1 The aim of the experimental work 23

2.2 Materials and methods 23

2.2.1 Seed sterilization and plant growth conditions 23

2.2.2 Genomic DNA isolation and purification 24

2.2.3 Gel-electrophoresis 25

2.2.4 DNA purification from gel 26

2.2.5 Cycle sequencing 26

2.2.6 *In silico* sequence analysis 27

2.3 Results and discussion 28

2.3.1 Historic set results regarding puroindoline-a and puroindoline-b 28

2.3.2 Biofortification-set results regarding puroindoline-a and puroindoline-b 29

2.3.3 HRM-PCR primers optimization 30

2.3.4 Historic set results regarding the puroindolineb-2 alleles 31

2.3.5 Australian set results regarding puroindolineb-2 alleles 32

2.3.6 Marker development for seed vitreousness and density 32

2.3.7 Marker development for wheat seed hardness 35

CONCLUSION ... 36
Terise tekstuuri molekulaarsete markerite uurimine harilikus nisus (*Triticum aestivum)* ... 38
REFERENCES ... 40
USED WEB ADDRESSES ... 45
APPENDIX I ... 46
APPENDIX II ... 47
AKNOWLEDGEMENTS ... 59
Non-exclusive licence to reproduce thesis and make thesis public ... 60

ABBREVIATIONS

+f – faint positive

AFLP – Amplified Fragment Length Polymorphism

B-set – Kazakhstan biofortification set

EndoC – endosperm collapse

EndoR – endosperm response

Ha- hardness locus

HRM-PCR – high resolution melting polymerase chain reaction

HRM-PCR- high resolution melting PCR

H-set – Kazakhstan historical set

MAS/MAB – Marker assisted selection/Marker assisted breeding

MQ – Milli-Q™ Millipore Corporation ultrapurifyed water

NIR – near infrared reflectance spectroscopy

NTC – no template control

PINA – protein of puroindoline a

Pina- puroindoline-a gene

PINB – protein of puroindoline b

Pinb – puroindoline b gene

Pinb-2 – puroindoline b-2 gene family

PuroA – bactericidial domain of PIN-a

PuroB – bactericidial domain of PIN-b

QTL – quantitative trait locus/loci

RFLP – Restriction Fragment Length Polymorphism

RHI – rheological hardness index

SKCS – single kernel characterization system

SNP – single nucleotide polymorphism

TKW – thousand kernel weight

TRD – tryptophan rich domain

PREFACE

Wheat is one of the worlds' most important crop and an important source of nutrients for human kind. A wide assortment of products is made from wheat such as bread, cakes and cookies, pasta and other. *T. aestivum* is an allohexaploid wheat containing three genomes A, B and D combining the diploid and tetraploid genomes of its ancestors. The size of the hexaploid bread wheat *(Triticum aestivum)* genome is 17 000 Mbp, which is 10-20 times bigger than other common crops. The complexity and the size of the bread wheat genome makes the research of it challenging, but necessary.

The research of wheat gives answers to breeders about the quality of the wheat and the end-product, which is made from it. Kernel hardness is one of most well researched characteristic of wheat kernels as it is perfectly controlled with genetics. It should be noted that apart from genetics environment also has effects on it, which makes science even more challenging. Research in the recent decades has found that proteins called puroindolines, coded by the puroindoline-a and puroindoline-b genes are the main determinants of the kernel hardness. The similarities between the electrophoretic, detergent fractionation and N-terminal sequence properties of two of the cold SDS-extractable starch friabilin polypeptides and those of puroindolines-a and -b strongly suggest that friabilin comprises a mixture of proteins in which the puroindoline polypeptides are important components. Although these genes affecting the wheat endosperm texture are close to the hardness *(Ha)* locus and the endosperm texture is referred to as kernel hardness, indeed the softness is the dominant trait. Wheat kernels are soft if both puroindolines are present as wild type. Any mutation either in *Pina* or *Pinb* results with hard kernel. Puroindolines and other molecular markers contribute to modern plant breeding.

The aim(s) of this work are(is) to study the hardness of the wheat kernel. We studied the genetic background determining the wheat endosperm texture focusing mainly on puroindolines. The practical part of this project on one hand was the molecular characterization and description of the diversity of puroindoline (PIN) genes and alleles. Sixty-one wheat varieties collected in Kazakhstan were involved in this project. One set contained 49 cultivars from a historical pool, while samples (12) in the other set were part of an experiment, carried out in another laboratory, used for biofortification studies On the other hand we focused on establishing and testing other DNA-based molecular markers regarding

kernel hardness, vitreousness and density. The markers were based on sequences published earlier and validated on Australian varieties. This marker development project is part of an international collaboration for finding new relationships for quality prediction.

The thesis work was conducted in Eötvös Lórand University (ELTE), Faculty of Science, Institute of Biology, Department of Plant Physiology and Molecular Plant Biology, HUNGARY under the supervision of Dr. László Tamás.

1 OVERVIEW OF LITERATURE

1.1 The importance of the wheat quality

There are several important characteristics which describe the quality of the bread wheat (*T. aestivum* L.). One of the most important amongst others is the hardness of the wheat endosperm which determines the end-use quality of the flour. To breed the best quality wheat – co-operation of scientists, breeders and millers is necessary. To ease the work of breeders it is important to use marker assisted breeding (MAB) or also mainly known as marker assisted selection (MAS). Marker assisted selection helps breeders to choose high quality varieties in the early stage which meet their expectations and needs and which are stable across environments. The 'appropriate' quality depends on the user of the wheat and on the product required from it. For miller the importance stands in the endosperm texture which influences the tempering, flow, sifting area and energy consumption of a flour mill (Bettge *et al*., 1995). For a baker who wishes to bake cakes, cookies and other low moisture content products it is important to use wheat which has a soft characteristic as its starch granules do not get damaged while milling and the water absorption of the granules is low so the result is more satisfying. In case of baking bread – hard bread wheat is advantageous. Its kernels suffer trough high mechanical damage during milling resulting higher endosperm starch granule fracturing. Due to the more damaged starch granules the flour has higher water absorption capacity and the starch molecules are more available for hydrolysis by α-amylase enzyme. This gives better quality bread, higher loaf volume, in case of making yeast leavened bread because yeasts have greater amount of fermentable sugar to use thanks to better starch hydrolysis (Bettge et al., 1995). Tetraploid durum wheat (*T. durum),* which has the hardest characteristics is perfect for making pasta-like products but not very good for bread making.

1.1 Hardness/texture of the wheat endosperm

Intensive screening on large population size is needed for breeding programs to get reliable results of the quality traits. As these procedures are quite expensive and time consuming, the varietal performance is predicted mainly on advanced breeding lines. Important field of studies are the identification of Quantitative Trait Loci (QTL) and genes influencing the specific end use quality traits such as endosperm texture (Tsilo *et al*., 2011). According to Edwards (2010) "QTL are stretches of DNA that are closely linked to the genes that underlie a quality trait". Hardness of the wheat kernel is one of the best researched quality attributes.

Wheat grain classes are mainly formed taking the morphology and texture of the kernel into consideration, like color, shape and length of the kernel (Campbell *et al.*, 1999). Generally, hard wheat kernels are narrow, translucent and long while soft wheat kernels are rounded, short and chalky (Campbell *et al.*, 1999). For efficient trading of wheats, classifications of wheat categories are developed so that some information about the wheat varieties, hybrids or landlines can be obtained by the name and without the need to test the samples before trading. Three main systems of classification are known: The United States, Canadian and Australian. The US classification is based on the color, hardness and the season of the wheat grown and harvested.

Table 1 Classification of U.S. wheat classes. Subcategories are marked in parentheses.

Code	**Official name**
HRSW	Hard Red Spring wheat (DNSW-*Dark Northern Spring;* NSW-*Northern Spring;* RSW-*Red spring)*
HRWW	Hard Red Winter wheat
SRWW	Soft Red Winter wheat
DW	Durum wheat (HADW – *Hard Amber Durum;* ADW – *Amber durum)*
WW	White wheat (HWW – *Hard white;* SWW –*Soft white;* Western; white Club*
UW	Unclassified wheat
MW	Mixed wheat

* - Club wheat is the special wheat produced from hexaploid *T. compactum* in the north-western part of the U.S. (Békés, 2001)

Canadian wheat classification system is based on the combination of endosperm texture and growing season in addition in some cases the growing region is also used as there are significant differences depending if the wheat is grown on the Eastern or the Western part. CWES is a special group as it is the best bread-wheat class in the world with perfect characteristics for baking high quality bread (Békés, 2001).

Table 2 Canadian wheat classification (Békés, 2001).

Code	Official name
CWRS	Canada Western Red Spring (hard)
CWES	Canada Western Extra Strong Red Spring (hard)
CWAD	Canada Western Amber Durum (hard)
CWRW	Canada Western Red Winter (hard)
CWSWS	Canada Western Soft White Spring (soft)
CPSR	Canada Prairie Spring Red (hard)
CPSW	Canada Prairie Spring White(hard)
CEWW	Canada Eastern White Winter (hard)
CERW	Canada Eastern Red Winter (hard)
CESWS	Canada Eastern Soft White Spring (soft)
CEMW	Canada Eastern Mixed Wheat (soft)
CFW	Canada Feed Wheat (forage)

Australia is the third biggest producer and exporter of wheat, also having its own classification system. The interesting point of Australian classification is that the classes are formed according to the usage of the wheat which makes it easy to export as it is known for the buyer what quality wheat was purchased.

Table 3 Australian wheat classification (Békés, 2001)

Code	Official name	Usage
APH	Australian Hard Prime	Yellow alkaline noodle, Japanes Ramen noodle, baking products, Wanton noodle, wheat with corrective qualities
AH1	Australian Hard	Baking products, Arabian pita bread, Chinese

		steamed products
APW	Australian Premium White	Hokkien and instant noodles, Arabian pita, Indian breads, Chinese steamed products
ASW	Australian Standard White	General usage
ANW	Australian Noodle	Udon noodle
AS	Australian Soft	Cookies, cakes, crackers
AD	Australian Durum	Extruded Pasta products

Generally, each country has its own classification, but knowing the classification of the main distributers of wheat helps the world market to be stable.

1.2 Puroindolines

1.2.1 The discovery and nomenclature of puroindolines

The discovery of 15 kDa protein named friablilin was a huge breakthrough in researching biochemical markers. This protein was found in association with soft wheat starch, found less association with hard wheat starch and no protein was found in durum wheat (Pasha et al., 2010). This 15kDa protein family is known as a biochemical marker for determining texture genotype of a single kernel. The appearance of friabilin on water-washed wheat starch granules is unrelated to the environmental affect and highly correlated to with grain hardness. (Bettge et al., 1995)

The discovery of puroindolines showed that puroindolines are the primary components of friabilin (Craig F Morris, 2002). Puroindolines have five disulphide bonds with tryptophan-rich domains. These proteins have unique tryptophan-rich region which has an indole ring. The name was first provided by Didier Marion and it refers to *puros* which means wheat and *indoline* which refers to the indole ring of tryptophan.(Blochet et al., 1993) (Pasha et al., 2010) Similar proteins have been reported in other cereals. In barley these similar proteins is named hordoindolines and secaloindoline-a and secaloindoline-b in rye.

1.2.2 Biochemical properties

The main task of PINs is the fact that they are the molecular basis for endosperm texture. With both puroindolines in their functional form causes soft and friable endosperm. In case of the absence or mutation of one of the puroindolines the texture of the endosperm is hard. Secondary, it has been proved *in vivo* and *in vitro* that PINs are antimicrobial proteins which participate in seed protection (Massa and Morris, 2006).

From the 15kDa biochemical marker friabilin, puroindolines form 12,8 kDa by molecular mass (Blochet et al., 1993). PINA and PINB comprehend cysteine backbone with 10 Cysteine residue. Cysteins form a tertiary structure of four alpha helixes which are separated by loops and stabilized by five disulphide bridges (Miao et al., 2012). Puroindolines have special tryptophan rich domain (TRD) which in case of *PINA* has five Trp residues and three Trp-s in PINB (Bhave and Morris, 2008b). PINA has better lipid binding and antimicrobial properties due to the fact that it has more tryptophan residues in TRD than PINB and other analogues. Often mentioned granule softening protein (GSP-1) has only two Trp residues in its TRD and this may not be enough for playing a role in binding lipids as much as puroindolines do.

The structure and function of the PIN proteins depends highly on the variation in the TRD. Massa and Morris (2006) adduced that based on nucleotide sequence and amino acid sequence predicted from it, the comparison showed a highly conserved *Pinb* domain (WPTKWWK). In the tryptophan rich region of the *Pina* (WRWWKWWK) found three mutations which concluded in amino acid alteration (R65Q, K68R, and K68T) in which the lysine-to-arginine or lysine-to-threonine alteration at position 68 may conclude in changes of the molecular structure of the protein (Massa and Morris, 2006).

