# European Cereals Genetics Co-operative Newsletter 2012

Proceedings of the 15th International EWAC Conference

7 – 11 November 2011

Novi Sad, Serbia


www.ewac.eu

Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany

and

Institute of Field and Vegetable Crops, Novi Sad, Serbia

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Edited by

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# Preface

A. Börner (Secretary, EWAC)

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From November 7 to 11, the 15th International EWAC Conference was held in Novi Sad, Serbia. The conference was jointly organised by EWAC and the Cereals Section of EUCARPIA, the ‚European Association for Research on Plant Breeding'. The co-operative returned to the place where 45 years ago the ' Proposal for the co-ordination of European work with wheat aneuploids' was made by Ralph Riley and Colin Law at the 'Fifth Yugoslav Symposium on Wheat Research' in 1966. More than 60 participants from 24 countries attended. EWAC became an international platform for researchers developing and using cereal stocks for genetic studies including the localisation and mapping of genes/QTLs by employing molecular techniques. Beside European participants contributors came from USA, Canada, Mexico, Egypt and Australia. Thirty lectures divided into four sessions were presented:

(1) Genetic stocks and alien introgressions
(2) Molecular gene mapping and breeding in wheat
(3) Molecular gene mapping and breeding in barley and oat
(4) New strategies/technologies for breeding and research

The oral presentations were supplemented by 34 posters. In addition a workshop on 'Adapting agriculture to climate change: collecting, protecting and preparing crop wild relatives' was organised by the 'Global Crop Diversity Trust'.

The Proceedings of the Conference are published as hard copy and at the EWAC web page (www.ewac.eu). Special thanks to Zbyněk Milec from the Crop Research Institute in Prague for running the internet portal.

The local organisation of the conference was excellent. Many thanks to Boris Kobiljski and his team for preparing and running the event. The atmosphere during the whole symposium was very kind and friendly. We did enjoy the days in Novi Sad very much.

The participants agreed to continue the successful series of EWAC Conferences and it was Krzysztof Kowalczyk who offered to organise the next symposium in Lublin, Poland. Considering other scientific events it was scheduled for June/July 2015.

We look forward to that 16th EWAC Conference.

# Cereal genetic stocks – examples of successful co-operation (2008 – 2011)

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## Introduction

Since EWAC (European Wheat Aneuploid Co-operative; later renamed: European Cereal Genetics Co-operative) was established in 1967 series of genetic stocks including monosomics, chromosome substitution lines, alloplasmic lines, single chromosome recombinant lines, introgression lines, etc. have been created. Analysing these genetic stocks many qualitative and quantitative inherited traits were associated to certain chromosomes, chromosome arms or introgressed segments. When molecular marker techniques became available, the knowledge gathered from the stock investigations often was the pre-requisite for the precise mapping. Today, cereal genetic stocks are supplemented by a huge number of genotyped mapping populations. Beside progenies of bi-parental crosses (doubled haploid lines, recombinant inbred lines, etc.) panels for association mapping were created recently. In our presentation we give examples for the successful co-operation within the frame of EWAC. Using both segregation and association mapping approaches, data on mapping of loci/marker trait associations for a range of different traits are presented.

## Segregation mapping

<u>*Grain quality in wheat (Russia, Germany):*</u> The International Triticeae Mapping Initiative (ITMI) mapping population was used to map quantitative trait loci (QTL) associated with individual characteristics of grain and flour quality investigating recombinant inbred lines (RILs) grown under contrasting environmental conditions. Overall, 22 QTL with various significances were detected on 10 chromosomes. Grain hardness and vitreousness were associated with three loci on chromosomes 5D, 6A and 3A, while the gluten content, with two loci on chromosomes 5B and 7A. Dough extensibility was associated with only one QTL localized in the region of *Glu-A1* locus. One of the loci determining flour and dough strengths is located in the region of *Gli-B1* and *Glu-B3* loci and the rest, in various regions of chromosomes 1B, 5D, and 4B, where no particular genes associated with grain quality have been yet found (Pshenichnikova et al. 2008a).

In another study activity of two enzymes of thiol-disulfide cell metabolism, lipoxygenase (LOX, EC 1.13.11.12) and disulfide-reductase (TPDO, EC 1.8.4.2) was investigated in recombinant inbred lines of bread wheat ITMI population. Their activity in the caryopsis may

be connected with the gluten quality, one of the most important traits significant for breeding. The activity of lipoxygenase under favorable and droughty environmental conditions was shown to be associated with a quantitative trait locus (QTL) located on chromosome 4BS near the structural gene of a subunit of this enzyme. However, no QTL common to this enzyme and any characteristic of gluten quality have been found. Four loci responsible for the activity of disulfide reductase were identified on chromosomes 4A, 5D, 6A and 7D. Previously, indicators of grain and flour properties, such as elasticity, flour strenght and grain hardiness were mapped at the same loci. This indicates that the given enzyme participates in the formation of the protein complex upon maturation of wheat grain. The detected QTL can be involved in further genetic studies designed to establish the regularities of gluten formation (Pshenichnikova et al. 2008b).

*Drought tolerance in wheat (Bulgaria, Germany):* Drought can affect growth at various stages of plant development. One of the most critical periods in wheat establishment is germination and early seedling growth. A major component of drought is osmotic stress, which generally causes growth inhibition. In the present study a QTL-approach was applied to dissect the complex genetic control of plant growth response to osmotic stress, induced by polyethylene glycol (PEG 6000). The 114 RILs of the wheat ITMI mapping population were subjected to 12% PEG osmotic stress from the onset of germination to the 8$^{th}$ day of seedling development. The growth of seedlings under stress, estimated by four parameters (root length, coleoptile length, shoot length, root length / shoot length ratio) was compared to that under control conditions. A total of 35 regions on 10 chromosomes contributed effects on seedling growth traits. Almost half of the QTLs (16) were detected under control conditions, 17 under osmotic stress conditions and 2 QTLs were determined for tolerance index. In regions on five chromosomes (1AS, 1BL, 2DS, 5BL and 6BL) the QTLs detected under stress co-mapped with QTLs for the same trait under control conditions, so they were classified as QTLs, affecting seed vigour *per se*. A wide chromosome region on chromosome 1AL, comprising 5 QTLs with major impact of markers *Glu1A* (LOD 3.93) and *Xksuh9d* (LOD 2.91), affected positively root length under stress and tolerance index for root length, respectively. A major QTL (LOD 3.60), associated with marker *Xcdo456a* in the distal part of chromosome 2DS was detected for tolerance index for shoot length. Three minor QTLs (LOD <3.0) for root length and root length / shoot length ratio under osmotic stress were identified in the distal parts of chromosomes 6DL and 7DL (Landjeva et al. 2009). Selected chromosomes are shown in figure 1.

*Flowering time in wheat (Romania, Russia, Germany):* Using a set of wheat single chromosome (7B) recombinant lines (SCRLs) developed from the cross 'Favorit' x 'Favorit/F26-70 7B' a new photoperiod response gene designated *Ppd-B2* was mapped. Mapping position of *Ppd-B2* was determined on chromosome arm 7BS, 8.8 cM distal to microsatellite marker *Xgwm0537* and 20.7 cM proximal to *Xgwm0255*. In contrast to the *Ppd-1* genes on the group 2 chromosomes, which are expressed under short day conditions, *Ppd-B2* was detectable only when the plants were exposed to long photoperiod. Earliness in flowering produced by *Ppd-B2* was correlated with an increase in grain protein content, and a major gene for this character was mapped 4.4cM proximal to *Ppd-B2* (Fig. 2). This gene does not affect grain size, and is therefore probably involved in nitrogen uptake and/or translocation (Khlestkina et al. 2009).

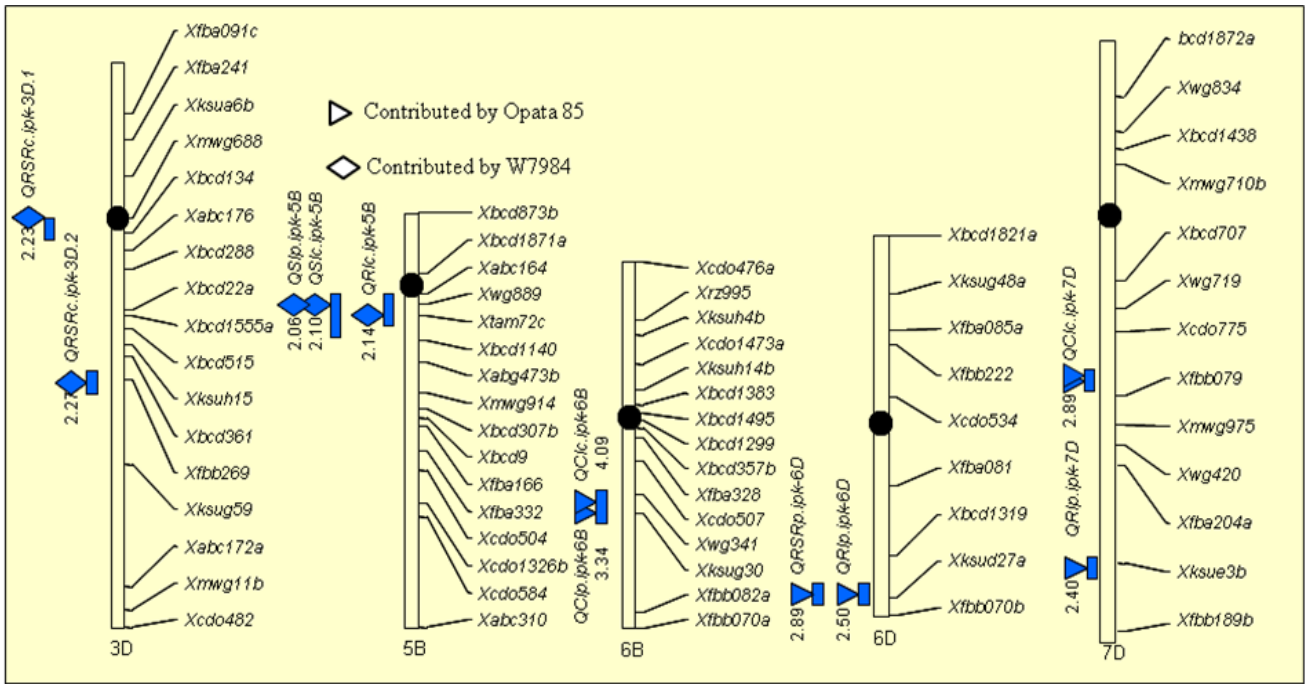


Fig. 1: Chromosomal regions affecting the growth characteristics of wheat seedlings