In the hardness of endosperm, the role of puroindolines is to bind lipids and insert into lipid bilayers in protein matrix. Due to this, the association of polar lipids and the surface of the starch granule is possible. Several researches have shown that probably the PINA binds to starch granules and PINB is assisting the binding. As mentioned before, PINA has greater influence on hardness. This is proved also by the fact that “null” allele *Pina-D1b* and SNP allele *Pina-D1m* have higher hardness values than the alleles of puroindoline-b (Bhave and Morris, 2008b). In general the hardness is determined by the strength of adhesion between starch granules and protein matrix as well as the other influences like the amount of free polar lipids play role. Increasing amount of lipids is reported to be correlated with increasing hardness (Pasha et al., 2010). Interestingly, it has been reported that the type of the granules also may affect the hardness as “puroindoline has a higher affinity for the surface of the type

A granules as these contribute least to bonding surface area in the endosperm composite structure" (Edwards, 2010). Three types of starch granules are known. The smallest is the C-type < 2 µm, following by the B-type granules with diameter 2µm-9,8µm. The biggest starch granule is the A-type with diameter > 9,8 µm (Dai, 2009).

1.2.3 The genetic background

Puroindolines were firstly isolated from wheat endosperm, but since then they have been reported in several other taxa of the tribe Triticeae including rye and barley (Gautier *et al*., 2000; Lillemo *et al*., 2002; Massa *et al*., 2004)

Allohexaploid means that bread wheat is an allopolyploid containing six copies of its seven chromosomes, which form sets of two and are inherited from three different species (2n=6x=42 AABBDD genomes) (Turnbull *et al.*, 2003). The DD of the genome is probably inherited from D diploid genome *Aegilops tauschii* and AB genome from tertraploid *Triticum turgidum* L. *ssp. dicoccum* Shrank ex Schübler (Massa and Morris, 2006). To look even further, the A-genome of wheat is mostly from *Triticum uratu.* Mostly, all three genomes contribute to the trait of interest, but that is not the case with kernel hardness (Turnbull et al., 2003). Due to the initial tetraploidisation event, the puroindoline genes were deleted from A- and B-genomes, so only a single locus of each of the puroindolines (*Pina-D1* and *Pinb-D1)* remained, but highly similar *Gsp-1* loci prevailed forming now *Gsp-1* loci from *Gsp-A1, Gsp-B1* and *Gsp-D1* (Massa and Morris, 2006). As mentioned previously grain softness is controlled by the hardness (*Ha)* locus which is located in the short arm of the chromosome 5D.

The hardness trait of the grain is determined by the expression of the proteins in the endosperm. As mentioned before, the protein associated to the starch granules is called friabilin and it consists of two separate polypeptides called PINA and PINB. According to Turnbull *et al*. (2003) the small part of GSP-1 is also a component of friabilin. The intronless coding regions of *Pina-D1* and *Pinb-D1* are 447 bp long and 70,2% identical (Bhave and Morris, 2008a). The genes are physically approximately 100bp far from each other (Edwards, 2010).

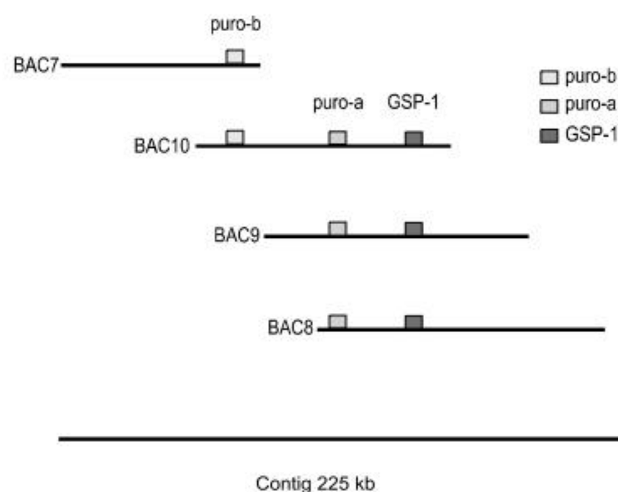

Figure 1 shows the physical order *Pinb* – *Pina* –*Gsp-1* within bacterial artificial chromosome (BACs) clones. Sequence overlap and maximum probable distance between the genes can be seen. (Turnbull et al. 2003)

Till this day 17 alleles of *Pina-D1 (Pina-D1a-q)* and 25 alleles of *Pinb-D1 (Pinb-D1a-17w,aa,ab)* have been reported in common wheat (Pasha et al., 2010). In modern cultivars, the allelic forms of puroindoline-b (*Pinb-D1b* and *Pinb-D1c*) are the most common. *Pinb-D1b* carries a mutation of G→A at the position 223 which results in Gly→Ser change. *Pinb-D1c* also has a single mutation at the pos. 60 which causes Leu→Pro. *Pina-D1b* allele has 15,360 bp deletion starting from 23rd nucleotide. *Pina-D1b* causes lack of puroindoline protein on SDS-page. This mutation was termed as “a-null”. The interpretation of a-null should be handled with caution as there may be other deletions which may disturb the transcription, translation or post-translational modification of the proteins (Morris and Bhave, 2008).

Gsp-1 is the third known gene located in the *Ha* locus. Although it has sequential similarity with puroindolines the role of *gsp-1* has remained unclear. *Gsp-1* is the gene coding grain softening protein. As it can be assumed from its name *gsp-1* has been related to the hardness of the wheat kernel. *Gsp-1* genes are reported to be very diverse but not even the mutations in the genes show remarkable differences in the hardness. The reason why this may be is that the GSP-1 has only two tryptophan in the TRD which may not be enough for binding the lipids and starch granules (Bhave and Morris, 2008b). The fact that most of wheat lines with identical *Pina* and *Pinb* alleles still vary in their hardness values (20-25 SKCS hardness units) although the plants were handled under the same conditions shows that there must be some other minor gene or QTL controlling the hardness and it might as well be the *Gsp-1* gene. Many scientists continue the research of the *Gsp-1* regarding its influence on hardness as its definite function remains unclear to this day.

1.2.4 Puroindoline b-2

Puroindoline b-2 is a novel puroindoline family with the possible effect on kernel hardness and grain yield among soft wheats and *Pinb-2v* proteins may possess antimicrobial activities.

The new *Pin* locus was first reported in 2008 by Mark Wilkinson who named it *Pinb-2* (Wilkinson *et al.*, 2008)(F. Chen *et al.*, 2010). *Pinb*-2 genes are located on group 7 chromosomes. *Pinb-2v1* settles in the long arm of 7D (7DL), *Pinb-2v2* resides on 7BL while puroindoline-b variant 3 (*Pinb-2v3)* also in 7BL and the fourth variant is located in 7AL (Chen *et al.*, 2010)(Geng *et al.*, 2013). The gDNA sequences of puroindoline b-2 families share 70% similarity with *Pinb-D1a* gene. (Geng *et al.*, 2013)

Puroindoline b-2 sequences show multiple variations, unlike the numerous single point mutation alleles in *Pina-D1* or *Pinb-D1*. Puroindoline b-2 families are similar to *Gsp-1* genes. Hence, the genes form haplotypes with each having a specific combination of SNPs that are inherited together as a single gene. According to this, sequences sharing identical SNPs form one haplotype and sequences differing by one or more nucleotides a separate one (haplotype). Based on this, 17 haplotypes were identified. Ramalingam, Palombo and Bhave (2012) previously reported haplotypes ***Pinb2v1*** (*Pinb-2v1*-1...5), ***Pinb2v2*** (*Pinb-2v2*-1...1), ***Pinb2v3*** (*Pinb-2v3*-1;3 and 4), ***Pinb2v4*** (*Pinb-2v4*-1..4), ***Pinb2v5*** (*Pinb-2v5*-1..2), ***Pinb2v6*** (*Pinb-2v6* 1...3). Based on latest article (Chen et al., 2013) the role of *Pinb-2v*-s is still unclear, although it must be emphesized that *Pinb-2v3* alleles give on average 5.4 SKCS hardness units higher results than other variants. *Pinb-B2v3* is divided into 4 alleles: *Pinb- B2v2, Pinb-B2v3a*, *Pinb-B2v3b* and *Pinb-B2v3c*. Allelic association analyses showed that varieties carrying *Pinb-B2v3b* allele had higher grain hardness compared to those with *Pinb-B2v3a* and *Pinb-B2v3c* in soft wheat (Chen *et al*., 2013) According to Chen *et al*. (2013) it is also possible that the higher SKCS HI with *Pinb-B2v3b* is due to relatively higher expression level of this allele.

Sequential variations of *Pinb-2* show that this novel puroindoline multigene family may possibly influence the membrane binding abilities and due to that also the grain texture and antimicrobial activity. Putative proteins of *Pinb-2* genes show changes in TRD as well as in basic and hydrophobic residues (Ramalingam *et al*., 2012). All *Pinb*-2 variants contain 10 conserved Cys backbones just like PINs, except PINB-2V3 and PINB-2V5 which have 11 residues (Chen *et al*., 2010). It is important to emphasize that PINB-2 variants also possess a part of TRD between 3rd and 4th Cys residue with only small Trp motif KWWK (Chen *et al*., 2010). As the lipid binding site is smaller than in puroindolines, it is highly likely that this explains the weaker effect of *Pin-2v* to kernel hardness.

Pinb-2v1 has been reported as minor QTL for hardness. *Pinb-2v2* has been reported to have a textural variation between soft and extra-soft wheats and to yield, while *Pinb-2v3* is allelic to *Pinb-2v2*. Also, *Pinb-2v4* as one of the latest discovered variations has probably some influence, but no clear statement about the function of this variation has been made as well as for *Pinb-2v5 and Pinb-2v6*. It is also known that *Pinb-2v* family has no influence on the hard group of bread wheat (Geng *et al*., 2013). The symbolization of Puroindoline b-2 family has been revised by Geng *et al*. (2013) where they suggest the following designation where *Pinb-2v2* and *Pinb-2v3* should be changed to *Pinb-B2a* and *Pinb-B2b* respectively, whereas they propose that *Pinb-2v1* and *Pinb-2v4* should be designated *Pinb-D2a* and *Pinb-A2a*, respectively. In this thesis, both symbolizations have been used parallel.

1.2.5 Antimicrobial activities of puroindolines

During germination and maturation, seeds are sensitive to fungal, bacterial and viral infections. Thionins were the first know proteins with antimicrobial activities. Thionins are low molecular weight basic cysteine rich proteins which are known to be toxic to several microorganism by binding to their membrane. Tryptophan has been found in several toxic sites of various animal toxins which interfere the membrane functions. As puroindolines behave quite similarly as purothionins at phase partitioning it suggested that puroindolines also might have antibacterial and antifungal properties (Blochet *et al.*, 1993). It has been suggested that PINs act synergistically with thionins and exert their antifungal-antimicrobial properties through the interaction with lipid cell membranes. Also, the larger number of tryptophan (Trp) and other positively charged amino acid residues which are common in puroA, may be the reason for its antimicrobial properties. PuroA (FPVTWRWWKWWKG-NH2) is the 13-residue fragment of wheat PIN-a which is known to act like a bactericidal domain in PIN-a as it has higher in vitro antimicrobial activity than the PIN-b (FPVTWPTKWWKG-NH2; puroB) peptide does. Both arginine (R) and lysine (K) can connect to tryptophan through the noncovalent molecular cation-π interaction, although the interaction of arginine is most likely. Caton-π interactions probably contribute to the stability of the protein (Massa and Morris, 2006). Antimicrobial tests regarding the PINB-2 group suggested that PINB-2V3 showed the least activity compared to PINB-2V1 and PuroA, but better compared to PuroB and PINB-2 peptides share the greatest activity against phytopathogenic fungi (Ramalingam *et al.*, 2012). Last but not least, Lys and Leu also exhibit strong antimicrobial activity (Ramalingam *et al.*, 2012).

The research of puroindoline-a evolution suggests that it has been under positive selection and fixation of the adaptive mutations to become plant defense-related gene and play role in plants immune systems (Massa and Morris, 2006). Tests *in vivo* and *in vitro* have been made with puroindolines. Antibacterial and antifungal activities *in vitro* against *S. aureus, Rhizoctonia solani, Collectotrichum graminicola, Fusarium oxysporum* have given proof about the antimicrobial activities (Ramalingam *et al.* , 2012)

1.3 Marker development for selection in wheat breeding

There are several benefits of MAB compared to traditional breeding. Due to the accuracy of the molecular DNA markers, it is possible to describe the characteristics of the kernel easily and quickly using only small amount of materials for example leaf. This test is considered to be cheap.

Classification of markers known divides them to linked markers and diagnostic markers. According to K. Gale (2005): "Linked markers have a probability of being co-inherited with the trait of interest that is dependent on the genetic proximity of the marker and the gene influencing the trait." Linked markers are used if the gene of interest is not known and therefore can not be targeted for the presence or absence of the gene or for the specific allele. In the opposite, way the diagnostic markers which are also known as perfect markers are directly and 100% connected with the gene which affects the variability of the trait of interest. Diagnostic markers are highly preferred as they have absolute linkage to the trait which is selected (Gale, 2005)

Traditional breeding is based on the phenotype and non-molecular markers like color and size. Later on physical measurements with certain equipment accrued. In case of physical measurements, usually large amount of seed material (approximately 5-50g) is needed to have reliable measurements. For example measuring test weight (TW), thousand kernel weight (TKW), particle size index (PSI) or using data generated by equipment like the single kernel characterization system (SKCS) and near infrared reflectance (NIR) - hundreds and thousands of kernels are needed, not to notice that some of these methods are destructive. In case of these measurements, the results of the phenotypic hardness can vary highly also if the same variety is grown on the same year or on different plots due to the environmental effect. (Bettge *et al*., 1995).