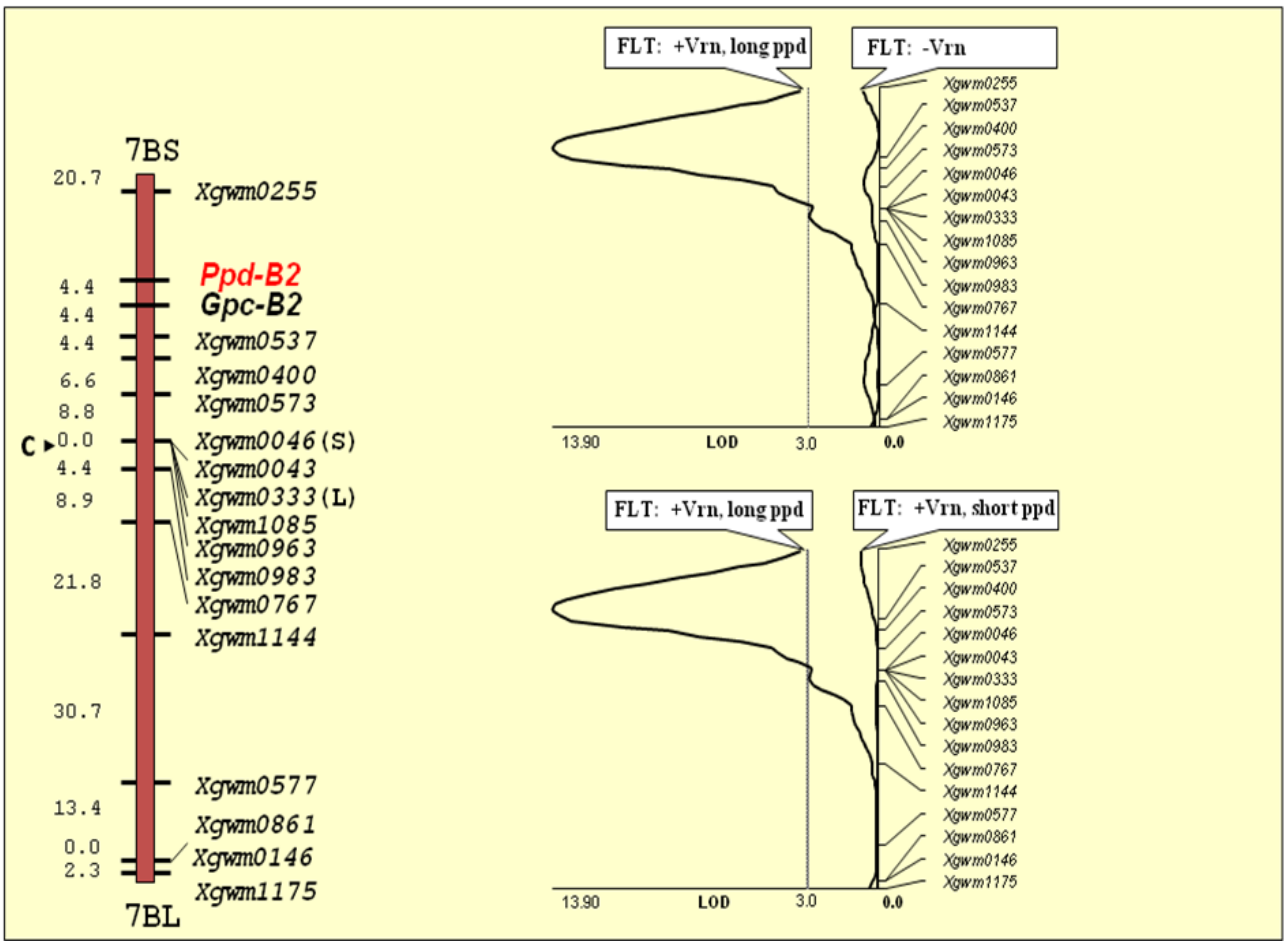


Fig. 2: Genetic map of chromosome 7B showing positions of *Ppd-B2* and *Gpc-B2* (left); QTL interval mapping for flowering time under different environments with respect to vernalisation and photoperiod (right)

*Agronomic traits in wheat (Serbia, Germany):* The ITMI population was used to detect quantitative trait loci (QTL) underlying key agronomic characters. Trait measurements were taken from five independent field experiments performed in Serbia. Stable across environment QTL involved in the determination of heading/flowering time and ear morphology/grain yield were detected on, respectively, chromosome arms 2DS and 4AL. These map locations are consistent with those obtained where the same population has been grown in contrasting geographical sites. However, as a result of QTL x environment interactions, not all these QTL are expressed in all environments. Nevertheless the (pleiotropic) effect on ear morphology (Fig. 3) appears to be expressed in almost all environments, and so represents a high value target for wheat improvement (Kobiljski et al. 2009).

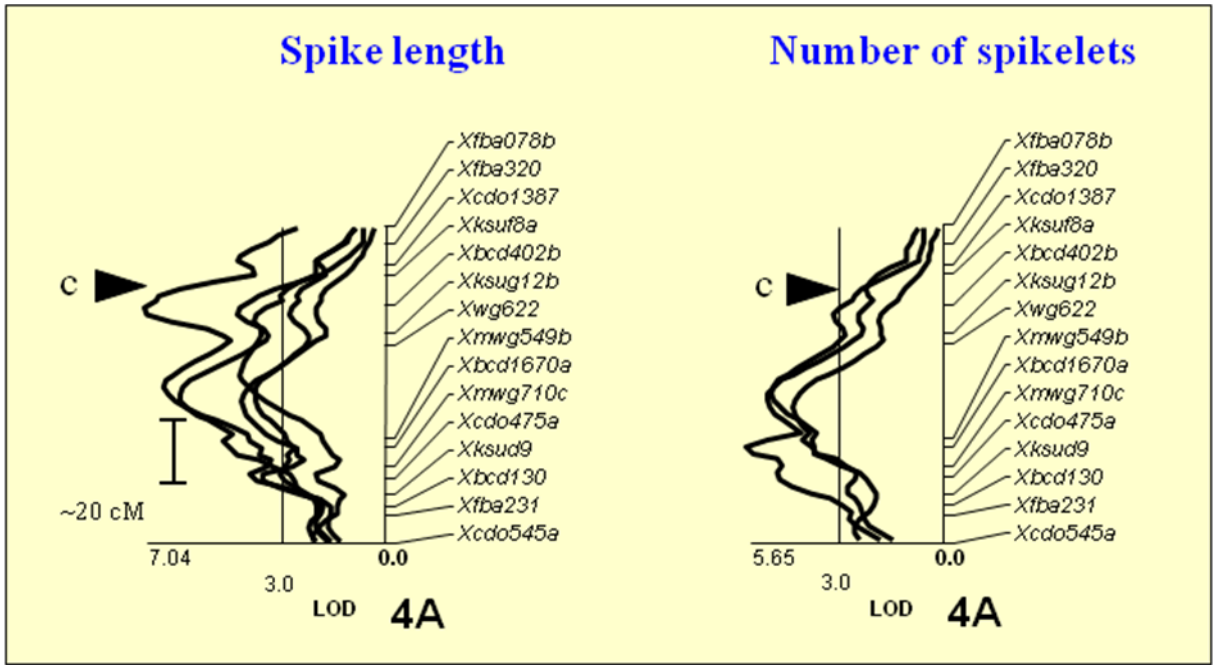


Fig. 3: QTL mapping along chromosome 4A showing the QTL intervals for ear length and spikelet number as detected in, respectively, five and three environments

*Septoria (Argentina, Russia, Germany):* The foliar wheat disease septoria tritici blotch can cause significant yield losses. A new source of resistance has been mapped on chromosome 7D of spelt wheat *Triticum spelta* (Fig. 4). The microsatellite-based genetic map was constructed from a set of 87 doubled haploid lines developed from the cross between the variety Chinese Spring and a Chinese Spring-based line carrying chromosome 7D from spelt wheat. Two regions of the chromosome were associated with a pathogen isolate-specific QTL expressed at both the seedling and the adult plant stage. One of these may be allelic to the major resistance gene *Stb4* present in the bread wheat variety 'Tadinia' (Simon et al. 2010).

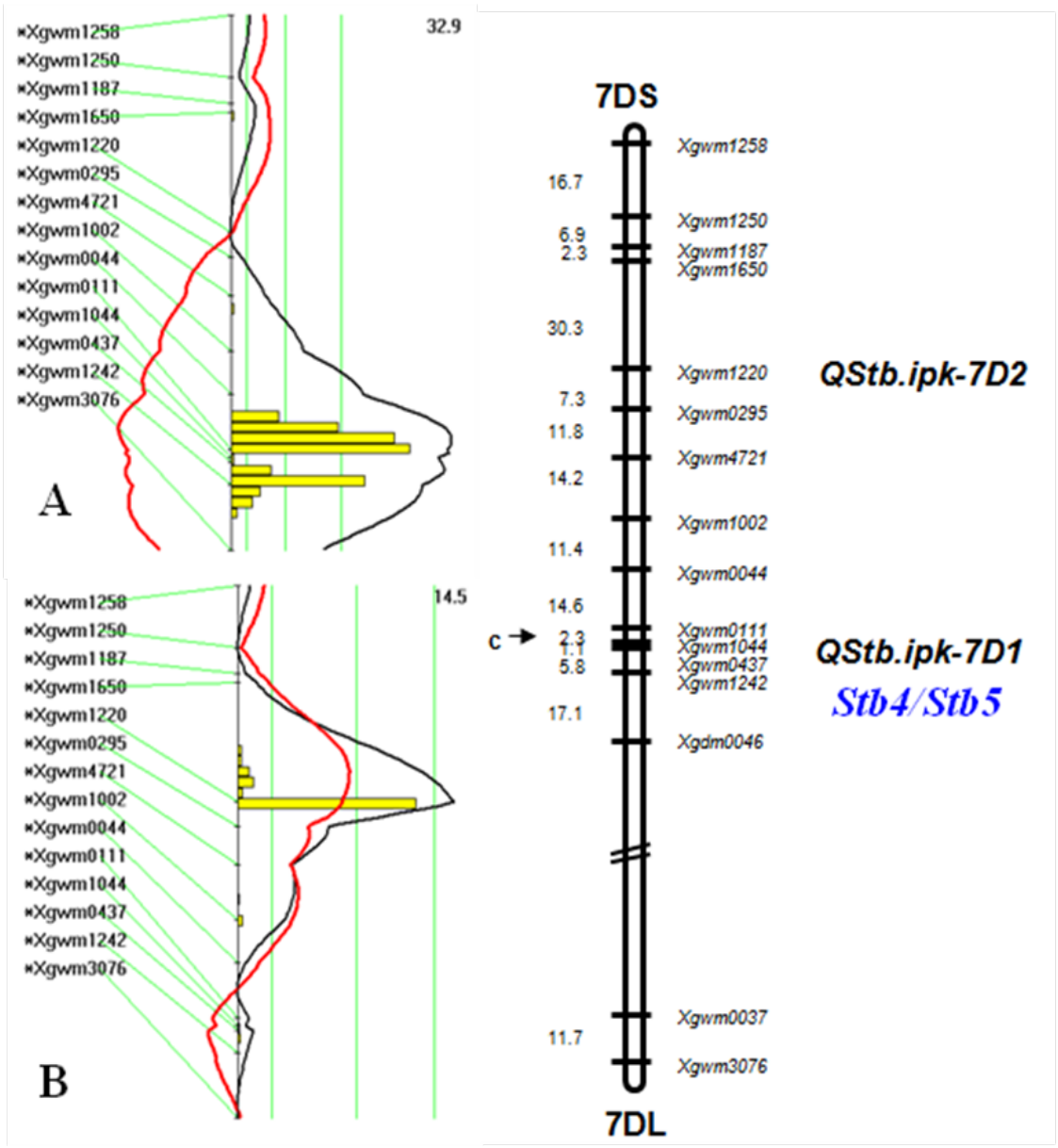


Fig. 4: Interval mapping of the seedling (A) and adult plant stage (B) resistances (left) and the genetic map of chromosome 7D. *QStb.ipk-7D1* is a locus for the seedling and *QStb.ipk-7D2* for the adult-plant stages of resistance

**Association mapping**

*Drought tolerance in barley (Hungary, Germany):* Studying drought tolerance interactions in several developmental stages to map QTL in bi-parental populations is often impossible due to low polymorphism (genetic or phenotypic) in at least one stage. We investigated a diverse spring barley collection for a genome-wide association study and examined extensive screenings in early and adult stages. The genotypes were selected for their differences in drought tolerance. Tests in germination and seedling stage were based on osmotic stress induced by polyethylene glycol. In adult stage, drought tolerance was tested using a rain out shelter, a foil tunnel and with the method of chemical desiccation using potassium iodide. The spring barley collection includes wild barley, landraces and cultivars. Diversity Array Technology (DArT) markers were used for genotyping. Population structure and extend of linkage disequilibrium (LD) were studied intensely. The marker-trait associations were calculated in TASSEL using different general linear and mixed linear models. The complexity of the interaction of relevant traits for drought tolerance could be seen on the genetic level. More than 200 loci were found for all traits, many loci are shared between the different

developmental stages. Often root traits were associated together with yield traits. Comparison with literature showed that many of the detected loci were involved in earlier studies, often associated with similar or the same traits. The results of this study enlight the difficulties in breeding for drought tolerance but also showed the value of deep phenotyping and of association mapping for such a complex trait (Neumann et al. 2011a).

*Agronomic trais in wheat (Serbia, Germany):* A genome-wide association study of wheat was initiated, in which a large number of diversity array technology (DArT) markers was used to genotype a winter wheat core collection of 96 accessions. The germplasm was structured into two sub-populations. Twenty agronomic traits were measured in field trials conducted over up to eight growing seasons. Correlations between traits across seasons were high in almost all cases. A total of 339 marker-trait associations was detected. Examples for flowering time and yield are given in figure 5. The intrachromosomal location of many of these coincided with those of known major genes or quantitative trait loci, but others were detected in regions where no known genes have been located to date. These latter presumptive loci provide opportunities for further wheat improvement, based on a marker approach (Neumann et al. 2011b).

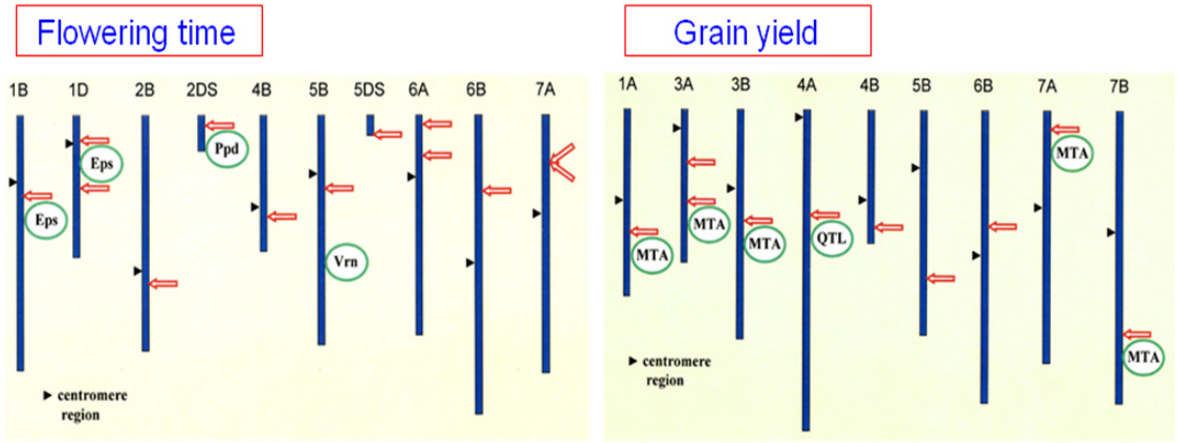


Fig. 5: MTAs for flowering time and grain yield are marked by arrows. Positions of comparable genes/QTLs/MTAs described earlier are indicated below arrows (ellipses)

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# Exploitation of Saratovskaya 29 (Janetzkis Probat 4D*7A) substitution and derivate lines for comprehensive phenotyping and molecular mapping of quantitative trait loci (QTL)

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In previous years large efforts were undertaken inside EWAC for the development of inter-varietal single chromosome substitution (ISCS) lines of bread wheat. This kind of genetic material played a significant role in the development of genetic research of this crop as it made possible a chromosomal location of genes for various traits of agronomic value.

At the 1st EWAC meeting in 1967 the problem of development and use of ISCS lines was discussed. Colin Law (1968) wrote in the first issue of EWAC Newsletter: "A wide range of wheat genetic problems can be resolved by the development of single chromosome substitution lines". However, at that time the lines only in the genetic background of cv. Chinese Spring (CS) were available. So, one of the first put aims of EWAC was to develop aneuploids and ISCS lines in other than CS backgrounds, in varieties which were adapted for environments of certain countries and which were well characterized for biological and agronomic traits.

Ten years later at the EWAC meeting in 1979 the list of genetic stocks was presented which contained the information about more than 20 full sets of substitution lines in different genetic backgrounds with numerous donors of chromosomes. The largest one was the collection from the Plant Breeding Institute Cambridge obtained by Colin Law and Tony Worland. Some of these lines are still used in our work.

Later, at the EWAC meeting in Martonvasar the application of molecular techniques for identification of new genes in bread wheat was discussed. The conclusion of presentation by John Snape (1987) was that aneuploids and substitution lines are valuable genetic materials for the development of wheat genetics using the new advanced and powerful methods of genome investigations.

In 1994 at the 9th EWAC conference in Gatersleben Colin Law reviewed the long-term work with substitution lines. He suggested using them for the development of homozygous recombinant lines which may be involved into precise mapping of new genes and QTLs and underlined that such lines should be tested in various environments (Law, 1995). At the same time he expressed a worry about the future of substitution lines and about many other obtained aneuploids as the interest of scientists at that time shifted to recombinant inbred populations. He hoped that EWAC will preserve this material for the future. At the following EWAC meeting in 1997 in Viterbo three unrelated sets of substitution lines were chosen for common investigations within EWAC. These were the lines CS (Synthetic 6x), Cappelle Desprez (Bezostya 1) and Saratovskaya 29 (Janetzkis Probat) (S29 (JP)). They were checked using molecular markers for authenticity of chromosome substitutions (Salina et al. 2003) and several wrong lines were detected. Some of them lately were re-developed; others were excluded from the future work.

The aim of our work was to present the example of comprehensive investigations of one substitution line from the set of S29 (JP) ISCS lines and derived substitution recombinant dihaploid (SRD) lines for different traits and under different environments for searching and mapping of responsible QTL.

Spring parental cultivars have different origins and are contrasting in the phenotype (Table 1). The substitution set was developed by R. Gaidalenok (1995). The line with 4D chromosome substitution and the derivate SRD lines were the main object of investigations in different environments of Russia (Novosibirsk), Bulgaria (Sofia) and Germany (Gatersleben). Yield components and flowering date were determined at all 3 sites, technological properties of dough were determined in grain grown in Bulgaria and Novosibirsk, resistance to sprouting was determined in Gatersleben.

Table 1: Comparative characteristics of the parental cultivars of S29 (JP) substitution set

| Cultivar | Origin | Agronomic characteristics |
|---|---|---|
| Saratovskaya 29<br>Recipient | Former USSR,<br>middle Volga<br>region | Middle early ripening<br>Low yield productivity<br>Comparatively large grain<br>Excellent elasticity of dough<br>Middle vitreousness associated with low milling yield<br>Highly drought tolerant |
| Janetzkis Probat<br>Donor | Germany<br>VIR collection<br>k-44923 | 3-5 days later then S29<br>More productive<br>Lower physical properties of dough than S29<br>High vitreousness<br>Sensitive to drought |

All the above mentioned traits are polygenic in nature; the controlling genes are situated at different chromosomes. Usually, sets of recombinant inbred lines derived from the inter-cultivar crosses are used for their investigation and precise mapping of associated QTL. However, part of the lines may simultaneously carry several loci determining the quantitative trait in their genomes. Assessment of the phenotypic contribution of separate loci in these cases may be difficult. The use of already created ISCS lines allows determining chromosome location of the responsible genes. On the second stage, after the development of SRD lines a precise mapping of one of the associated QTL on this chromosome is conducted.

During the previous investigation of the substitution lines (Khlestkina et al. 2010) it was unexpectedly found that in spite of 8 backcrosses the '4D-line' line still carry a fragment of chromosome 7A of the donor in addition to the 4D donor chromosome. So, the derived lines were recombinant for two chromosomes. Both chromosomes were genotyped with microsatellite markers and, additionally, on chromosome 7A genes for anthocyanin pigmentation of different plant organs were mapped (Khlestkina et al. 2010).

Observations of flowering date of ISCS lines in Europe and Siberia have showed that the line with 4D substitution delayed flowering in compare to the recipient S29 by 5-10 days. Study of the derived RSD lines under the same conditions allowed detecting two QTL in 4DS and 7AS chromosomes associated with the trait manifestation. The locus on 4DS chromosome was located near the marker *Xwmc0720* and manifested only in Bulgaria and Germany. Another

locus on chromosome 7AS was observed in all environments and mapped to the interval between microsatellite markers *Xgwm0060* and *Xgwm0974*. The differences in mapping results between two sites may be explained by different growing conditions. In Novosibirsk, the period from sowing till flowering (May-July) is uniform for day length. At the same time, in Germany and Bulgaria the first developmental phases of plants fall into March and April, when the day length is increasing. It may be supposed that QTL found on chromosome 4DS has a photoperiodic nature.