Most used non-molecular methods for measuring the hardness of the wheat kernel are single kernel characterization system (SKCS), near infrared reflectance (NIR) spectroscopy, pearling

index (PI) and particle size index (PSI) (Pasha et al., 2010). NIR is used on wavelength 1100-2500 nm and it is depending on the reflectance signal which is associated to the particle size. Larger particle size is positively correlated to the NIR absorption and particle size increases with hardness. Thanks to this, NIR can be used in all classes of whole grain wheats. (AACCI Method 39-70.02) NIR hardness categories are the following: extra hard >84; very hard 73-84; hard 61-72; medium hard 49-60; medium soft 37-48; soft 25-36; very soft 13-24 and extra soft <13 (Hrušková and Švec, 2009).

Single kernel characterization system (SKCS) generates data from measuring and crushing 300 kernel samples which takes approximately only 3 minutes. Individual kernels are weighed and then crushed between the toothed rotor (Gaines et al., 1985). Weight, width and moisture content of the single kernel is measured before crushing. Following to non-destructive measuring, crushing measures the force needed to crush the kernel recorded as individual crush response profile (iCRP). All these measurements are taken into consideration while mathematically calculating the SKCS HI (hardness index). SKCS HI is non-rheological computed interpretation of the hardness and due to that highly variable between kernels. Hard-medium-soft categorization is used in SKCS HI as following: supersoft <25; soft 25,1-45; medium hard 45,1-65; hard 65,1- 85 and premium hard from 85,1 to 100 (Haraszi et al., 2013).

Recently, a new hardness index has been developed by Réka Haraszi et al. named rheological hardness index (RHI), which provides better categorization of hardness than SKCS hardness indexes do. SKCS RHI is based on the rheological phenotype phases of the averaged crush response (aCRP). RHI takes endosperm response (EndoR) and endosperm collapse (EndoC) into consideration providing more information about the fracturing of the hard and soft wheats. RHI is calculated by EndoR/EndoC. New RHI also brings new categorization as following: soft < 1,5; medium hard 1,5-2,1, hard 2,1-4.0 and extra hard >4.0. It is even suggested by the authors that hard category should be divided into two subcategories: RHI=2,3-3.0 and RHI= 3.0-4.0 (Haraszi *et al*., 2013)

1.3.1 Protein markers

Different protein markers have been used since 1970s. Main protein markers are storage proteins like prolamin and globulin storage proteins of cereals. For different protein markers like gluten proteins (gliadins and glutenins) in wheat, Sodium Dodecyl Sulphate Polyacrylamide Electrophoresis (SDS-PAGE) is used mainly for analysis. Gliadins are monomeric proteins while glutenins are polymers forming several subunits connected by

disulphide bonds (Lafiandra et al., 2007). SDS-PAGE separates glutenin to High Molecular Weight Glutenin Subunits (HMW-GS), Low Molecular Weight Glutenin Subunits (LMW-GS). Modified forms of same protein or slightly different structure variants like gliadin groups (ω-gliadins) are tested with Acetic Acid-Urea Polyacrylamide Electrophoresis (A-PAGE), which at pH=3.0 is able to separate proteins with similar size, but different charge (Institute of molecular development, LLC, 2001)(Morell and Gale, 2002). Protein markers for different members of the starch biosynthetic pathway, like granule bound starch synthases (GBSSI or waxy proteins) which are related to amylose and amylopectin ratios of starch can also be tested using SDS-PAGE (Lafiandra et al., 2007). A-PAGE and SDS-PAGE usually complete each other and are invaluable methods in seed storage protein research.

1.3.2 Antibody-based marker assays

Immonassay is based on the presence or concentration of a macromolecule like protein in the solution. There is a small number of antibody based marker assays available regarding the wheat quality. These diagnostic antibody markers are usable with high throughput enzyme-linked immunosorbent assay (ELISA). Antibody-based marker assays for granule-bound starch synthase-1 (GBSS-1), amylase expression levels in wheat grain for the prediction of starch quality and tests for gluten-free foods are known. Most interesting to our part, antibody-based detection for bread wheat contamination with durum wheat based on friabilin detection has been developed (Gale, 2005)

1.3.3 DNA- based markers

Since early 1990s, several DNA-based molecular markers (RFLPs, RAPDs, AFLPs and microsatellites) have been developed and used for the genetic analysis of quantitative and qualitative characteristics of wheat (Blanco et al., 1996). Main molecular markers used in wheat MAB are resistance markers for leaf rust, stripe rust, fusarium head blight (FHB) and other plant diseases to help create resistant lines of wheat (Mammadov et al., 2012). The main advantage of DNA-based assays is that DNA can be extracted from any tissue and the results are not depending on the plant growth and development conditions (Morell and Gale, 2002).

The simple molecular marker methods are usually divided into two groups: PCR-based techniques such as microsatellites (SSR), identification of SNPs and AFLPs and non-PCR based techniques like RFLPs. PCR-based techniques can be divided in two subcategories: linked markers like SSR and AFLPs. Secondly, direct markers are known. Direct DNA-markers are genes and their testing is based on the presence/absence of the gene or are

targeted to the fragment of the gene for a specific allele. Insertion/deletion mutations can be targeted with allele specific (AS) PCR to identify SNPs (Morell and Gale, 2002). In case of molecular PCR methods, a few milligrams of leaf or seed could be enough for extraction of DNA and conducting as much PCR tests as needed. As the PCR assay recognizes specific molecular regions in the sequence (whole genes or gene fragments, microsatellites, SNPs) the results give specific and detailed knowledge about the hardness genotype.

Each of the molecular methods also have their advantages and disadvantages. RFLPs require large amounts of clean, not degraded DNA and the technique is quite complex, slow and it cannot be automatized. AFLPs are useful for creating high density maps, but the low information content of each data point makes them not easy to use on other populations, so this non-correlation is the main disadvantage of the use of AFLP markers. Microsatellites are widely used in mapping and breeding. The advantages of SSRs are the high portability from one germplasm to another and that the high degree of polymorphism provides efficient marker systems. However, previous genetic information is needed.

1.3.3.1 ***Puroindolines as markers for hardness***

Although the trait of interest is named kernel hardness, actually the softness is the dominant characteristic in puroindolines meaning that any mutation in the gene causes harder kernel. Puroindolines are perfect markers as the genes can be easily targeted with specific primers and tested using PCR. Usually the presence or absence of the wild type *(Pina-D1a)* is tested by puroindoline-a. In case of puroindoline-b wild type *(Pinb-D1a)* and the most common alleles containing mutations are tested. For example *Pinb-D1b* allele-specific primer is used to detect mutation at nucleotide position 233 causing glycine to serine change, resulting with hard phenotype. Conclusions are made based on the PCR results. If both puroindolines are wild type (*Pina-D1a/Pinb-D1a*) the phenotype of the endosperm is always soft. In case of the genotypes *Pina-D1a/Pinb-D1b; Pina-D1a/Pinb-D1d; Pina-D1b/Pinb-D1a* and *Pina-D1b/Pinb-D1b* the hardness characteristics are mixed, but they are definitely in the hard category. Haplotypes *Pina-D1b/Pinb-D1b* give the hardest trait. Durum wheat lacks of puroindolines because of the absence of the D-genome, hence it is always hard. As several puroindoline alleles have been sequenced, it is possible to design more allele-specific primers for unreported mutations and used for screening. Although, it has to be kept in mind that wheat itself is quite a complex subject of research as its hexaploid nature makes developing of allele-specific markers quite difficult due to the highly homologous genes and homologous loci it is possible to amplify areas not in our interest (Morell and Gale, 2002). So, while designing primers to amplify the specific allele or region to our interest, several controls of

the sequences should be completed. For example, in case of designing primers for puroindoline-a also the puroindoline-b and granule softening protein-1 should be checked as they share high nucleotide sequence similarities.

1.3.3.2 *Other markers related to the endosperm texture*

Several studies have showed that although puroindolines of the main hardness (*Ha)* locus on chromosome 5D are major determinants of the wheat endosperm texture, the puroindolines do not explain all the variation of endosperm hardness. As found by Bettige and Morris (2000) in soft wheats the variation of the hardness was related o cell-wall associated pentosan fraction. Reported regions contributing to kernel hardness are found in chromosomes 2A, 2D, 5B, 6D and a major QTL found in 1A plus adding the regions which have indirect influence such as in chromosomes 5A, 6D and last but not least 7A (Wang et al., 2012). No such relation had been noticed by hard wheats. Besides the major *Ha* locus, other chromosomes influence the hardness, but till this day, minor effects of other chromosomes have been noticed between hard and soft wheats (Crepieux et al., 2005). Identifying the additional genes and QTL which control this trait is important for plant breeding (Tsilo et al., 2011). It is also a possibility, that the hardness is controlled by the two main genes – puroindoline-a and puroindoline-b, but modified by one or more minor genes.

The main parameters which indirectly measure hardness are density and virtuousness (Faqir M Anjum and Walker 1991). Therefore not only molecular markers of endosperm texture are the only importance, but to also find molecular markers for density and vitreousness. Vitreousness is expressed as a percentage and usually was determined visually by cutting the wheat kernel in half (Ouafi, 2001). The percentage of vitreousness shows the percentage of the transparent kernels (Gómez-Becerra et al. 2009). Based on the US Official Standards of Grain, subclasses for Hard Red Spring and Durum wheat are based on the percentage of the vitreous kernels (Morris and Beecher, 2012). Finding molecular markers for this trait would make plant breeding and classification easier and faster. So far, the vitreousness of the kernel was mainly tested on tetraploid *T. durum,* but lately there have been more and more reports to test this feature also on hexaploid *T. aestivum* as some parts of the durum wheat genome may be also found in bread wheat as they share a common ancestor.

Kernels which are vitreous have continuous endosperm from which the light refracts in a way that the kernel looks “glassy” or also called “vitreous”. In case of the non-vitreous kernels the structure of the endosperm is discontinuous and contains small airspaces (Morris and Beecher, 2012). The opaque or mealy non-vitreous kernel is related to softness and low protein content

while vitreous kernel is known to be related to hardness and high protein content (Anjum and Walker, 1991). Higher N fertility results with higher proportion of vitreous kernels and also with higher grain protein content (Morris and Beecher, 2012). So, both hard and soft wheats can be vitreous or non-vitreous. This is probably because of the environmental effect as vitreousity is influenced by nitrogen fertility.

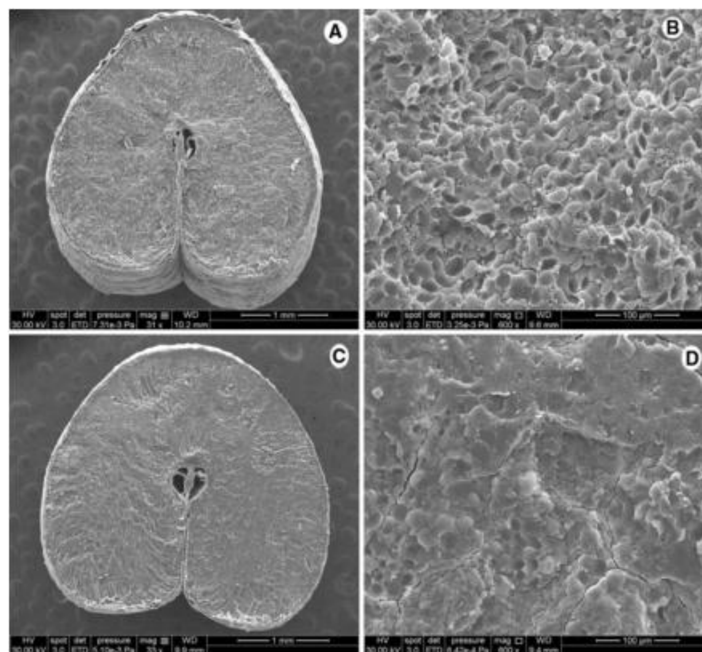

Figure 2 Field emission scanning electron micrographs. a, b A soft wheat kernel shows the non-vitreous kernel morphology and high degree of free starch granules. c, d A hard wheat kernel shows the vitreous kernel morphology and continuous endosperm matrix with no free starch granules. (Morris and Beecher 2012)

According to work of Morris and Beecher (2012) the connection between the hardness of the kernel and vitreousness is noticeable, although it may not be the direct result of the puroindoline proteins, but some other genes located in the distal end of the 5DS which are deleted or carry a mutation in hard wheats.

Previously mentioned hardness is a mechanical property, vitreousness an optical one and density is physical (Samson *et al.*, 2005). Higher density is related to higher protein content where accumulation of gliadin is preferred. Unit of density is g/cm^3. Density is highly related to moisture content, hence this should be taken into consideration while measuring density with physical methods (Peters and Katz, 1962). Markers for density are also in interest as they are related to the hardness indirectly.