Physical properties of dough were studied in the parental cultivars and 4D*7A substitution line in Bulgaria and Russia. At both sites, the line demonstrated lower dough strength and lower tenacity (Table 2). SRD lines grown under the field conditions showed a wide variation for these traits with positive and negative transgressions. One major QTL in the same position for both traits was found on chromosome 4DL near to the marker *Xgwm 0165*. The donor of high physical properties of dough was S29.

Table 2: Physical properties of dough of cvs. S29, JP and ISCS line S29 (JP 4D*7A)

| Genotypes | Physical properties of dough | |
|---|---|---|
| | Dough strength | Tenacity |
| S29, Recipient | 496±43 | 160±8 |
| JP, Donor | 282±39*** | 98±12*** |
| S29(JP 4D*7A) | 254±29*** | 96±5*** |

***P<0.001, comparing to S29

Donor and recipient cultivars significantly differ in vitreousness of grain (81.0% vs. 89.9%, correspondingly). SRD lines were evaluated for this trait and a variation was found which was associated with QTL on 7AL chromosome in the region of the marker *Xgwm0870*. Interestingly, earlier the novel QTL was detected on chromosome 7AL for grain hardness using two wheat mapping populations (Wilkinson et al. 2008). Hardness and vitreousness of grain are known to be highly positively correlated.

Evaluation of S29 (JP 4D*7AL) SRD lines for resistance of seeds to sprouting was made in Gatersleben in the field under wet weather conditions. The parental cultivars did not differ significantly but the lines demonstrated variation for sprouting level (Table 3). Integration of phenotypic data into the linkage map resulted in obtaining the QTL in the region of centromere near the markers *Xwmc0048* and *Xwmc0720* associated with resistance to sprouting. The donor of high resistance to sprouting was the recipient cultivar S29. It should be mentioned that in ITMI mapping population of bread wheat QTL for this trait was identified on the homoeological chromosome 4AL in comparable position in the region of centromere near the marker *Xbcd402d* (Lohwasser et al. 2005).

An extensive phenotyping for yield was made at all three sites of growing in different years. Final yield evaluation consisted of the following separate components as plant height, main spike length and productivity, TGW, productivity of secondary tillers, etc. Several QTL common for remote growing sites were revealed using S29 (JP4D*7A) RSD lines. Donor and recipient are known to differ significantly for spike parameters (Table 4). The corresponding QTL associated with these two traits was detected in the region of the marker *Xgwm0974* on 7AS chromosome. The locus was significant in Novosibirsk, Sofia and Gatersleben. Another one was found in the region of genes for anthocyanin pigmentation of stem and leaf blade. It was associated with TGW and manifested in Novosibirsk and Sofia.

Table 3: Sprouting level of seeds in the parental cultivars and SRD S29 (JP 4D*7A) lines under field conditions in Gatersleben

| Resistance to sprouting | Cultivars and line | | |
|---|---|---|---|
| | S29, Recipient | JP, Donor | RSD lines, Range |
| Scores | 2.5 | 3.0 | 1.2-3.0 |

Table 4: Spike parameters of parental cultivars and ISCS line S29 (JP 4D*7A)

| Spike traits | Cultivars and line | | |
|---|---|---|---|
| | S29, Recipient | JP, Donor | S29 (JP 4D*7A) |
| Spike length, cm | 7.7±0.4 | 9.8±0.2** | 8.6±0.3* |
| Spikelet number | 13.4±0.5 | 17.5±0.3*** | 14.9±0.6* |

*P<0.05; **P<0.01; ***P<0.001, comparing to S29

Additionally, the full set of ISCS lines S29 (JP) is involved into phenotyping for activity of cell enzymes relevant to protection from oxidative stress and for verification of earlier detected QTL for grain quality and root system.

Many sets of ISCS lines were obtained in the previous century around the world. This kind of genetic material was obtained on the basis of recipient and donor cultivars well-investigated for their performance. Now the lines may be again involved into comprehensive phenotyping for different traits and the derived recombinant lines will be useful for mapping of new genes in wheat.

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# A new range of wheat precise genetic stocks application: insights into gene function

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The development of new and the exploitation of existing bread wheat precise genetic stocks such as aneuploid and chromosome substitution lines as well as introgression and near-isogenic lines is primarily aimed at gene localization. The rapid accumulation of methabolic pathways data (including genes, sequences, enzymes, metabolites etc.) provides the possibility of studying functions of wheat genes determining traits of interest. Precise genetic stocks may be suitable models to investigate just this issue and to establish functional differences between alleles having different effect on phenotype. In the current report, we demonstrate usefulness of the existing bread wheat precise genetic stocks and their derivatives for investigation of functional role of alleles determining coloration of different wheat organs by flavonoid pigments.

## Materials and methods

Analysis of the flavonoid biosynthesis structural genes transcription by RT-PCR (as described in Khlestkina et al. 2008) was performed in different tissues of wheat stocks carrying different alleles in the loci determining coloration of wheat organs: wheat near-isogenic (Arbuzova et al. 1998), substitution (Kuspira and Unrau, 1958; McFadden and Sears, 1947) and introgression lines (Pestsova et al. 2006); wheat-rye (Silkova et al. 2006) and wheat-barley (Trubacheeva et al. 2008, Pershina et al. 2009) chromosome substitution lines; wheat-Aegilops (Friebe et al. 1993, 1995, 2000) and wheat-rye (Driscoll and Sears 1971) chromosome addition lines.

## Results and discussion

Comparative analysis of expression of the key flavonoid biosynthesis structural genes (*F3h* and *Chi*) in glumes of near-isogenic and introgression lines having different *Rg* (red glume) alleles has led to a conclusion that the *Rg* gene encodes a factor which may play a regulatory role in biosynthesis of 3-deoxyanthocyanidin and phlobaphene pigments in glumes (Fig. 1). It was observed that the *Rg-A1c* and *Rg-D1b* alleles affected expression of the *Chi* gene, increasing level of its transcription in colored glumes, whereas *F3h* was expressed neither in white nor in colored glumes (Fig. 1). Chalcone-flavanone isomerase encoded by the gene *Chi* is involved in biosynthesis of several classes of pigmented flavonoids, from those only the 3-deoxyanthocyanidin/phlobaphenes branch doesn't involve flavanone-3-hydroxylase (Winkel-Shirley 2001, 2002). Similarly, investigation of near-isogenic lines differing in anthocyanin pigmentation of the coleoptile (*Rc*), culm (*Pc*), leaf sheath (*Pls*), leaf blade (*Plb*) and grain pericarp *Pp* (purple pericarp) suggested the genes *Rc*, *Pc*, *Pls*, *Plb*, and *Pp* to encode tissue-specific transcription factors regulating transcription of a number anthocyanin biosynthesis structural genes (Khlestkina et al. 2008, 2009, 2010; Tereshchenko et al. unpublished). A

regulatory role of the *R* genes (red grain) for transcription of some structural genes of flavonoid biosynthesis pathway in the seed coat was demonstrated (Himi et al. 2005; Himi and Noda 2005; Himi et al. 2011) using wheat near isogenic lines developed by Dr. S.F. Koval (1997).

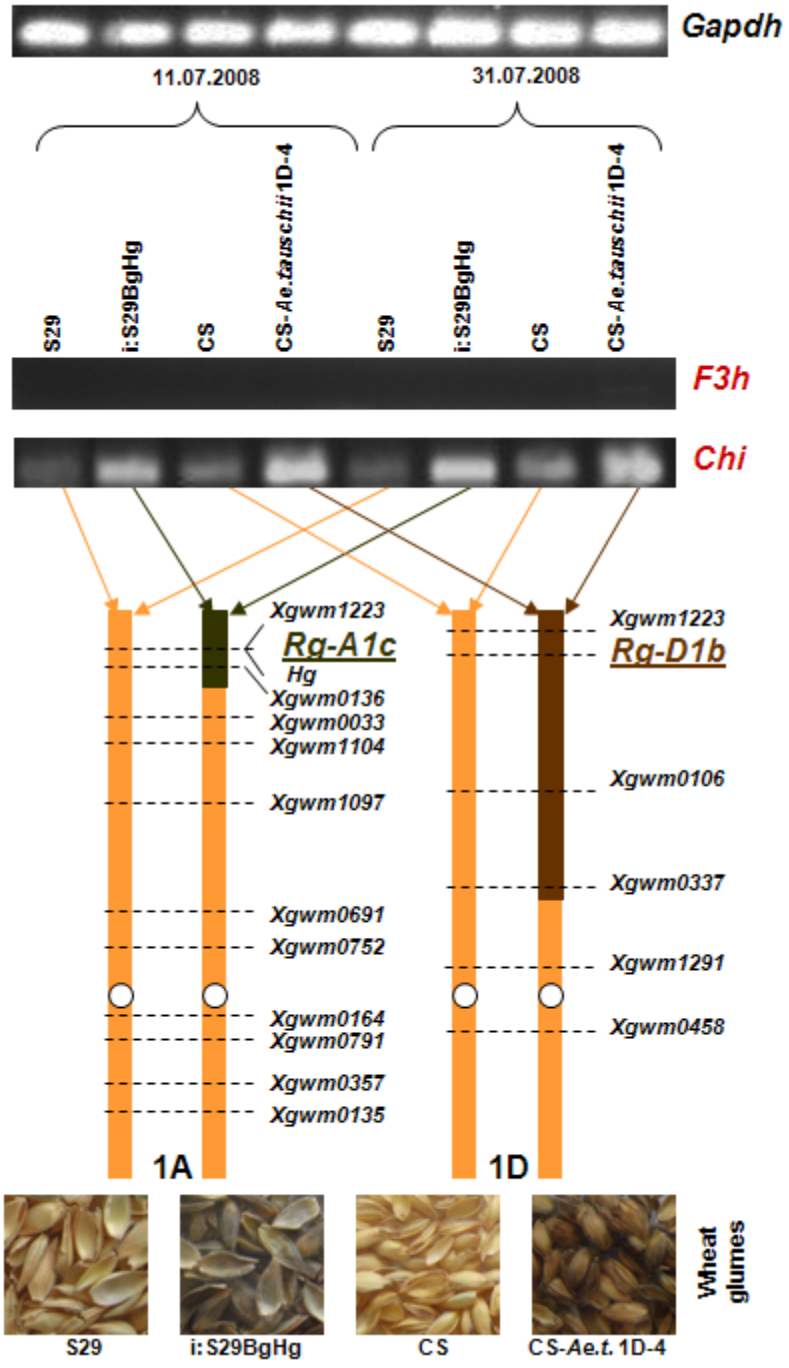


Fig. 1: Patterns of glume coloration and transcription of wheat *Chi* and *F3h* genes in glumes of near-isogenic and introgression lines. *Gapdh* - control for the RNAs amounts standardization. (S29 = 'Saratovskaya 29; i:S29BgHg = near-isogenic line carrying allele determining black glume color in S29 background; CS = 'Chinese Spring'; CS-Ae.t.1D-4 = introgression line CS-*Aegilops tauschii* 1D-4 carrying allele determining red glume color in CS background)