1.4 High resolution melting analysis

Although standard PCR method is a well working way for screening it is not always able to give the necessary information. In 2002 a new method was introduced named High Resolution Melting DNA analysis which is based on a special HRM-PCR technique (Reed et al., 2007). HRM is a good method for genotyping, sequence matching and mutation scanning. It is based on the melting temperature of the PCR products and melting profile which is depending on the GC%, length of the PCR product and heterozygosity. Mutation scanning is depending on the forming heteroduplexes which change the profile of the melting curve (Reed et al., 2007). New generation saturating dyes like EvaGreen are used while disassociation of dsDNA samples is measured for generating melting curve. New generation dyes are used as they do not inhibit PCR reaction and bind equally to GC- and AT- rich regions (Lochlainn et al., 2011). This technique is also applicable for identifying SNPs, the sensitivity of this method with products up to 400 bp is 100%. For that reason, primers used for HRM-PCR are usually maximally 300 bp long including the primers. As there is no need for further processing after HRM-PCR, samples containing mutation can be used for sequencing. Main benefits of this system is the high throughput and closed tube system, by which lots of samples can be analyzed and the risk of contamination is small.

2 EXPERIMENTAL WORK

2.1 The aim of the experimental work

The main aim of this work is the molecular characterization and description of puroindolines in different varieties. Forty-nine (49) historical and 12 varieties of biofortification was tested in Kazakhstan group. The aim for Kazakhstan group was to screen the puroindoline genes. From Australian group 106 varieties was used. As Australian group had been tested before for puroindoline-a and puroindoline-b, the aim for this group was to test and develop new markers for later comparison with SKCS RHI. The other purpose of this work was to test the reliability of new molecular markers which were in QTL loci and exceeded the significance threshold as reported in literature. As mentioned before, the most common alleles in modern cultivars are *Pina-D1a*, *Pinb-D1b* and *Pinb-D1c*. This lead to the idea to test puroindolines on the Kazakhstan historical group (in this thesis).

This thesis is a part of international collaboration where these samples were tested also for protein content, patterns for different storage protein groups (HMW-GS, LMW-GS, gliadins, mixing parameters, size distribution of storage proteins in the flour) and NIR. The molecular background for hardness has been assessed in this work.

2.2 Materials and methods

2.2.1 Seed sterilization and plant growth conditions

The seeds of the Kazakhstan lines sets H (historical) and B (biofortification) were used. The seeds were separately placed to previously marked 100 ml Erlenmeyer flasks and sterilized in 3% hydrogen peroxide (H_2O_2) for 15 minutes. Flasks were placed under running tap water for rinsing overnight.

Hydroponic method for growing plants with 1/4 strength modified Hoagland solution (Appendix 1) was used. Seeds on marked beakers were placed to the dark chamber for germination for 2 days. After the dark period seeds were placed to climate controlled growth chamber (Fitotron, SANYO) for 8-12 days with 14/10 hours day/night photoperiod with light intensity of 200 µmol $m^{-2} s^{-1}$. Temperature regime was 24°C/18°C at day/night, respectively.

2.2.2 Genomic DNA isolation and purification

Plant leaf tissue was harvested and plant genomic DNA (gDNA) was extracted/isolated using AquaGenomic™ kit from MultiTarget Pharmaceuticals. Manufacturers' instructions were followed with some modifications. The concentration and quality of the nucleic acid sample was measured by using NanoDrop® ND-1000 Spectrophotometer. Special attention was drawn to the absorption curve and to 260/280, 260/230 ratio. The stock samples were stored at -20°C. Working solutions were adjusted to 40 µl/ml using MQ water.

2.2.2.1 *Aqua Genomic Plant Protocol –pestle and mortar*

25mg of plant tissue was harvested from upper 1/3 of the leaf from 14 days old plants. Small amount of sterilized quartz sand and according to the larger amount of plant tissue used, 250µl AquaGenomic Solution was added. During homogenization at room temperature, 1/10 volume (30µl) of 100% 2-propanol was added to reduce the foaming. The samples were transferred to 1,5 ml Eppendorf tube and VORTEXed vigorously for 30 seconds and incubated at 65°C for 15 minutes. The mixture was rotated gently in every 5 minute during the incubation. Five minute centrifugation at 12,000 x *g* was used to pellet the debris. Clear lysate (approximately 185µl) was transferred to a clean 1,5 ml Eppendorf tube. 1 volume 100% 2-propanol was added to the lysate and VORTEXed vigorously for 60 seconds. To pellet the DNA Eppendorf tube was centrifuged at 12,000 x *g* for 5 minutes and the supernatant was carefully discarded . After the pellet was washed twice with 70% ethanol and air-dried it was dissolved in 100µl 1xTE buffer.

For further DNA extraction, if it was necessary extra tissue was collected and lyophilized. 100mg tissue was put into Eppendorf tube, closed with Parafilm M® Laboratory Film and holes were made on it using needle. Lyophilization was carried out in a SpeedVac®Plus, Savant equipment. The freeze dried tissues were stored at +4°C.

2.2.2.2 *AquaGenomic Plant Protocol – Eppendorf method*

Because the Australian samples were delivered as lyophilized tissue a modified method was developed for DNA extraction. Only 8 mg dry tissue was grinded with sterilized quartz sand in Eppendorf tube using glass rod with cone end. 100 µl AquaGenomic solution was added to the powder and mixed for another 5 minutes. After homogenization 15µl of 2-propanol was also added and the tube was incubated at 65°C for 10 minutes. Samples were centrifuged at 12, 000 x *g* for 10 minutes (Heraeus Biofuge Pico, KENDRO). Lysate was transferred to new Eppendorf tube and 1 volume (approximately 15µl) 2-propanole was added followed by vigorous VORTEXing for 60 seconds. Samples were centrifuged at 12,000 x g for 5 minutes.

The discarding of the supernatant and centrifugation was completed followed by two washing steps with 70% ethanol. DNA was dissolved in 50 µl 1xTE buffer.

All PCR reactions were carried out in the volume of 10µl. For general PCR and optimization procedure, determining the annealing temperature and the appropriate $MgCl_2$ concentration the following general reaction mixture was used as starting point:

0,5 µl of *polymerase* (*Taq* or *Pfu,* Fermentas; Thermo Scientific)

1µl of 10X buffer (-$MgCl_2$) (Fermentas; Thermo Scientific)

0,5 µl of 10µM (0,5µM) Forward primer (Mycrosynth)

0,5 µl of 10µM (0,5 µM) Reverse primer (Mycrosynth)

0,5 µl of 10 mM dNTP (0,5 mM) (2,5 mM each deoxynucleoside phosphate)

0,6 µl of 25mM (1,5mM) $MgCl_2$ (Fermentas; Thermo Scientific)

2 µl (80ng) of DNA template

4,4µl MQ water

The following general PCR programs was used:

Pre-denaturation: 95°C for 5 minutes

Denaturation: 95°C for 30 seconds

Annealing: APPENDIX II

Extension: 72°C for 12-35 seconds

X 36

Post-extension: 72°C for 2 minutes

PCRs were conducted with thermocyclers ICycler™ Version 2.033 (Bio-Rad). Additional information on all the Primers used during my work can be found under APPENDIX II.

2.2.3 Gel-electrophoresis

Gel-electrophoresis was used to separate the DNA molecules using 1-2% (w/v) agarose gel depending on the size of the product. Agarose was made with 1x Tris-acetate (TAE) buffer. For electrophoresis RunOne™ Eletrophoresis Cell (EmbiTec) was used. Samples were loaded using bromophenol blue or xylencianol as loading dye. GeneRuler™ 1kb DNA Ladder

(Fermentas, Thermo Scientific) was used to assess the length of the DNA fragment. Samples were run in 1x TAE buffer and stained in ethidium bromide solution (0,5 µg/ml) for 10 minutes and the bands were visualized under UV-light.

2.2.4 DNA purification from gel

For DNA purification from gel QIAquick Gel Extraction Kit microcentrifuge protocol was used with minor modifications. All the centrifugation steps were carried out with 12,000 x *g*, unless mentioned otherwise. After visualization under UV-light, the expected DNA fragment was cut out, weighed and placed to 2ml Eppendorf tube. Three (3) volumes of Buffer QC was added to 1 volume of gel (~100mg). The gel slice was incubated at 50°C until the gel was dissolved completely. Add 1 gel volume 2-propanole and mix gently. QIAquick spin column was placed to 2ml collection tube. For binding the DNA, the sample was applied on the QIAquick column and centrifuged for 1 minute. Flow-trough was discarded. As the DNA was used for direct sequencing, 0,5 ml Buffer QG was added to QIAquick column and centrifuged again for 1 min. In the washing step, 0,75 ml of Buffer PE was added to the column and left for 5 minutes at room temperature, followed by 1 min centrifugation. The flow-trough was discarded and additional centrifugation for 1 min was completed. The column was placed into new 1,5 ml Eppendorf tube. For DNA elution, 50 µl of 2 mM Tris buffer (pH=8) was added to the center of the column membrane instead of elution buffer (EB). Centrifugation at 13,000 x *g* for 1 minute was followed. Concentration and quality of the gel-extracted DNA was measured on NanoDrop-1000.

2.2.5 Cycle sequencing

For asymmetric PCR sequencing Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) was used. Final reaction volume for sequencing was 20 µl in which 200 ng of template DNA, 3,2 µl of primer (1 µM), 2 µl of BD reaction mixture and 4 µl of Big Dye sequencing buffer was used. The rest of the volume was sterile MQ water. Cycle sequencing was conducted on thermocycler Icycler™ (Bio-Rad) with following program: initial denaturation at 96 °C for 1 minute followed by 25 cycles of 96°C for 10 seconds, 55°C for 20 seconds, 60°C for 4 minutes and infinitive hold at 4°C. Amplified DNA (20 µl reaction) was ethanol precipitated with the addition of 0.5 µl of 5M EDTA and 2 µl of 10M ammonium acetate (pH=5) 55 µl of 96% ethanol. Sample was centrifuged 13,000 x *g* (Sigma 3-18K) at 4°C for 30 min. Supernatant was carefully removed and pellet was washed with 70% ethanol,

supernatant was removed and sampled was dried at 37°C. The tubes containing pellet and purified DNA were sent to adequate laboratory for sequencing product analysis.

2.2.6 *In silico* sequence analysis

Sequences were analysed with Chromas Lite v2.01 (Technelysium Pty Ltd) program and alignments were made with Blast® (NCBI). Unclear nucleotides in the sequence were controlled manually by checking the chromatogram. For analysis of amino acid changes, nucleotide sequence was translated into amino acid sequence with ExPASy Bioinformatics Resource Portal.

2.3 Results and discussion

All PCR reactions were optimized to the highest possible annealing temperature using gradient PCR as different final $MgCl_2$ concentration (1,5mM; 2mM or 2,5mM) was also determined to get the most specific and more trustable information.

2.3.1 Historic set results regarding puroindoline-a and puroindoline-b

In Kazakhstan H-set 49 varieties were tested (Table 10). For screening this set 6 primer pairs were used. For detecting *Pina* Primpa1-3 was used and the results were checked with PAF-PAR if needed. For *Pinb* wild type PBF and PBR was used. *Pinb* was also tested for specific mutation allele *Pinb*-D1b with PBKF-PBKR. PCR reaction conditions and used primers can be found in Table 8. Forty-four (44) varieties (89,80%) out of 49 were tested positive for *Pina* (*Pina-D1a* allele) meaning that 44 varieties are *Pina* WT. Five (5) varieties (H10; H12;H37;H38;H39) were tested as negative for puroindoline-a gene by PCR testing (puroindoline null).

Table 4 Historical set results regarding *Pina* and *Pinb*

Puroindoline	***Pina***		***Pinb***		
Result	+	**-**	+		**-**
Validation	WT	null	mut	WT	null
Frequency in pool	44	5	43	3	3
Frequency %	**89,80%**	**10,20%**	**87,76%**	**6,12%**	**6,12%**

Regarding the results of *Pinb* three (3) varieties (H12; H19; H44) were tested positive for *Pinb-D1a* allele. Forty-three (43) varieties were tested positive as puroindoline-b mutants. These samples were checked with PBKF-PBKR primer pair which is *Pinb-D1b* allele specific. PBKF-PBKR amplifies fragment with the size of 356 bp of *Pinb*-D1b allele. According to the results from these tests it can be assumed that these mutants are *Pinb-D1b* allele, which is the most frequent mutation of puroindoline-b gene. There is also a possibility that there are mutants that share the same SNP as *Pinb-D1b* allele (Gly 46→Ser) does plus some other mutation(s) in the amplified region. Irrespective of the mutation, based on this information, the kernels with mutation should be harder. Three (3) varieties (H37;H38;H39)

were tested as *Pinb* null. The phenotypes of hardness based on the *Pina* and *Pinb* markers are shown in the Table 5.

Table 5 Genotypes found in testing and their frequencies in the pool of Kazakhstan H-set 49 samples

Genotype	*Pina*-D1a/*Pinb*-D1b	null/*Pinb*-D1b	null/*Pinb*-D1a	*Pina*-D1a/*Pinb*-D1a	null/ null
Phenotype	hard	hard	hard	soft	hard
Frequency in pool	42	1	1	2	3
Frequency %	85,71%	2,04%	2,04%	4,08%	6,12 %

The most frequent (85,71%) genotype was *Pina-D1a/Pinb-D1b* which gives hard phenotype. Only two varieties have soft genotype with the frequency of (4,08%) in the H-set. Only varieties carrying puroindoline-a and puroindoline-b alleles have soft phenotype as any other changes like mutation or lack of the gene results with hard phenotype.

2.3.2 Biofortification-set results regarding puroindoline-a and puroindoline-b.

B-set refers to biofortification. Biofortification is the development of crops in means to increase the nutritional value (Food *et al.*, 2006). Traditional selective breeding or genetic engineering is used to get these results. The samples of the B-set are nutritionally improved varieties with fortified Zn+ content.

In Kazakhstan group B-set (Table 11) varieties were tested for *Pina* and *Pinb*. All 12 varieties were tested as positive for *Pina* meaning that they carry the WT *Pina-D1a* allele. For *Pinb* all 11 varieties were tested as *Pinb* mutants and one WT (Eritrospermium-32), meaning that only this variety has soft phenotype in this group based on DNA molecular markers for puroindoline proteins. Five varieties from 12 were selected randomly for sequencing. For sequencing the PCR product: Big Dye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems) was used. Whole puroindoline-b gene (477 bp) was sequenced using PBF-PBR. Sequencing of one variety was carried out twice. Asymmetrical sequencing with PBF and PBR primers was used. This way, the often “unreadable” first 50 nucleotides can be read from the other end of the chromatogram. The sequence chromatogram was controlled manually for possible mismatches from both strands. Sequence analysis showed that variant B7

(GVK1369-2) and B-26 (Golubovskaya) are *Pinb-D1b* allele containing mutation at position 223 resulting with Gly to Ser substitution. In case of B-6 (Stepanya 1), B-12 (Lutescens 53-95) and B-31 (Iren) new alleles were found. B-6 has an A to G substitution at the position 128 plus G to A leading to Gly to Ser change and at 223. This is common for *Pinb-D1b* allele, but allele with these two mutations in one sequence has not been reported taking the latest review into consideration (Morris and Bhave, 2008). B-12 has substitution in position 382 where C changes to T which concludes with Gln being substituted with STOP codon. This C to T substitution has been reported in *Pinb-D1ab* allele catalogued in 2007 (Morris and Bhave 2008). Additionally in B-12 position 257 a G to A change was found which results in Cys to Tyr substitution. In case of B-31 in nucleotide position 22 a G to T (Ala to Ser) as in *Pinb-D1v* and 266 T to C (Pro to Leu) like in *Pinb-D1c* was found, plus in position 63 C→T without change in amino acid sequence.

From information gathered from sequencing B-set *Pinb* mutants it can be concluded that many alleles carry the frequent substitution in position 233 plus some other SNPs. Based on this, SNP identification in other locations using high resolution melting PCR (HRM-PCR) or sequencing should be done on remaining B-set and H-set puroindoline-b mutants which tested positive for *Pinb-D1b* allele but may also carry double SNPs

2.3.3 HRM-PCR primers optimization

Primers found in **Error! Reference source not found.** were designed specifically for HRM-CR which requires quite short product size (max 250-300 bp including primers). Overlapping primers were designed for HRM-PCR with the aim to identify puroindoline polymorphisms. Designed primers were tested using standard PCR method. With primer pair Pura-J1F and Pura-J1R several optimization reactions of annealing temperature and $MgCl_2$ concentration were carried out, since there was no clear amplification at 56°C as designed. After the optimization the optimal annealing temperature of 59°C and 2,5mM $MgCl_2$ was determined for this primer pair. In case of the Purb-J1F – PurbJ1Rd the optimal annealing temperature was 63°C with final $MgCl_2$ concentration of 2mM. Letter d in the end of the PurbJ1Rd refers to degenerative primer in which S=G+C in the sequence for the primer to be able to amplify all alleles needed. No further optimization was needed with the other primers and optimal annealing temperatures are shown in the Table 13**Error! Reference source not found.** All eight primers were tested on 9 samples [B-6; B-12; D-21; D-23; H-36; H-42; K-22, K-123 and MV-SUBA (+ control)] and all were tested positive, meaning that these oligonucleotides

are able to anneal to the sequence. In future, these primers will be tested with HRM-PCR for detecting polymorphisms

2.3.4 Historic set results regarding the puroindolineb-2 alleles

Screening of puroindolineb-2v alleles was conducted as *Pinb*-2 variants have been reported as minor QTL for hardness (Wilkinson *et al*., 2008). Based on the information gathered from the screening of H-set with *Pina* and *Pinb*, we could group the varieties as soft and hard. For finer categorization among soft group, *Pinb*-2v alleles were tested. Primers and conditions used for screening can be found in Table 9. General PCR program was used.

In Kazakhstan group, only historical (H-set) was tested for puroindolineb-2v alleles. Forty-nine (49) samples were tested in this group (Table 10). Regarding *Pinb-2v1 (Pinb-D2a)* all 49 varieties were tested positive, in which H49 was tested positive, but had an extra band on 500 bp and H54 had a faint positive (+f WT) band.

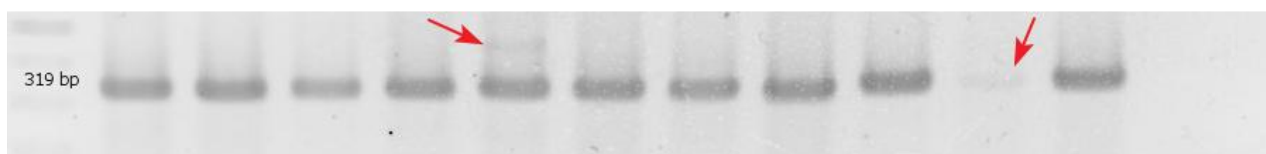

Figure 3 *Pinb-2v1* H-set. Lane 1 MWS, lane 2 H43; lane 3 H44; lane5 H47; lane 6 H48; lane 7 H49; lane 8 H50; lane 9 H51; lane 10 H52; lane 11 H53; lane 12 H54;lane 13 B6 (+ **control); lane 14 NTC.**

Pinb-2v2 (*Pinb-B2a*) had no WT *Pinb-2v2* variants. All 40 varieties were tested as *Pinb-2v2* negative, meaning that they are a "null" type of *Pinb-2v2* as there was no amplification. The 50th sample, positive control Chinese Spring was tested as WT *Pinb-2v2* as expected. *Pinb-2v3* (*Pinb*-B2b): 47 samples were tested as WT *puroindoline-2v3* (*Pinb-B2b* allele). Two samples (H7 and H48) gave a "null" amplification. Chinese Spring was used as negative control. *Pinb-2v2* and *Pinb-2v3* have been reported to be allelic and not to exist together. In this case it can be said that this is not the case in this group as varieties H7 (Omskaya 26) and H48 (Omskaya 28) were both tested as *Pinb-2v2* and *Pinb-2v3* negative**.** In case of *Pinb-2v4* (*Pinb-A2a*) all 49 samples were tested positive as wild-type *Pinb*-A2a.

According to this, no variation was detected in *Pinb-2v1* and *Pinb-2v4* as they have been detected in all common wheat varieties tested. Based on this data, it can be concluded that 45 samples out of 47 had the following genotype: *Pinb-2v1/ Pinb-2v3/Pinb-2v4*. Two samples H7 (Omskaya 26) and H48 (Omskaya 28) having a genotype *Pinb-2v1/Pinb-2v4* lacking the

both *Pinb-2v2* and *Pinb-2v3* allele. According to this it can be said that variants with genotype *Pinb-2v1/ Pinb-2v3/Pinb-2v4* are probably harder than those containing other alleles.

2.3.5 Australian set results regarding puroindolineb-2 alleles

Australian set was also screened for *Pinb*-2 variants. PCRs were carried out only on soft varieties as *Pinb-2v2* marker is reported to make difference among soft wheats. As *Pinb-2v3* was reported allelic and as a dominant haplotype, tests with *Pinb-2v3* were conducted. The Australian group was tested only on 42 varieties. Results can be seen in Table 12. Six samples were tested negative for *Pinb-2v3*. All the tests were completed twice and the same results were gained. Later on, the negative samples were tested separately with *Pinb-2v3* and *Pinb-2v2* markers. Varieties with *Pinb-2v3* should be harder in soft group than those containing *Pinb-2v2*. It is likely that these 6 varieties (Bungulla, Chinese Spring, Isis, Kondut, Macquarie, Tatiara) are softer among soft group. The results of SKCS RHI are needed for comparison and conformation.

According to works of Chen and Zhang (2011), Geng and Beecher (2012), *Pinb-2v3* is the predominant and most frequent allele. Our work with Australian group confirms that *Pinb-2v3* is more frequently occuring than *Pinb-2v2*. According to our Australian group results among soft wheats, *Pinb-2v2* variant was detected in 6 samples (14,28%) and the occurance of *Pinb-2v3* was 36 samples – (85,72%) from the pool of 42 samples. Statistical analysis for sample variance was not carried out as the number of samples is not adequate.

Based on the hypothesis of Chen *et al.,* (2013) that the higher SKCS HI can be correlated with the higher expression level of *Pinb-B2v3b* allele, the author of this thesis suggests that *Pinb-2v3*b allele expression level measurments should be carried out in the future.

2.3.6 Marker development for seed vitreousness and density

New molecular markers with promising QTL were searched from the articles. From five markers selected, *Xgwm107* (vitreousness) was chosen as it was only microsatellite in our group of markers which could be tested by PCR. This QTL is known to be located in the long arm of the 4B chromosome. Relative importance of the this QTL in influencing a trait (QTL R^2 %) is reported to be 12 %. (Ouafi, 2001)

Vitreousness marker was tested on different hardness category durum wheat samples provided by Martonvasar Research Institute, Hungary. During the optimization following program was used 95° for 5 min, 95° for 30 sec, 65° for 30 sec, 72° for 30 sec and lastly 72° for 5 min, 10°

hold for ∞, repeated 40 cycles. The annealing temperature was raised till dissapearing of the products at 67°C. Optimal annealig temperature was determined to be 65 °C with 2mM $MgCl_2$ concentration.

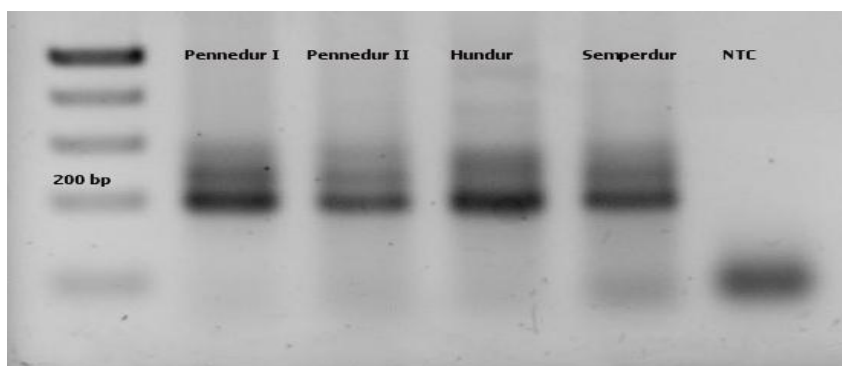

Figure 4 Agarose gel-electrophoresis 1,5% gel with different hardness type durum wheats. Lane 1 MWS, Lane 2 Pennedur I, lane 3 Pennedur II, lane 4 Hundur, lane 5 Semperdur and lane 6 NTC.

Table 6 *T.durum* samples used in vitreousness testing with Xgwm107F-R, 65°C. The information about durum wheat vitreousness types was known previously and selected for marker control

Variety	Vitreousness	Result
Selyemdur	chalky, mealy	++
Makaroni	good vitreous (opaque in case of watery environment)	-+
Pennedur	good vitreous	++
Hundur	good vitreous	- /-+
MV-Vitaldur	very good vitreous	++
Svevo (Italian)	spring wheat, international control	No data
Semperdur (Austrian)	spring wheat, international control	-+

According to the tests on these samples, even after PCR optimization, this marker could not make clear difference between different hardness index varieties. Due to that, this marker was

not tested further on Australian group. The testing for this marker will be continued. Search for better molecular marker identifying vitreousness characteristics in wheat kernels should be continued.

From five (5) density markers, an STS marker was chosen for testing. As no PCR markers were found for wheat, marker *Mwg770* with the R^2 of single marker effect =10% was used (Campbell *et al.,* 1999). According to the GrainGenes this marker has been used on barley. This marker was tested on samples of wheat for the reason that many conserved regions has been reported in cereals. Following varieties from soft group (20) were used: Bencubbin, Bolero, Bungulla, Capelle deprez, Capitole, Centauro, Chinese Spring, Ghurka, Insignia, Insignia 49, Kewell, Libero, Mersey, Pinnacle, Ranee, Robin, Summit, Tincurrin and Zenith. Density marker was also tested on three (3) hard wheats: Soissons, Golia and Wildcat – all tested positive.

Even after optimization, this marker gave two products after gel-electrophoresis imaging. One at the expected size of 195 bp and another approximately at 250 bp. First of all this marker was tested on barley to check whether it results with double-band also.