Using such precise genetic stocks as wheat-alien chromosome substitution and addition lines we investigated how genes function in a foreign background. In the wheat-rye chromosome substitution line 2R(2D), instead of wheat gene *F3h-D1* (2D) the rye *F3h-R1* gene (2R) is present, and anthocyanin pigmentation in the coleoptiles is controlled by the wheat *Rc* gene. Wheat *Rc* activated rye *F3h-R1* in coleoptiles along with wheat *F3h-A1* and *F3h-B1* (Khlestkina et al. 2009). Wheat-*Aegilops* chromosome addition 'Chinese Spring' ('CS')+*Ae. speltoides* 7S, 'CS'+ *Ae. longissima* 7S, 'CS'+ *Ae. searsii* 7S, and substitution 'CS'(*Ae.tauschii* 7D) lines, wheat-rye chromosome addition line 'CS'+ 'Imperial' 4R, and wheat-barley chromosome substitution line 'P28'(*H. marinum* 7Hm(7D)) have alien chromosomes carrying dominant alleles of the *Rc* gene and therefore their coleoptiles are colored (Fig. 2). Wheat 'CS' and 'P28' ('Pyrothrix 28') or control hybrid lines with additional chromosomes distinct from 7S or 4R have green coleoptiles (Fig. 2). It was shown that each of the alien *Rc* genes (if dominant) is able to activate wheat *F3h* gene (Fig. 2) (Khlestkina 2010). It can be concluded that within Triticeae species, alien homoeoalleles can compensate function of the missing wheat fuctional allele.

Thus, precise genetic stocks are suitable models to investigate molecular functions of wheat genes determining traits, to establish functional differences between alleles having different effect on phenotype and to investigated gene expression in a foreign background.

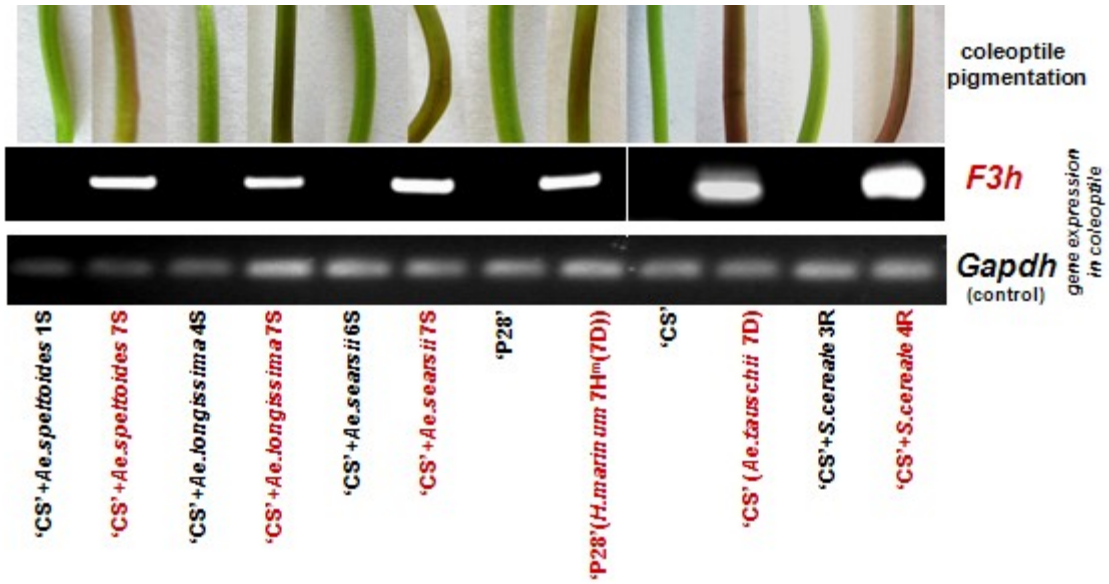


Fig. 2: Patterns of the coleoptile coloration and transcription of wheat *F3h* gene in wheat and wheat-alien lines. *Gapdh* - control for the RNAs amounts standardization


## Acknowledgements

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# Analysis of GA 2-oxidase gene transcription in isogenic lines of common wheat ‘Bezostaya 1’ with different *Rht* genes

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## Introduction

Lodging is one of the major problems in wheat production. It makes harvesting difficult and subsequently lead to poor grain quality and high yield losses. The main solution use for prevention from lodging is application of the chemical growth regulators and cultivation of the short-straw wheat forms. The most effective way of lodging resistant cultivars production is introduction of the dwarfing genes into wheat genetic background. The most popular genes utilized for straw length reduction in wheat breeding programmes worldwide are GA-insensitive *Rht-B1b* and *Rht-D1b* genes derived from ‘Norin 10’ and GA-sensitive gene *Rht8* derived from ‘Akakomugi’ (Borojević and Borojević 2005).

Examinations showed that some of the important dwarfing genes present in cereals genomes encoding modified enzymes involved in gibberellins biosynthesis pathway. One of the examples is *sd1* gene, which determines rice plants dwarfism. Plants containing this gene contain lower level of gibberellins in comparison to the wild type and are sensitive to

exogenous application of $GA_3$. Cloning and sequence analyses showed that *sd1* gene is a mutated allele of the GA20ox-2 gene, which encodes GA20 oxidase – one of the most important enzymes of the bioactive gibberellins biosynthesis (Ashikari et al. 2002). Recently it was shown that similar phenomenon is probable for *sdw1*/*denso* gene in barley (Jia et al. 2009).

Gibberellins (GAs) are one of the most important group of hormones determining plant development, especially important for stem elongation (Olszewski et al. 2002). Biosynthesis of the active gibberellins is a complex process, which proceed in three phases and is catalised by seven different enzymes found in chloroplasts, endoplasmic reticulum and cytosol (Yang et al. 2009). Biosynthesis of all plant gibberellins starts with conversion of the *trans*-geranylgeranyl diphosphate into *ent*-copalyl diphosphate by *ent*-copalyl diphosphate synthase (CPS), and afterwards into *ent*-kaurene by *ent*-kaurene synthase (KS) (Aach et al.1997, Yang et al. 2009). *Ent*-kaurene is subsequently transformed in number of oxidation reactions occur in cytosol into bioactive forms of gibberellins ($GA_1$, $GA_3$, $GA_4$, $GA_7$). The major enzymes involved in this process are GA20 oxidase (GA20ox) and GA3 oxidase (GA3ox). Important role in GAs biosynthesis plays also GA2 oxidase (GA2ox). This enzyme is responsible for transformation of the bioactive gibberellins into their non-bioactive forms by means of 2β-hydroxylation (Hedden and Phillips 2000).

Biological activity of gibberellins is dependent also on DELLA proteins, which repress plant reaction for bioactive GAs. Recent studies revealed that primary donors of cereals dwarfing genes contained spontaneous mutations within DELLA proteins encoding genes sequences (Hartweck 2008). Peng et al. (1999) showed that sequences of the wheat dwarfing genes *Rht-B1b* and *Rht-D1b* contain substitution caused occurrence of stop codon in the fragment encoding DELLA region. As a result plants show stable dwarf phenotype, independent on GA presence.

Hitherto majority of genes involved in gibberellins biosynthesis and metabolism have been identified and described (e.g. Hedden and Phillips 2000, Khlestkina et al. 2010). Nowadays many studies concerned their functional analysis and interactions. It is known that on gibberellins biosynthesis pathway genes expression is regulated by many different factors. The main way of regulation of gibberellins homeostasis in cell is feedback reaction. High concentration of gibberellins represses expression of genes encoding GA20ox and GA3ox enzymes and enhances the expression of the gene encoding GA2ox (Hedden and Phillips 2000).

The aim of presented examinations were determination of the differences in the level of GA2 oxidase encoding gene between isogenic lines of ‘Bezostaya 1’ wheat containing different dwarfing genes in genetic background.

## Material and methods

<u>*Plant material:*</u> Analyzed plant material contains a set of common wheat (*Triticum aestivum* L.) isogenic lines with different dwarfing genes in the genetic background of ‘Bezostaya 1’ cultivar. In examination four different dwarfing genes were included: *Rht-B1b*, *Rht-B1d*, *Rht-B1e* (GA-insensitive) and *Rht12* (GA-sensitive). As a control form we used tall ‘Bezostaya 1 *rht*’ line.

Kernels of analyzed forms were sterilized and placed on moisture filter paper on Petri dishes. Dishes were transferred to incubator (25ºC) for five days. All samples were prepared in two independent biological replications.

*RNA extraction:* Before isolation all plastic and glass equipment was rinsed with 0.1% DEPC solution. For RNA extraction whole five-day-old wheat seedlings were used. Immediately after harvesting plant material was frozen in liquid nitrogen and homogenized with mortar and pestle. For total RNA extraction a commercial kit 'GeneMATRIX Universal RNA Purification Kit' (EURx) was used according manufacturers protocol. The integrity of obtained RNA samples was checked by means of electrophoresis in 2% agarose gel with ethidium bromide. Obtained clear and sharp bands characteristic for 25S and 18S ribosomal RNA proved absence of nucleic acid degradation during extraction procedure. Concentration and purity of RNA were analyzed with NanoDrop 2000 spectrophotometer (Thermo Scientific). After measurement all samples were diluted to equal RNA concentration 100 ng/µl.

*Reverse transcription:* For reverse transcription of RNA to cDNA a commercial kit 'TaqMan® Reverse Transcription Reagents' (Applied Biosystems) was used. Reaction procedure was carried out according to the protocol suggested by reagents manufacturer with utilization of random hexamer primers. Concentration of obtained cDNA were checked with NanoDrop and equalized to 100 ng/µl.

*Real-time PCR:* Quantitative analysis in real-time PCR was based on SYBR® Green I fluorescent dye. Moreover for accuracy improvement additional reference dye ROX was utilized. All reactions were prepared with use of 'Brilliant II SYBR® Green QPCR Master Mix' (Agilent Technologies) kit according to protocol supplied with reagents. In our examinations we used Mx3005P apparatus with MxPro software (Agilent Technologies). Each sample was analyzed in three technical replications. Our studies were based on relative gene expression analysis according to $\Delta\Delta C_T$ method by Livak and Schmittgen (2001). As a template 100 ng of previously obtained cDNA were used and as a reference glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was utilized. For amplification a two sets of primers derived from previously published data were used. Sequences of primers used for real-time PCR were as follows; for GA2ox gene: 5'-TCGCTGCGCCTAAGCCACAG-3' and 5'-CTGCAACTCAAGCAGTCATCCCTC-3' (Zhang et al. 2007), and for GAPDH gene: 5'-CAACGCTAGCTGCACCACTA-3' and 5'-TTCCACCTCTCCAGTCCTTG-3' (Yin et al. 2009). Single reaction contains 45 cycles and SYBR® Green fluorescence measurement was conducted in each cycle after primers annealing step. After last cycle melting curve analysis was carried out to confirm specificity of obtained product. For both analyzed genes a single, explicit peaks were obtained what indicate at specific amplification.