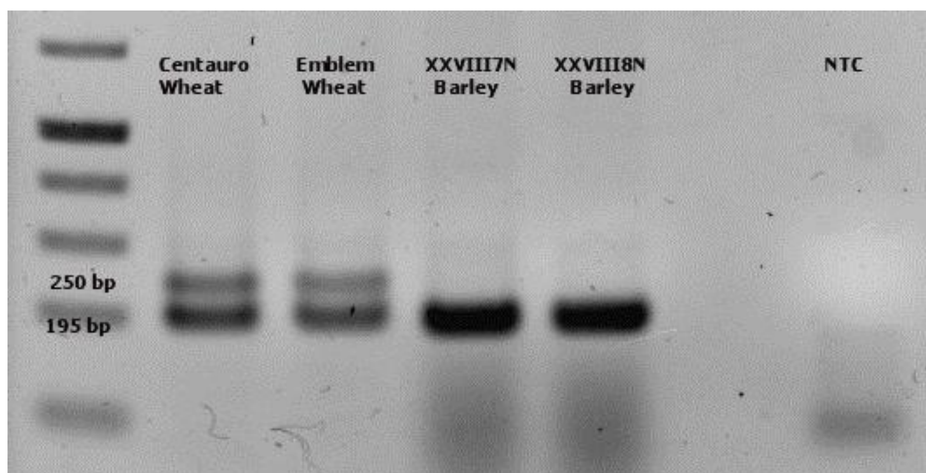

Figure 5 Lane 1 MWS, lanes 2 and 3 wheat, lanes 4and5 barley, lane 6 empty, lane 7 NTC. On this photo, it can be seen clearly that barley gives one strong band at 195 bp, but wheat samples give a double band, one on 195 bp and other on approximately 250 bp.

For finding out if the longer product was caused by the D-genome, the same marker was also tested on durum wheat. The same double-band appeared also in durum wheat samples. According to this, the amplification of the longer product was not because of the D-genome as it is absent in durum wheat. As it can be seen on Figure 5 the barley samples give perfect products at 195 bp when wheat samples are a bit longer. This suggests that the amplification

of the wheat products is not perfect as both bands are higher. This marker needs to be tested further whether it would be suitable to use as wheat density marker before using it on larger pool of samples.

2.3.7 Marker development for wheat seed hardness

A new hardness marker was chosen from 42 markers found in the literature. A hardness marker *Xcfa2153* located in the 1AL chromosome was reported significant (Arbelbide and Bernardo, 2006). This marker had not been associated with hardness previously. The marker was chosen because of its location in the end of 1AL as in this region more markers and QTL have been reported related to kernel hardness.

Optimal annealing temperature was 60°C for 25 seconds with 2mM $MgCl_2$ concentration. The extension time in 72°C was decreased to 12 seconds as tests in the workgroup showed that *Taq polymerase* (Thermo Scientific) can extend the chain faster than 1000 bp/min. As 200 bp product was expected, the PCR samples were run on 1,5% (w/v) agarose gel. After clear amplifications the marker was tested on all 106 Australian hexaploid bread wheat and tetraploid durum wheat varieties where clear differences could be seen. Further specific determination of the length of every single product should be determined to make conclusions.

Figure 6 PCR-results using Xcfa2153F-R primers. 200 bp fragment was expected, but fragments from 210 bp to 280 bp were gotten.

As it can be well seen on Figure 5, this marker gives as several different length products referring that it may be marker with fragment length polymorphism. The exact size of these bands was determined by calculating the distance of the bands using program Phoretix 1D (Phoretix International). The sizes were compared with the GeneRuler 1kb (Thermo Scientific) ladder and the possible lengths of the products were calculated logarithmically using MS Excel. Based on this, the products could be divided into 4 or 5 groups as following: 190 bp, 200 bp, 210 bp and 250 bp and 280 bp. According to these results the author suggests that further research and development for this marker is needed. Probably *Xcfa2153* could give us a marker with size difference to detect kernel hardness.

CONCLUSION

The use of molecular markers closely related to QTLs of interest allows precise and fast breeding processes. That makes usage of molecular markers simple and perfect way to improve modern plant breeding. Although, molecular methods cannot replace the traditional methods completely as the environmental influences play a huge roll in the selection of wheat lines. Puroindolines have been proved to be perfect markers for seed endosperm texture as they determine the hardness of the kernel genetically and no environmental influence is known.

Plants were grown from seeds to extract genomic DNA from plant tissue. Clean gDNA was necessary for the PCR reactions. In the Kazakhstan group puroindoline-a and puroindoline-b WT and mutant genes were screened with PCR. Optimizations of the PCR reactions were conducted by varying by $MgCl_2$ concentration and modifying annealing temperatures. In Kazakhstan group H-set our results proved that *Pina-D1a* is the dominant allele occurring 89,90% in the set. *Pina-D1b* was the second most common allele also referred as “null” allele. In case of puroindoline-b, mutant allele *Pinb-D1b* is the frequent allele (87,76%) as reported also by others. Three *Pinb-D1* nulls and four *Pinb-D1a* alleles were detected. B-set of Kazakhstan group was also tested for *Pina* and *Pinb*. All 12 varieties were tested as WT puroindoline-a. In case of *Pinb*, only one variety B-32 (Eritrospermum 78) was tested as WT, 8 alleles were established as *Pinb-D1b* mutant and 3 alleles have not been reported, so possibly new alleles were identified by sequencing.

In the second part of this work, four (puroindoline b-2, *Mwg770, Xgwm107* and *Xcfa2153*) other markers were tested with the aim to develop a new marker and for using them as indirect markers for hardness. Puroindoline b-2 gene family has been reported previously as a significant marker and was used for screening. The aim was to use and develop markers which would be able to separate the mixed category of endosperm hardness or to make difference inside the wheat hardness soft categories (soft and extra-soft). From four new markers, *Xcfa2153* seems the most promising as it gives several different products around 200 bp. Based on testing this marker on 106 Australian varieties, it can be assumed that *Xcf2153* may be a marker with fragment length polymorphism. Regarding this marker, comparisson with SKCS HI/RHI and statistical analysis should be carried out. Unfortunately, the author has to admit that in this work the developing and testing of new QTL markers had unexpected difficulties and more tests are needed to obtain significant information. In the future the *Pinb-*

2v3 variant needs to be tested more specifically and expression levels of *Pinb-2v3b* should be determined and compared with the SKCS HI results.

The author would like to emphasize the importance and the reliability of using puroindoline markers in wheat breeding in means of kernel hardness. In the future perspective more molecular DNA markers should be developed for different traits connected to kernel hardness to provide more specific information about the traits of the lines to breeders.

Terise tekstuuri molekulaarsete markerite uurimine harilikus nisus (*Triticum aestivum)*

Jaanika Udam

Resümee

Hariliku nisu (*Triticum aestivum* L.) endospermi tekstuuri mõjutavate tegurite väljaselgitamine on oluline soovitud omadustega nisutoodete valmistamist võimaldavate sortide aretamisel. Kaasaegses sordiaretuses on geneetiliste markerite tuvastamine kvantitatiivsete tunnuste lookustes (QTL) ja nende kasutamine muutunud üha olulisemaks. See lihtsustab soovitud tunnustega nisusortide saamist. Puroindoliinid on oluliseimad markerid määramaks nisu terise kõvadust endospermi tekstuuri muutuste kaudu.

Käesoleva bakalaureusetöö kirjanduse ülevaates on käsitletud puroindoliinide geenide poolt kodeeritud valkude osa nisu endospermi tekstuuri määramises, samuti on antud ülevaade puroindoliinide geneetikast ja nende geenijärjestuste mitmekesisusest. Puroindoliin-a (PINA) ja puroindoliin-b (PINB) metsiktüüpi valgud põhjustavad pehme ja rabeda endospermi tekstuuri, sest tärkliseterad on nõrgalt seotud valgulise maatriksiga. Jahvatamisel eralduvad tärkliseterad tervetena, tagades hea kvaliteediga jahu, mis on sobilik küpsiste, kreekerite, tortide ja teiste madala niiskusesisaldusega toodete valmistamiseks. Mutatsioonid *Pina-D1* või *Pinb-D1* puroindoliinide geenides tekitavad kõvema terise. Jahvatamisel saavad tärkliseterad kahjustatud, seovad hilisemal kasutamisel paremini vett ja seetõttu sobib kõvema terisega harilik nisu paremini leivatoodete valmistamiseks. Lisaks on kirjeldatud uut puroindoliinide *b2v* multigeenset perekonda. Tutvustatud on nisuteriste kõvaduse kaudsete tunnuste nagu klaasilisus ja tihedus molekulaarseid markereid.

Töö eksperimentaalses osas kasutati kahte erinevat katsetaimede rühma: Kasahstani rühm ja Austraalia rühm. Kasahstani rühm omakorda on jagunenud kaheks: ajalooline H- (historical) alarühm 49-st sordist ja B-(biofortification) alarühm 12 sordiga. Enamus markeritest on kasutatavad pehme fenotüübiga sortidega. Seetõttu valiti Austraalia rühma 106-st sordist 43 sorti põhiliseks markerite testimiseks ja jäeti *Triticum durum* ja enamus terise kõva fenotüübiga sorte kõrvale. *Xcfa2153* oli ainus marker mida testiti kõigi 106 sordi peal.

Katsed tõestasid, et puroindoliin-a puhul on enimlevinud metsiktüüp (*Pina-D1a)* ja "null" alleel (*Pina-D1b).* Mutatsioonid puroindoliin-a alleelis esinevad harva. Olukord puroindoliin-b puhul on vastupidine. Kõige levinumaks on mutatsiooniline alleel *Pinb-D1b*, mille puhul punktmutatsiooni tõttu nukleotiidses järjestuses, mille tõttu valgus glütsiin 46 positsioonis on

asendunud seriiniga. Kasahstani rühma B-alarühmast tuvastati sekveneerimisel kolm puroindoliin-b alleeli, mida pole varem kirjanduses mainitud.

Puroindoliinidele sarnase aminohappelise järjestusega ja sarnaste omadustega *Pinb-B2* variatsioonide puhul näitasid tulemused, et *Pinb-2v1* ja *Pinb-2v4* esinesid kõikides sortides. Variante *Pinb-2v2* ja *Pinb-2v3* on peetud alleelseteks, kuid selles töös leiti ka kaks sorti, mis olid nii *Pinb-2v2* kui ka *Pinb-2v3* null, ega sisaldanud kumbagi alleeli. *Pinb-2v3* esineb sagedamini kui *Pinb-2v2*. Kõige suuremat mõju terise tugevusele *Pinb-B2* perekonnas on täheldatud *Pinb-2v3* alleelil (Geng *et al.*, 2013). Selle alleeli täpne toimimismehhanism on veel teadmata, kuid lisaks alleelispetsiifikale võib tegu olla erinevatest ekspressioonitasemetest põhjustatud endospermi tekstuuri muutustega. Sellest tulenevalt tuleks jätkata selle geneetilise markeri uurimist ekspressioonianalüüside kaudu.

Uute terise klaasilisuse ja tiheduse molekulaarsete markerite katsetamine ei andnud üheselt mõistetavaid tulemusi. Vajalikud on edasised katsed ja SKCS (*single kernel characterization system*) kõvadusindeksi andmed. Lisaks klaasilisuse ja tiheduse markeritele testiti ka uut kõvaduse markerit *Xcfa2153*. Uutest markeritest on *Xcfa2153* kõige sobivam kasutamiseks, sest annab PCR analüüsil 4 kuni 5 erineva pikkusega produkti. *Xcfa2153* puhul võib tegu olla fragmendi pikkuse polümorfisimi sisaldava molekulaarse markeriga. Edasised uuringud on vajalikud, määramaks markeri usaldusväärsust pehme ja väga pehme fenotüübiga sortide eristamisel.

Töö autor soovib rõhutada molekulaarsete markerite uurimise ja kasutamise olulisust kaasaegses sordiaretuses. Molekulaarsed DNA-põhised markerid annavad kiire ja usaldusväärse võimaluse määrata sordi genotüüpi välistades seejuures keskkonnategurite mõju.

REFERENCES

Anjum, F. M. and Walker, C.E., (1991). Review on the Significance of Starch and Protein to Wheat Kernel Hardness *. Journal of the Science of Food and Agriculture, 56: 1–13.

Arbelbide, M. and Bernardo, R., (2006). Mixed-model QTL mapping for kernel hardness and dough strength in bread wheat. Theoretical and applied genetics, 112: 885–90.

Békés, F. 2001. A búza endospermium szerkezetének szerepe néhány minőségi búzát termelő országban, p. 25-35. *In* B. Zoltan, B. Géza, and M. János (ed.), *A jó minőségű keményszemű búza nemesítése és termesztése*. MTA-MKI, KITE Mezőgazdasági szolgáltató és Kereskedelmi Rt., Gabonatermesztési Kutató Közhasznú Társaság. Mártonvásár; Nádudvar; Szeged, Magyarorszag.

Bettge, A. D., Morris, C. F. and Greenblatt, G. A., (1995). Assessing genotypic softness in single wheat kernels using starch granule-associated friabilin as a biochemical marker. Euphytica, 86: 65–72.

Bhave, M. and Morris, C. F., (2008a). Molecular genetics of puroindolines and related genes: allelic diversity in wheat and other grasses. Plant molecular biology, 66: 205–219.

Bhave, M. and Morris, C. F., (2008b). Molecular genetics of puroindolines and related genes: regulation of expression, membrane binding properties and applications. Plant molecular biology, 66: 221–231.