## Results and discussion

Our analysis revealed that for all analyzed isogenic 'Bezostaya 1' lines with different *Rht* genes noticed GA2 oxidase transcript level was higher than in tall control line 'Bezostaya 1 *rht*'. The strongest expression of analyzed gene was observed for 'Bezostaya 1 *Rht12*' line – 1.22-fold increase in comparison to control form. The least GA2ox gene expression within tested lines was found for 'Bezostaya 1 *Rht-B1e*' line – 0.19-fold increase compared to control. Enhancement of analyzed gene expression in comparison to control tall line amounted to 0.95-fold change for 'Bezostaya 1 *Rht-B1b*' and 1.08-fold change for 'Bezostaya 1 *Rht-B1d*' (Figure 1).

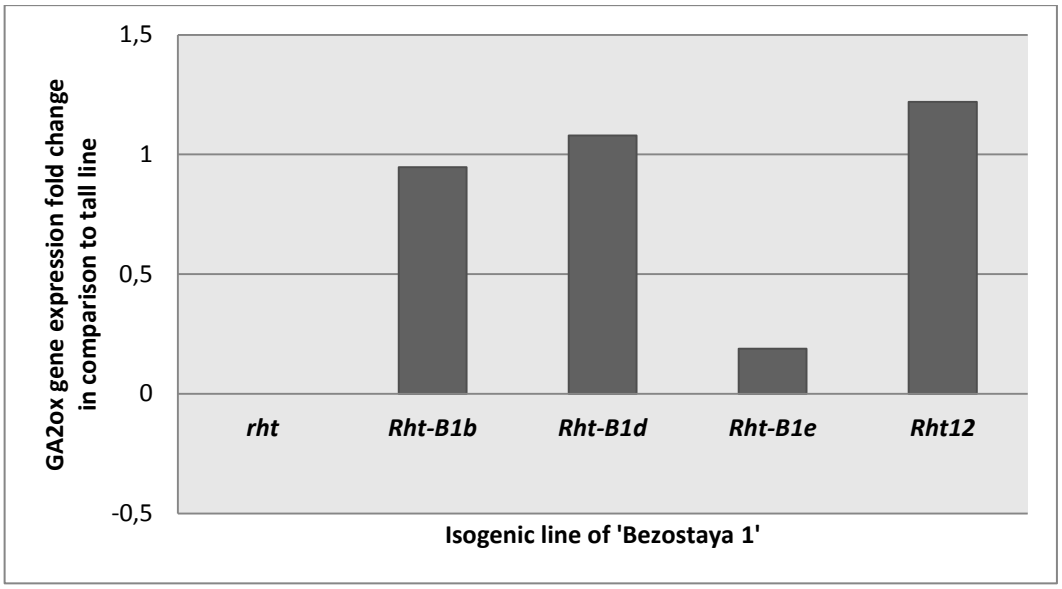


Fig. 1: Fold change of GA2 oxidase gene expression in 'Bezostaya 1' isogenic lines containing different dwarfing genes (in comparison to tall line)

Obtained results indicate that presence of the dwarfing genes in common wheat genome is connected with alteration of the gibberellin biosynthesis pathway genes expression. We showed that GA2 oxidase encoding gene transcription pattern was similar for analyzed wheat lines with exception of isogenic line containing *Rht-B1e* gene. Enhancement of expression of the GA2ox gene and subsequent enzyme production can play an important role in degradation of bioactive gibberellin forms by 2β-hydroxylation in wheat genotypes with *Rht-B1b*, *Rht-B1d* and *Rht12* genes. Relationship between expression of the GA2ox gene and dwarf phenotype has been previously revealed. Sakamoto et al. (2001) showed that expression of GA2ox encoding gene cDNA in transgenic rice plants caused deficiency in endogenous GA1 and inhibition of stem elongation. Recently Zhou et al. (2012) also revealed that over-expression of gibberellins 2-oxidase gene from *Arabidopsis thaliana* in *Brassica napus* lead to plant dwarfism.

## Conclusion

Our results support hypothesis that some dwarfing genes can be a modified genes encoding enzymes involved in gibberellin biosynthesis pathway. The activity of these enzymes can be based either on inhibition of bioactive gibberellins formation or their conversion into non-active forms what cause dwarf plant phenotype.

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## Evaluation of morphological and agronomic traits of wheat/barley introgression lines developed in Martonvásár

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Wheat (*Triticum aestivum* L.) and barley (*Hordeum vulga*re L.) are both important cereals worldwide. The intergeneric hybridization of these species makes it possible to transfer agronomically useful genes (drought tolerance, salt tolerance, earliness, nutritional parameters) from barley into wheat. The first successful hybridization between wheat and barley was reported by Kruse (1973) and not much later a set of wheat/barley addition lines was developed by Islam et al. (1978). The production of other wheat × barley hybrids was reported by several authors, but in most cases no backcross progenies were obtained. Barley has great genetic diversity for many agronomically important traits (spring or winter habit, two-rowed or six-rowed, tolerance to abiotic stresses, yield ability, earliness, quality, adaptation, etc.). In order to utilise the useful agronomic traits of barley cultivars it is necessary to produce wheat/barley addition and introgression lines with agronomically adaptable two- and six-rowed winter barley cultivars.

The aim of the experiments was to produce new wheat/barley addition lines with winter barley cultivars. In order to transfer chromosome segments from barley into wheat it was planned to select wheat/barley translocation lines. The identification of the introgresssion lines was carried out by combination of genomic *in situ* hybridization (GISH), fluorescent *in situ* hybridization (FISH) and molecular markers. The effect of the incorporated barley chromosomes (segments) on various morphological and agronomic traits was analysed under controlled environmental conditions in the phytotron and in the field. The plant height, tillering, spike characteristics, the salt-, Al- and drought tolerance, and nutritional parameters, especially the β- glucan content were analysed.

**Production and identification of new wheat/barley addition and translocation lines**

Wheat/barley addition lines were produced in two cultivar combinations: the 2H, 3H, 4H, 6HS, 7H disomic and 1HS isochromosomic Mv9kr1/Igri (German two-rowed winter barley cultivar) lines and the 2H, 3H, 4H, 6H and 7H Asakaze komugi/Manas (Ukrainian six-rowed winter barley cultivar) disomic addition lines (Molnár-Láng et al. 2005, Szakács and Molnár-Láng 2007, 2010; Molnár-Láng et al. 2012).

The presence of the barley chromosome pair in the wheat genome was detected using GISH. The barley chromosomes were identified using FISH by means of repetitive DNA probes (HvT01, pTa71, GAA, Afa family) (Fig. 1 a, b). The cytological identification was confirmed with molecular markers previously mapped on barley. The addition lines were multiplied in the phytotron and in the field. Most of the addition lines were genetically stable except the 1HS isochromosome Mv9kr1/Igri and the 3H Asakaze/Manas disomic additions. The reason of the instability of the 3H Asakaze/Manas addition is not yet known, and will be further investigated. The 3HS.3BL, 2DS.2DL-1HS, 6BS.6BL-4H, 7DL.7DS-5HS, 4D-5HS, 4BS.7HL, 5DL.4HL wheat/barley translocation lines were selected and identified with combination of GISH, FISH (Fig. 2 a,b) and SSR markers (Molnár-Láng et al. 2000, Nagy et al. 2002). New translocations carrying chromosomes segments of 3H, 6H and 7H were selected from the progenies of the Asakaze × Manas hybrids.

**Evaluation of various morphological and agronomic traits of wheat/barley introgression lines**

*Morphological characters:* Several morphological characters of the wheat/barley addition lines in both cultivar combinations (Mv9kr1/Igri; Asakaze/Manas) were evaluated in the phytotron and in the field. The 4H additions had the best fertility and 7H the lowest. The 2H addition line had a lax spike structure in both cultivar combinations. The 3H addition had the shortest, most compact spike of all the addition lines. The 4H addition line had the tallest plants and 3H the shortest. The 6H and 7H additions were shorter than the 4H. These characters were similar to that of the CS/Betzes addition lines reported by Islam et al. (1978). Unfortunately the 5H addition could not be selected neither from the Mv9kr1/Igri nor from the Asakaze/Manas combination, as this chromosome was eliminated most frequently from the backcross progenies (Molnár-Láng et al. 2005).

*Heading and flowering time of the addition lines:* The heading and the flowering time of the wheat/barley addition lines in two cultivar combinations (Mv9kr1/Igri, Asakaze/Manas) were studied in the phytotron and in the field. The wheat/barley addition lines were grown in the phytotron under three different environmental conditions, short day (12 hours) and long day (16 hours) illumination and without vernalization. The temperature was 15 °C during the day, and 10° C during the night until stem elongation, after that it was increased by 2°C. The 7H additions were the earliest in flowering and the 4H additions were the latest both in the field and in the phytotron. The difference in the flowering time between the 7H and the 4H Asakaze/Manas and the Mv9kr/Igri additions were 6 and 7 days in the field, respectively, which increased into 16 and 32 days under long day illumination in the phytotron. The difference in the flowering time of the 7H and 4H additions increased to 44 and 52 days, respectively, under short day illumation. The flowering time of 2H, 3H, 6H additions were between that of the 4H and 7H additions.

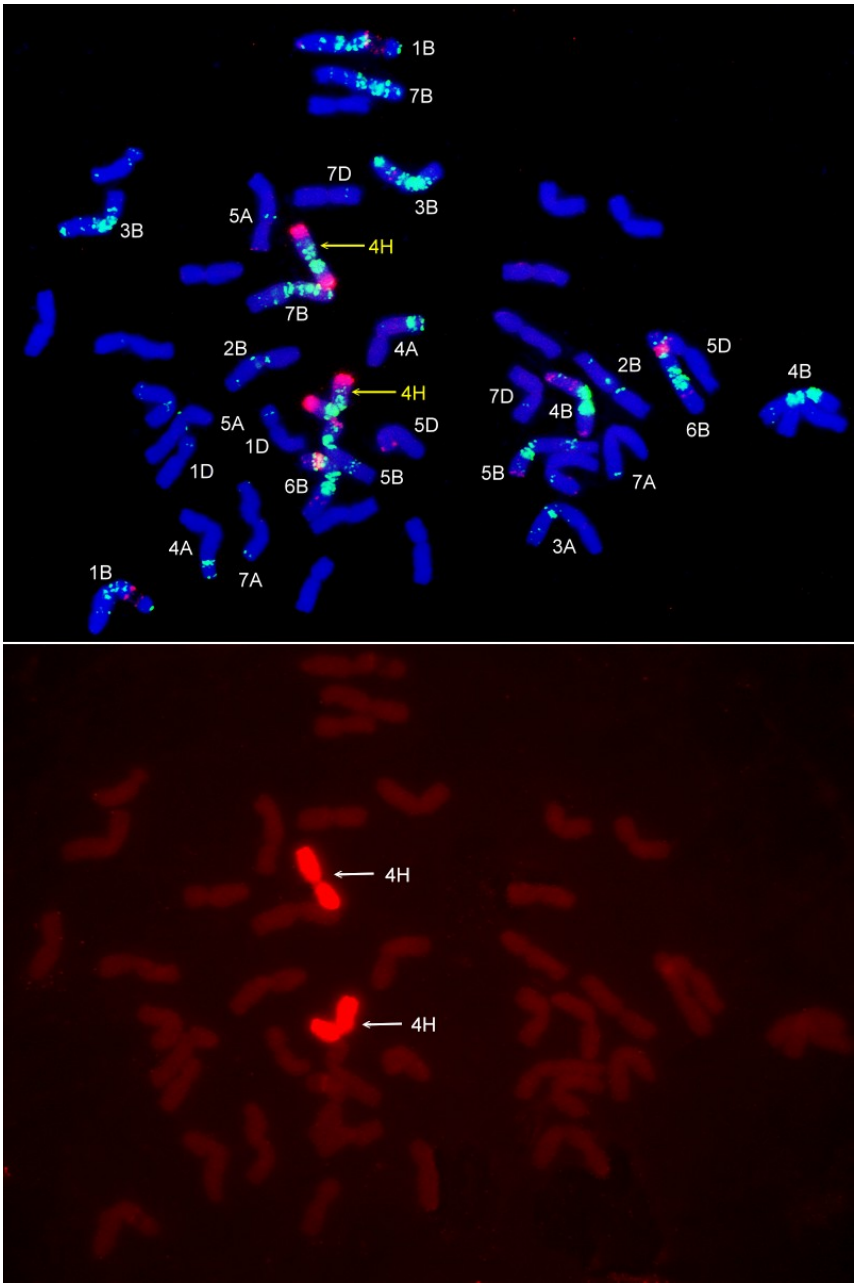


Fig. 1: Sequential FISH and GISH on mitotic chromosomes of 4H Mv9kr1/Igri wheat-barley disomic addition line.
a, Identification of the 4H barley chromosomes using DNA probes GAA (green), HvT01 (red) and pTa71 (orange) on the FISH image.
b, Barley chromosomes are red as a result of labelling the barley DNA with digoxigenin and were detected with antidig-Rhodamin on the GISH image. 4H barley chromosomes are indicated by arrows. Wheat chromosomes are unlabeled

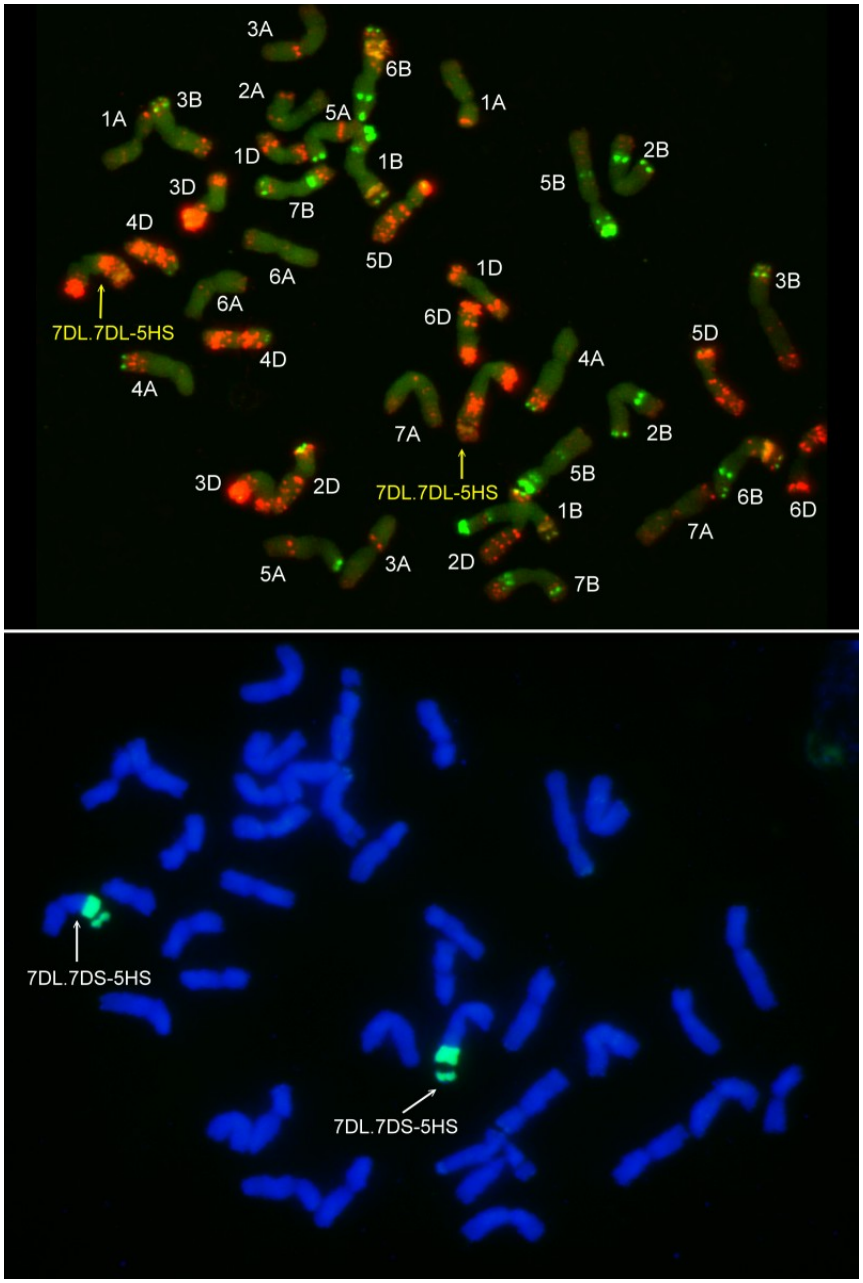


Fig. 2: Sequential GISH and FISH on mitotic chromosomes of 7DL.7DS-5HS wheat-barley disomic translocation line.
a, Identification of the wheat chromosomes using FISH with DNA probes pSc119.2 (green), Afa family (red) and pTa71 (yellow). Disomic 7DL.7DS-5HS translocated chromosomes are indicated by arrows.
b, Detection of barley chromosome segments (green) in the translocation chromosome pairs using GISH. Total barley DNA was labelled with biotin, and detected with streptavidin-FITC (green). The translocated chromosomes are indicated by arrows. The wheat chromosomes are blue as a result of counterstaining with DAPI

*The drought-, Al-, and salt-tolerance of the wheat/barley introgression lines:* The 2H, 3H, 4H Mv9kr1/Igri, 4H Asakaze/Manas, 2H Asakaze/Betzes and 6H Mv9kr1/Betzes addition lines, the 4H(4D) Mv9kr1/CS/Betzes substitution line, the 3HS.3BL, 2DS.2DL-1HS, 6BS.6BL-4HL Mv9kr1/Betzes and 7DL.7DS-5HS Mv9kr1/Igri translocation lines, and the wheat and barley parental genotypes were sown under a rain shelter simultaneously in Martonvásár and in Keszthely (UP Georgikon Faculty) for three years (2008-2010). Ten seeds of each genotype were sown in three replications in Martonvásár. Eleven genotypes were sown in a 15-m row and half of each row was covered with a plastic rain shelter from April until harvest in Keszthely. Heading time, plant height, tillering, fertility, thousand grain weight, seeds/main spike and seeds/plant and the root/shoot ratio were evaluated. The environmental factors, heavy storms and bird damage had a great influence on the results in Martonvásár, so the deviation was very large making it difficult to evaluate the data. The root length and the root/shoot ratio were evaluated in Keszthely. The 7D-5HS translocation and the 4H(4D) substitution line had the most favourable root/shoot ratio of the 11 lines analysed in Keszthely in the first year of the experiments. (Hoffmann et al. 2010).

The Al tolerance of the 17 lines also tested for drought tolerance in Martonvásár and of two control wheat cultivars (cv. Atlas, Scout) and two new Asakaze/Manas addition lines (6H and 7H) was studied by determining root growth in a solution containing 75 μM $AlCl_3$ at pH 4.0 and by root regrowth after haematoxilin staining. The results were compared to those obtained without $AlCl_3$ at pH 4.0 and 5.5. Manas had better salt and Al tolerance than Betzes or Igri. This was manifested in the Asakaze/Manas addition lines analysed (4H, 6H, 7H). The 2DS.2DL-1HS translocation line exhibited higher Al tolerance than the parental wheat line Mv9 kr1 (Darkó et al. 2010).

Salt stress was induced gradually in four-week-old plants through the addition of sodium chloride (100, 200 and 300mM NaCl $L^{-1}$) to the hydroculture medium. The photosynthetic $CO_2$ fixation, chlorophyll fluorescence quenching and relative water content (RWC) were measured to compare the salt tolerance of the genotypes. The 7H Asakaze/Manas addition line, similarly to the parental cv. Manas, was able to retain its $CO_2$ fixation rate during salt stress (up to 200 mmol $L^{-1}$ NaCl) with relatively high stomatal conductance, suggesting it had better tolerance to salt stress than the wheat genotypes (Dulai et al. 2010). The 2H, 3H and 4H Mv9kr1/Igri addition lines were very salt-sensitive.

*The protein, β-glucan content and amino acid composition of the wheat/barley introgression lines:* Various nutrional parameters (protein content, amino acid composition, β-glucan content) of the introgression lines were analysed. The protein content of the 2H, 3H, 4H Mv9kr1/Igri, the 4H Asakaze/Manas additions, the wheat/barley translocation lines (3HS.3BL, 2DS.2DL-1HS, 6BS.6BL-4H, 7DL.7DS-5HS) and the 4H(4D) substitution were studied. The protein content of the 3H Mv9kr1/Igri addition was higher than that of the 2H addition in two consecutive years. The amino acid composition of the 2H, 3H, 4H, 7H Mv9kr1/Igri and 4H, 6H, 7H Asakaze/Manas and the wheat/barley translocation lines together with the parental wheat and barley genotypes were analysed. The lysine content of Manas barley was higher than that of the wheat genotypes. The lysine content of the 7H Asakaze/Manas addition was also higher than that of the wheat parents. It is planned to repeat these measurements in the next future to make final conclusions from the data. The β-glucan content of the introgression lines was also determined. The average β-glucan levels were four times higher in the barley cultivars than in the wheat cultivars. The β-glucan level was significantly higher in the 7H addition and 4BS.7HL translocation lines containing the *HvCslF6* gene than in the control wheats (Cseh et al. 2011).