Blanco, A., Di Fonzo, N., Laddomada, B., Pasqualone, A., Simeone, R., Troccoli, A. 2000.Variation for grain protein content and identification of QTLs by molecular markers in tetraploid wheats, p. 455-461 *In* : Royo, C., Nachit, M., Di Fonzo, N., Araus, J.L. (ed.). *Durum wheat improvement in the Mediterranean region: New challenges* . Zaragoza: CIHEAM. Zaragoza (Spain).

Blochet, J.E., Chevalier, C., Forest, E. *et al.* (1993). Complete amino acid sequence of puroindoline, a new basic and cystine-rich protein with a unique tryptophan-rich domain, isolated from wheat endosperm by Triton X-114 phase partitioning. FEBS letters, 329: 336–40.

Campbell, K.G., Bergman, C.J., Gualberto D.G. *et al.* (1999). Quantitative Trait Loci Associated with Kernel Traits in a Soft x Hard Wheat Cross. Crop Science, 39: 1184–1195.

Chen, F., Xu, H.-X., *et al*. (2010). Physical mapping of puroindoline b-2 genes and molecular characterization of a novel variant in durum wheat (Triticum turgidum L.). Molecular Breeding, 28:.153–161.

Chen, Feng *et al*. (2013). Allelic variation and distribution independence of Puroindoline b-B2 variants and their association with grain texture in wheat. Molecular Breeding, *May*. DOI: 10.1007/s11032-013-9879-z

Chen, F., Zhang, F. *et al*. (2010). Association of Puroindoline b-B2 variants with grain traits, yield components and flag leaf size in bread wheat (Triticum aestivum L.) varieties of the Yellow and Huai Valleys of China. Journal of Cereal Science, 52: 247–253.

Chen, F., Beecher, B. S. and Morris, C. F. (2010). Physical mapping and a new variant of Puroindoline b-2 genes in wheat. Theoretical and applied genetics,120: 745–51.

Crepieux, S., Lebreton C., Flament, P. and Charmet, G. (2005). Application of a new IBD-based QTL mapping method to common wheat breeding population: analysis of kernel hardness and dough strength. Theoretical and applied genetics, 111: 1409–1419.

Dai, Z. (2009). Starch Granule Size Distribution in Grains at Different Positions on the Spike of Wheat (Triticum aestivum L.). Starch - Stärke, 61: 582–589.

Edwards, M.A. (2010). Morphological features of wheat grain and genotype affecting flour yield Morphological Features of Wheat Grain and Genotype Affecting Flour Yield. PhD thesis, Southern Cross University, Lismore, NSW.

Food, S. *et al*. (2006). Symposium: Food Fortification in Developing Countries. The journal of nutrition, 136: 1064–1067.

Gaines, C.S., Finney, P.F., Fleege L.M. and Andrews L.C. (1985). Predicting a Hardness Measurement Using the Single-Kernel Characterization System. Cereal Chemistry, 73: 278–283.

Gale, K.R., 2005. Diagnostic DNA markers for quality traits in wheat. Journal of Cereal Science, 41: 181–192.

Geng, H., Beecher, B.S., Pumphrey, M., Zhonghu, H., Morris, C.F. (2013). Segregation analysis indicates that Puroindoline b-2 variants 2 and 3 are allelic in Triticum aestivum and that a revision to Puroindoline b-2 gene symbolization is indicated. Journal of Cereal Science, 57: 61–66.

Gómez-Becerra, H.F. *et al*. (2009). Phenotypic correlations, G × E interactions and broad sense heritability analysis of grain and flour quality characteristics in high latitude spring bread wheats from Kazakhstan and Siberia. Euphytica, 171: 23–38.

Haraszi, R., Sissons, M., Tamas, L., Juhasz, A., Rakszegi, M., Anderssen, R.S. (2013) Rheological Hardness Index for Assessing Hardness of Hexaploids and Durums. *Cereal Chemistry*. Accepted for publication. DOI: 10.1094/CCHEM-10-12-0133-R

Hrušková, M. and Švec, I. (2009). Wheat Hardness in Relation to Other Quality Factors. Czech Journal of Food Science, 27: 240–248.

Lafiandra, D., Sanguineti, M.C., Maccaferri, M. and Deambrogio, E. 2007. Molecular markers and QTL analysis for grain quality improvement in wheat, p 25-50. *In* Varshrey, R.K. and Tuberosa, R. (eds.), *Genomics-Assisted Plant Iprovement*, vol.2. Springer.

Lochlainn, S.O. *et al*. (2011). High Resolution Melt (HRM) analysis is an efficient tool to genotype EMS mutants in complex crop genomes. Plant methods, 7:43.

Mammadov, J., Aggarwal, R., Buyyarapu, R. and Kumpatla, S. (2012). SNP markers and their impact on plant breeding. International Journal of Plant Genomics, vol. 2012, Article ID 728398, 11 pages. DOI:10.1155/2012/728398

Massa, A.N. and Morris, C. F. (2006). Molecular Evolution of the Puroindoline-a, Puroindoline-b , and Grain Softness Protein-1 Genes in the Tribe Triticeae. J.Mol. Evol, 63: 526–536.

Miao, Y. *et al*. (2012). Expression, purification and antimicrobial activity of puroindoline A protein and its mutants. Amino acids, 43: 1689–1696.

Morell, M.K. and Gale, K.R. (2002). Use of perfect markers in wheat quality. Acta Agronomica Hungarica, 50: 263–274.

Morris, C. F. (2002). Puroindolines: the molecular genetic basis of wheat grain hardness. Plant Molecular Biology, 48: 633–47.

Morris, C. F. and Beecher, B.S. (2012). The distal portion of the short arm of wheat (Triticum aestivum L.) chromosome 5D controls endosperm vitreosity and grain hardness. TAG, 125: 247–54.

Morris, C. F. and Bhave, M. (2008). Reconciliation of D-genome puroindoline allele designations with current DNA sequence data. Journal of Cereal Science, 48: 277–287.

Ouafi, I. (2001). Quantitative Trait Loci (QTL) determination of grain quality traits in durum wheat (Triticum turgidum L . var . durum) PhD. Thesis, Cordoba, Spain.

Pasha, I., Anjum, F. M. and Morris, C. F. (2010). Grain hardness: a major determinant of wheat quality. Food science and technology international = Ciencia y tecnología de los alimentos internacional, 16: 511–22.

Peters, W.R. and Katz, R. (1962). Using A Density Gradient Column To Determine Wheat Density. Cereal Chemistry, 39: 487-493

Ramalingam, A., Palombo, E. A. and Bhave, M. (2012). The Pinb-2 genes in wheat comprise a multigene family with great sequence diversity and important variants. Journal of Cereal Science, 56: 171-180.

Reed, G.H., Kent, J.O. and Wittwer, C.T. (2007). High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics, 8: 597–608.

Samson, M. F., Mabille, F. *et al.* (2005). Mechanical and Physicochemical Characterization of Vitreous and Mealy Durum Wheat Endosperm. Cereal Chemistry, 82: 81–87.

Tsilo, T.J.,Simsek, S. *et al.* (2011). Quantitative trait loci influencing endosperm texture, dough-mixing strength, and bread-making properties of the hard red spring wheat breeding lines. Genome / National Research Council Canada = Génome / Conseil national de recherches Canada, 54: 460–70.

Turnbull, K., Turner, M. *et al*. (2003). The organization of genes tightly linked to the Ha locus in Aegilops tauschii, the D-genome donor to wheat. Genome / National Research Council Canada = Génome / Conseil national de recherches Canada, 338: 330–338.

Wang, G., Leonard, J.M. *et al*. (2012). Identification of genetic factors controlling kernel hardness and related traits in a recombinant inbred population derived from a soft × “extra-soft” wheat (Triticum aestivum L.) cross. Theoretical and applied genetics, 124: 207–21.

Wilkinson, M., Wan, Y. *et al*. (2008). Identification and genetic mapping of variant forms of puroindoline b expressed in developing wheat grain. Journal of Cereal Science, 48: 722–728.

USED WEB ADDRESSES

ExPASy: Bioinformatics Resource Portal - http://web.expasy.org/translate/

FastPCR - http://primerdigital.com/fastpcr.html

GrainGenes: a Database for Triticaceae and *Avena*- http://wheat.pw.usda.gov/GG2/index.shtml

NCBI: National Center for Biotechnology information - http://www.ncbi.nlm.nih.gov/

Primer3: http://frodo.wi.mit.edu/

Reverse complement: http://www.bioinformatics.org/sms/rev_comp.html

IDT: Integrated DNA technologies Oligoanalyzer: http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx

Institute of molecular development, LLC: http://www.molecularinfo.com/MTM/G/G1/G1-3.html

APPENDIXES

APPENDIX I

Table 7 Modified Hoagland nutrient solution after Dr. Cseh Edit workgroup, Dep. Plant physiology and Molecular Plant Biology, ELTE).

Hoagland nutrient solution (modifyed)				
Macronutrients	**Final concentration (mol/dm^3)**	**For 100 ml stock solution (g)**	**Amount of stock solution for 1 L nutrient solution**	**For ¼ Hoagland (8L)**
KNO_3	0,00125	12,6375	1 ml	8ml
$Ca(NO_3)_2$	0,00125	29,5188	1 ml	8ml
$MgSO_4$	0,0005	12,323	1 ml	8ml
KH_2PO_4	0,00025	3,4023	1 ml	8ml
Fe-EDTA	0,00001	1,468	1 ml	8ml
Micronutrients				
H_3BO_3	0,00001156	0,2859	250 µl	2ml
$MnCl_2 \cdot 4H_2O$	0,0000046	0,3642	250 µl	2ml
$ZnSO_4 \cdot 7H_2O$	0,00000019	0,0219	250 µl	2ml
$Na_2MoO_4 \cdot 2H_2O$	0,00000012	0,0116	250 µl	2ml
$CuSO_4 \cdot 5H_2O$	0,00000008	0,008	250 µl	3ml*

* - 3ml of CuSO4•5H2O was used as CuSO4•5H2O precipitates easily, therefore based on the practice +1ml was used. For stock solutions, macro- and micronutrients were diluted in deionized water. For preparing ¼ strength solution, the dilution to 8 L was made using destilled water.

APPENDIX II

Table 8 Primers used with Kazakhstan H- and B-set. Optimal annealing temperature given.

	Primer	Sequence 5'→3'	Annealing t°C	Annealing time (sec)	Extension time at 72°C	Fragment size (bp)	Use
1	PAF	ATTCATCTCCACCTGACC	61	30	35	535	*Pina* WT (*Pina-D1a)*
2	PAR	ACACGCAGTGGTATGTGAC					
3	Primpa1	GCCCTCTTCCTCATAGGAC	55	20	30	420	*Pina* WT (*Pina-D1a)*
4	Primpa3	TGCCGGGGATGTTGCAGGG					
5	PBKF	CCCACAAAATGGTGGAAGA	58	25	25	356	*Pinb* mutant (*Pinb-*D1b)
6	PBKR	GAACACAGATCAATATACAAGG					
7	Primpb1	ACCTTATTCCTCCTAGCTC	58	25	30	422	*Pinb* WT (*Pinb-D1a)*
8	Primpb3	GGGAACTTGCAGTCGGCGC					
9	PBF	ATGAAGACCTTATTCCTCCTA	55	20	30	447	*Pinb* WT (*Pinb-D1a)*
10	PBR	TCACCAGTAATAGCCACTAG					

Tartu 2013

Table 9 Primers for puroindoline-B2v after Chen *et al.,* (2010). Optimal annealing temperature is given

	Code	Sequence	Annealing t°C	Annealing time (sek)	Product size (bp)	Use
1	Pb2v1F	GGTTCTCAAAACTGCCCAT	57	25	319	purB-2 variant1(*Pinb*-D2a), wheat kernel hardness
2	Pb2v1R	ACTTGCAGTTGGAATCCAG				
3	Pb2v2F	CTTGTAGTGAGCACAACCTTTGCA	65	30	401	purB-2 var.2 (*Pinb*-B2a), kernel texture among soft and very soft wheats
4	Pb2v2R	GTATGGACGAACTTGCAGCTGGAG				
5	Pb2v3F	GCAGAAAAAGCCATTGCACCTA	65	30	528	purB-2 var.3 (*Pinb*-B2b), kernel texture among soft and very soft wheats
6	Pb2v3R	CATTAGTAGGGACGAACTTGCAGCTA				
7	Pb2v4F	CCTTTCTCTTGTAGTGAGCACAACCA	65	30	403	purB-2 var.4 (*Pinb*-A2a)
8	Pb2v4R	GACGAACTTGCAGTTGGAATCCAA				

Table 10 Results regarding Kazakhstan H-set. Variety, pedigree and year/cross of selection was known previously.

Nr	code	Variety	Pedigree	Year of cross/selection	***Pina***		***Pinb***			**Genotype**	**Phenotype**
					+	-	+		-		
							mut	**WT**			
1	H2	Smena	selection from local (Siberia) variety	1919	*		*			*Pina*-D1a/*Pinb*-D1b	hard
2	H3	Lutescens 956	selection from local (Siberia) variety	1919	*		*			*Pina*-D1a/*Pinb*-D1b	hard
3	H4	Irtyshanka 10	Skala/Saratovskaya 36	1964	*		*			*Pina*-D1a/*Pinb*-D1b	hard
4	H6	Omskaya 21	Spontaneous hybrid (Canada)/Novosibirskaya 22	1986	*		*			*Pina*-D1a/*Pinb*-D1b	hard
5	H7	Omskaya 26	Novosibirskaya 22 / W.W.16151 (Sweden)	1986	*		*			*Pina*-D1a/*Pinb*-D1b	hard
6	H8	Pamyaty Azyeva	Saratovskaya 29 / Lutescens 99-80-1	1987	*		*			*Pina*-D1a/*Pinb*-D1b	hard
7	H9	Omskaya 32	Lutescens 162-84-1 / Chris (USA)	1989	*		*			*Pina*-D1a/*Pinb*-D1b	hard
8	H10	Kazanskaya yubileynaya	Omsraya 20 / Lutescens 204-80-1//Lutescens 3-86-6	1992		*	*			null/*Pinb*-D1b	hard

9	H11	Tulunskaya 12	Biryusinka / Bezostaya 1	1970	*		*			*Pina*-D1a/*Pinb*-D1b	hard
10	H12	Altayskaya 92	Novosibirskaya 67 / Lutescens 4029	1981		*		*		null/*Pinb*-D1a	hard
11	H13	Rosinka	Physical mutant from Sibakovskaya 3,	1979	*		*			*Pina*-D1a/*Pinb*-D1b	hard
12	H14	Selenga	Buryatscaya 34 / Buryatscaya 79	1978	*		*			*Pina*-D1a/*Pinb*-D1b	hard
13	H16	Strada Sibiri	Rang/ Hibryd 21// Irtishanka 10/4/к-54049/3/Line 1633	1986	*		*			*Pina*-D1a/*Pinb*-D1b	hard
14	H17	Omskaya 34	Omskaya 21 / Lutescens 4979c / 1553	1993	*		*			*Pina*-D1a/*Pinb*-D1b	hard
15	H18	Tarskaya 6	Lutescens 89-87-29 / Narskaya 5	1993	*		*			*Pina*-D1a/*Pinb*-D1b	hard
16	H19	Omskaya 36	Lutescens 150-86-10 / Runar (Norway)	1994	*			*		*Pina*-D1a/*Pinb*-D1a	soft
17	H20	Tarskaya 7	Lutescens 89/87-29 / Omskaya 26	1993	*		*			*Pina*-D1a/*Pinb*-D1b	hard
18	H22	Omskaya 27	Omskaya 17 // Atlas 66 / Lutescens 1140	1983	*		*			*Pina*-D1a/*Pinb*-D1b	hard
19	H23	Albidum 3700	selection from local (Siberia) variety	1925	*		*			*Pina*-D1a/*Pinb*-D1b	hard
20	H24	Sibiryashka	Sibiryashka 2 / Saratovskaya 29	1966	*		*			*Pina*-D1a/*Pinb*-D1b	hard

21	H25	Omskaya 17	Lutescens 1138-166 / Red River 68 (USA)	1972	*		*			*Pina*-D1a/*Pinb*-D1b	hard
22	H26	Omskaya 19	Lutescens 1138-70 / Lutescens 1210	1973	*		*			*Pina*-D1a/*Pinb*-D1b	hard
23	H27	Dias 2	Novosibirskaya 67 / Rang (Norway)	1973	*		*			*Pina*-D1a/*Pinb*-D1b	hard
24	H28	Omskaya 20	Irtishanka 10 // Graecum 114 / Kavkaz	1980	*		*			*Pina*-D1a/*Pinb*-D1b	hard
25	H29	Omskaya 29	Lutescens 204-80-1 / Lutescens 99-80-1	1987	*		*			*Pina*-D1a/*Pinb*-D1b	hard
26	H30	Omskaya 33	Lutescens 137-87-39 / Omskaya 28	1992	*		*			*Pina*-D1a/*Pinb*-D1b	hard
27	H31	Saratovskaya 29	Lutescens 91 / Sarroza // Lutescens 55-11	1938	*		*			*Pina*-D1a/*Pinb*-D1b	hard
28	H32	Tselinnaya 26	Shortandinskaya 25 /FKN 25 (USA)	1983	*		*			*Pina*-D1a/*Pinb*-D1b	hard
29	H33	Sybakovskaya 3	Bezostaya 1 / Saratovskaya 29	1965	*			*		*Pina*-D1a/*Pinb*-D1a	soft
30	H34	Niva 2	PS -360/76 / Irtishanka 10	1982	*		*			*Pina*-D1a/*Pinb*-D1b	hard
31	H35	Rosinka 2	Chemical mutant from Tselinnaya 21,	1979, 1987	*		*			*Pina*-D1a/*Pinb*-D1b	hard
32	H36	Slavyanka Sibiri	Chemical mutant from Lutescens	1984, 1986	*		*			*Pina*-D1a/*Pinb*-D1b	hard

			65,								
33	H37	Sonata	Tselinnaya 20 /Tertsiya	1986		*			*	null/null	hard
34	H38	Tuleevskaya	Olivatseva / Vendel // Lutescens 105 (WW)	1989		*			*	null/null	hard
35	H39	Svetlanka	Omskaya 23 / Tselinnaya 26	1987		*			*	null/null	hard
36	H40	Marquis	Hard Red Calkytta / Red Five	1892	*		*			*Pina*-D1a/*Pinb*-D1b	hard
37	H41	Omskaya 20	Irtishanka 10 // Graecum 114 / Kavkaz	1980	*		*			*Pina*-D1a/*Pinb*-D1b	hard
38	H42	Milturum 321	selection from local (Siberia) redspike variety	1913	*		*			*Pina*-D1a/*Pinb*-D1b	hard
39	H43	Milturum 553	Milturum 321 / Citchener (Canada)	1927	*		*			*Pina*-D1a/*Pinb*-D1b	hard
40	H44	Cesium 94	Caesium 117 / Western Polba	1923	*			*		*Pina*-D1a/*Pinb*-D1a	soft
41	H45	Omskaya 9	Bezostaya 1 / Saratovskaya 29	1964	*		*			*Pina*-D1a/*Pinb*-D1b	hard
42	H47	Omskaya 24	Lutescens 1594 / Sibiryashka 8 // Krasnodarskaya 39	1977	*		*			*Pina*-D1a/*Pinb*-D1b	hard
43	H48	Omskaya 28	Lutescens 19 / Hibryd (Canada)	1985	*		*			*Pina*-D1a/*Pinb*-D1b	hard
44	H49	Omskaya 30	Omskaya 20 / Lutescens 204-80-1	1987	*		*			*Pina*-D1a/*Pinb*-D1b	hard

45	H50	Omskaya 35	Omskaya 29 / Omskaya 30	1994	*		*			*Pina*-D1a/*Pinb*-D1b	hard
46	H51	Omskaya 37	Lutescens 61-89-100 / Lutescens 350-89-9	1997	*		*			*Pina*-D1a/*Pinb*-D1b	hard
47	H52	Tertsiya	Saratovskaya 36 / I 428010 (Canada)		*		*			*Pina*-D1a/*Pinb*-D1b	hard
48	H53	Karagandinskaya 70	Chayka (WW) / Irtishanka 10	1979	*		*			*Pina*-D1a/*Pinb*-D1b	hard
49	H54	Eritrospermum 59	ANK 1 / ANK 2 // ANK 3 /3/ ANK 7A	1980	*		*			*Pina*-D1a/*Pinb*-D1b	hard

Table 11 Results of biofortification set; *Pina* and *Pinb* PCR and sequencing results.

Code	Variety	Biofortification	*Pina*	*Pinb*	Comment
B-1	Aktobe 32	Zn+	wild (+band)	mutant, may be *Pinb*-D1b allele	
B-6	Stepnaya 1	Zn+	wild (+band)	mutant sequenced (no name)	in position 128 A→ G (Glu→ Gly); plus in position 223 G→A (Gly→Ser) like in *Pinb*-D1b
B-7	GVK 1369-2	Zn+	wild (+band)	mutant sequenced (*Pinb*-D1b allele)	in nucleotide position 223 G→A(Gly→Ser)
B-12	Lutescens 53-95	Zn+	wild (+band)	mutant sequenced extra Stop codon (no name)	in 382 position C →T (Gln→ Stop codon) like in *Pinb*-D1ab variant; plus in position 257 G →A (Cys→ Tyr)
B-13	Astana	Zn+	wild (+band)	mutant, may be *Pinb*-D1b allele	
B-18	Shortandinskaya 95	Zn+	wild (+band)	mutant, may be *Pinb*-D1b allele	
B-19	Chelyaba	Zn+	wild (+band)	mutant, may be *Pinb*-D1b allele	

B-24	Lutescens 148-97-16	Zn+	wild (+band)	mutant, may be *Pinb*-D1b allele	
B-25	Fora	Zn+	wild (+band)	mutant, may be *Pinb*-D1b allele	
B-26	Golubkovskaya	Zn+	wild (+band)	mutant sequenced (*Pinb*-D1b allele)	in nucleotide position 223 G→A (Gly → Ser)
B-31	Iren	Zn+	wild (+band)	mutant sequenced (no name)	in nucleotide position 266 T→C (Pro→Leu) like in *Pinb*-D1c; plus in position 22 G → T (Ala→Ser) like in *Pinb*-D1v; plus in position 63 C→T but no change in amino acid, (Tyr).
B-32	Eritrospermum 78	Zn+	Shortady	wild (+band)	wild (+band)

Table 12 Results regarding Pb2v2 and Pb2v3 in Australian group. Puroindoline-a and puroindoline-b genotypes were screened earlier, SKCS HI known previously.

	Genotype	**Grain texture**	**SKCS HI**	**Pin a**	**Pin b**	**Pinb2-v3**	**Pinb-2v2**	**Validation based on *Pinb-2***
1	Bencubbin	soft		a	a	+		hard
2	Bolero	soft	28	a	a	+		hard
3	Bungulla	soft		a	a	-	+	softer
4	Capelle Desprez	soft		a	a	+		hard
5	Capitole	soft		a	a	+		hard
6	Centauro	soft	21	a	a	+		hard
7	Chinese Spring	soft	33	a	a	-	+	softer
8	Declic	soft		a	a	+	-	hard
9	EGRET	soft		a	a	+		hard
10	Emblem	soft		a	a	+		hard
11	Galahad	soft		a	a	+		hard
12	Gamenya	soft		a	a	+f		hard
13	Ghurka	soft		a	a	+f		hard
14	Glenwari	soft		a	a	+f		hard
15	Gluclub	soft		a	a	+		hard
16	Gluyas	soft		a	a	+		hard
17	Insignia	soft		a	a	+		hard
18	Insignia 49	soft		a	a	+		hard
19	Isis	soft		a	a	-	+	softer

20	Jabiru	soft		a	a	+		hard
21	Kalkee	soft		a	a	+		hard
22	Kewell	soft		a	a	+		hard
23	Kondut	soft		a	a	-	+	softer
24	Libero	soft	35	a	a	+		hard
25	Macquarie	soft		a	a	-	+	softer
26	Matong	soft		a	a	+		hard
27	Mengavi	soft		a	a	+		hard
28	Mersey	soft		a	a	+		hard
29	Mokoan	soft		a	a	+		hard
30	Olympic	soft		a	a	+		hard
31	Orca	soft		a	a	+		hard
32	Pinnacle	soft		a	a	+		hard
33	Radja	soft		a	a	+		hard
34	Ranee	soft		a	a	+		hard
35	Robin	soft		a	a	+		hard
36	Summit	soft		a	a	+		hard
37	Tatiara	soft		a	a	-	+	softer
38	Teal	soft		a	a	+		hard
39	Tincurrin	soft		a	a	+		hard
40	Wren	soft		a	a	+		hard
41	Zenith	soft		a	a	+		hard
42	Heron	soft		a	a	+		hard

Table 13 Primers designed for HRM-PCR. Fragment locations in the sequence based on the nucleotide location counting based on the information in NCBI using the accession number in the parentheses.

	Primer	Sequence 5'→3'	Annealing t °C	time (sek)	Extension time at 72°C (sec)	Fragment size (bp)	Use
1	PuraJ1F	AACCACACTGACAACATGAAG	59	25	20	245	For HRM-PCR, puroindoline-a polymorphism detection. Locations 836 -1080 (DQ363911.1).
2	PuraJ1R	TTGACAACCTCCCTTCC					
3	PuraJ3F	AAGGGAGGTTGTCAAGAGC	60	25	20	231	For HRM-PCR, puroindoline-a polymorphism detection. Locations 1066-1297 (DQ363911.1)
4	PuraJ3R	ATCACCAGTAATAGCCAATAGTG					
5	PurbJ1F	AACATGAAGACCTTATTCCTCCT	63	28	20	250	For HRM-PCR, puroindoline-b polymorphism detection. Locations 271-527 (DQ363913.1)
6	PurbJ1Rd	TTCTCSCGAACCTCATGC					
7	PurbJ2F	TGTGAGCATGAGGTTCG	59	25	20	240	For HRM-PCR, puroindoline-b polymorphism detection. Locations 502-742 (DQ363913.1)
8	PurbJ3R	GTCACGAATAGAGGCTATATCA					

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