## Conclusion

The development of new wheat/barley addition lines with two-rowed and six-rowed winter barley cultivars (Igri, Manas) makes it possible to increase the allelic variation in wheat/barley introgression lines. The selection of ditelosomic additions from these lines is in progress, and those can be subjected to chromosome sorting by flow cytometry, thus allowing barley chromosome arms with an allele composition different from Betzes to be sequenced. The wheat/barley introgression lines made it possible to study the effect of the introgressed barley chromosomes (regions) on various morphological and agronomic characters of wheat.


## Acknowledgements

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# *Aegilops geniculata* chromosome introgressions into bread wheat and their effects on plant physiological responses to abiotic stress


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The wild relative of wheat *Aegilops geniculata* Roth (syn. *Ae. ovata* L.) is an allo-tetraploid species (2n=4x=28, genome formula $M^gM^gU^gU^g$) native to the Mediterranean region,

Southern Europe, the Aegaeis, North Africa, southern parts of Crimea, Near and Middle East, and western arc of the Fertile Crescent (van Slageren 1994). It is a potentially important source of genes for improving wheat earliness, disease resistance and tolerance to abiotic stress, including drought and mineral deficiencies (Zaharieva et al. 2001; Neelam et al. 2010). The establishment of wheat-alien chromosome substitution lines allows the study of genetic effects of the individual alien chromosomes in wheat background and their ability to compensate missing wheat chromosomes for phenotypic and physiological traits (Molnár et al. 2007). This work describes the production of wheat-*Aegilops geniculata* chromosome substitution lines, their cyto-molecular characterization, and the physiological responses of seedlings to induced osmotic stress and Fe-deficiency stress.

**Development of lines**

The development of wheat-*Ae. geniculata* substitution lines started with the production of a $F_1$ hybrid between a Dt5AL line of wheat cultivar Chinese Spring (CS) and an *Ae. geniculata* accession, kindly provided by the former Plant Breeding Institute, Cambridge, now John Innes Centre, Norwich, UK. Following treatment of $F_1$ plants with colchicine, an amphiploid was produced, which after few selfings was backcrossed twice or three times with CS (Ganeva et al. 1992). Several single or double monosomic additions were selected from which the corresponding additions were recovered. These were characterized cytologically using Giemsa N-banding (Landjeva and Ganeva 1999). After several self-pollinations of the addition lines, each time after selection for spike morphology, three lines with spontaneous wheat-alien substitutions were isolated (ADL-18, ADL-73 and ADL-107).

**Cyto-molecular and phenotypic characterization of lines**

To identify the alien chromatin in wheat-*Ae. geniculata* hybrid lines, sequential multicolour fluorescence *in situ* hybridization (FISH) and genomic *in situ* hybridization (GISH) were applied on mitotic metaphase chromosome spreads. FISH was carried out using three DNA repetitive sequences: pSc119.2 (labelled with digoxigenin-11-dUTP), Afa-family (labelled with biotin-16-dUTP) and pTa71 (labelled with a combination of digoxigenin-11-dUTP and biotin-16-dUTP). To determine the genomic affiliation of the alien chromosomes, GISH was carried out on the same chromosome preparations using total genomic DNA of the two diploid progenitor species, *Ae. umbellulata* (labelled with digoxigenin-11-dUTP) and *Ae. comosa* (labelled with biotin-16-dUTP), as U- and M-genomic probes, respectively.

*Line ADL-73:* Line ADL-73 (2n=42) was identified as $2M^g$(2A) disomic chromosome substitution. The wheat chromosome 2A, characterized with weak Afa signals at the telomeric and centromeric sites on the short arm was absent, and two alien submetacentric chromosomes with specific FISH patterns were identified in all plants analyzed (Fig. 1a). The alien chromosomes had strong pSc119.2 and slight pTa71 telomeric signals on both short and long arms, subtelomeric Afa signals on the long arm and an intercalary Afa band on the short arm. The overall distribution of the hybridization signals was similar to those of chromosomes 2M of *Ae. geniculata* and *Ae. comosa* described earlier (Molnár et al. 2011) and to that of the added alien chromosome in the wheat-*Ae. geniculata* $2M^g$ disomic addition line (kindly provided by Dr. B. Friebe, Kansas State University, USA). The GISH analysis verified the M-genome affiliation of the substituted chromosome. The phenotypic effects of the wheat-alien $2M^g$(2A) chromosome substitution were evidenced in the altered spike and seed morphology. The spike of the substitution line was shorter and more compact in the upper half, compared

with that of wheat CS. The seed had specific characteristics: the lemma, and especially the palea, tended to adhere slightly to the pericarp epidermis of the grain. The plants of the substitution line headed earlier, produced more tillers, but shorter culms and spikes, and had fewer spikelets per spike and poorer fertility as compared to wheat CS (Landjeva et al. 2012).

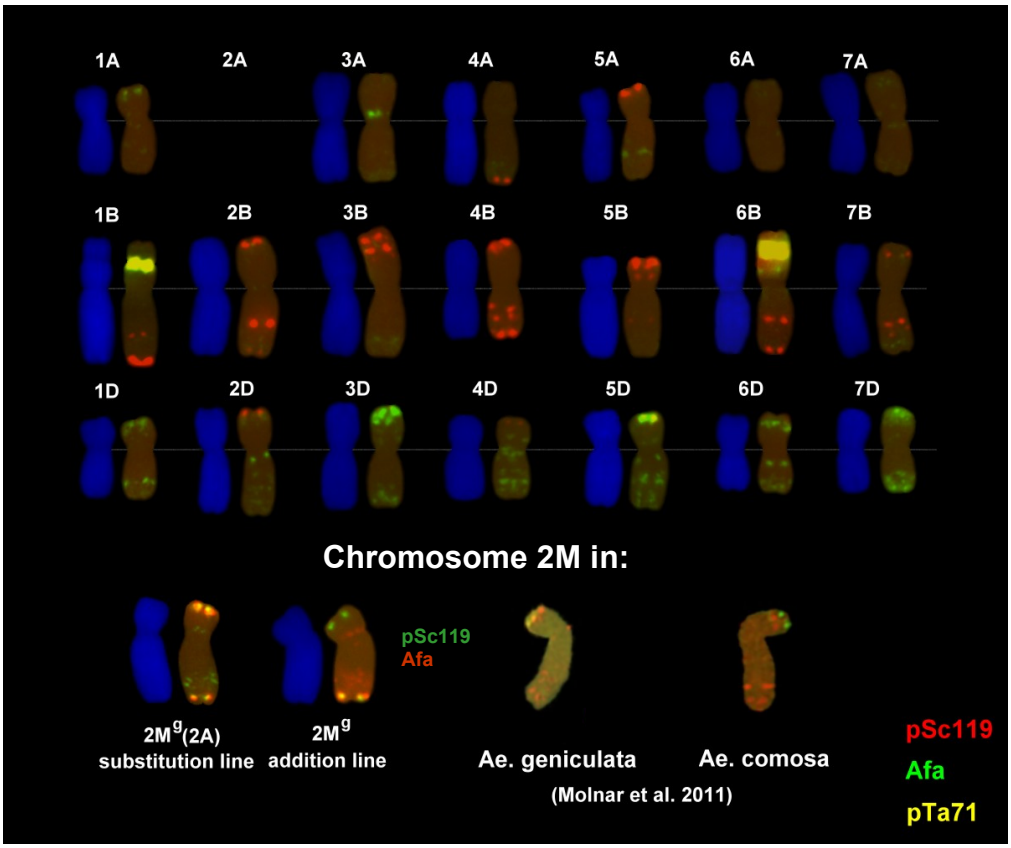
*Line ADL-107:* Line ADL-107 was a mixture of plants with $5U^g$(5B) disomic substitution (2n=42), and plants with incomplete $5U^g$(5B) substitution (2n=42+t) (Fig. 1b). The alien pair of chromosomes was identified as $5U^g$ based on the chromosome morphology (submetacentric with satellites on the short arms) and FISH pattern (strong pTa71 signals on the satellites). The U-genome affiliation was further confirmed using GISH. The wheat chromosome 5B was absent, but some plants (2n=42+t) carried a 5BL telocentric. In some plants, a wheat-wheat translocation involving chromosomes 6D and 7A was suggested based on their FISH patterns as compared to the standard 6D and 7A of wheat CS (Fig. 1b). The spikes of the $5U^g$(5B) substitution line were lax at bottom and compact at top with highly reduced fertility. Increased susceptibility to naturally occurring powdery mildew was consistently observed on plants of this line.

*Line ADL-18:* Line ADL-18 was earlier described as a $3U^g$(3D) disomic substitution based on Giemsa N-banding analysis (Landjeva et al. 1998) and biochemical diagnostic approach (Stoilova 2002). After several self-pollinations of ADL-18, three sub-lines (18-1, 18-3 and 18-6) were isolated, all having different spike characteristics regarding shape, length, spikelet density and presence of awns. The sequential FISH and GISH of the three sub-lines did not detect any alien chromosome or wheat-alien translocations. However, rearrangements in wheat chromosomes 2B, 4A and 5D were suggested based on the FISH patterns (Fig. 2). Compared to CS, the terminal pSc119 signal in 2BL was absent in either one (18-3) or in both homologues of chromosome 2B (18-6). Also, in two sub-lines (18-1 and 18-3), the integrity of chromosome 4A was disturbed and one telocentric 4AL was observed in all studied plants of these sub-lines. In sub-line 18-3, the pTa71 signal on 5DS was extremely strong compared to CS and the rest of sub-lines.

**Physiological responses to osmotic stress**

Much of the injury to plants experiencing drought stress, especially at early developmental stages, is associated with osmotic stress. At cellular level, this triggers oxidative stress, which is indicated by the accumulation of hydrogen peroxide ($H_2O_2$) and malondialdehyde (MDA), and is manifested as dysfunction of cell membranes and ion leakage from damaged tissues. Seedlings of line ADL-73 and the wheat cultivar CS (as control) were treated with 15% PEG dissolved in nutrient solution for 8 days (Landjeva et al. 2012). Compared to wheat CS, the plants of the wheat-alien line retained higher values of RWC in the leaves, suggesting that the replacement of wheat chromosome 2A with $2M^g$ led to retention of better water balance in the leaves. The less pronounced increase in MDA in the line implied reduced lipid peroxidation and less severe membrane damage. The greater increase in $H_2O_2$ concentration accompanied by lower MDA accumulation in stressed leaves of the substitution line compared to CS suggested differences in the efficiency of the antioxidant defence system in the two genotypes. The latter observation was consistent with the significantly lower cell membrane injury index in the substitution line compared to that in CS.

a)




b)

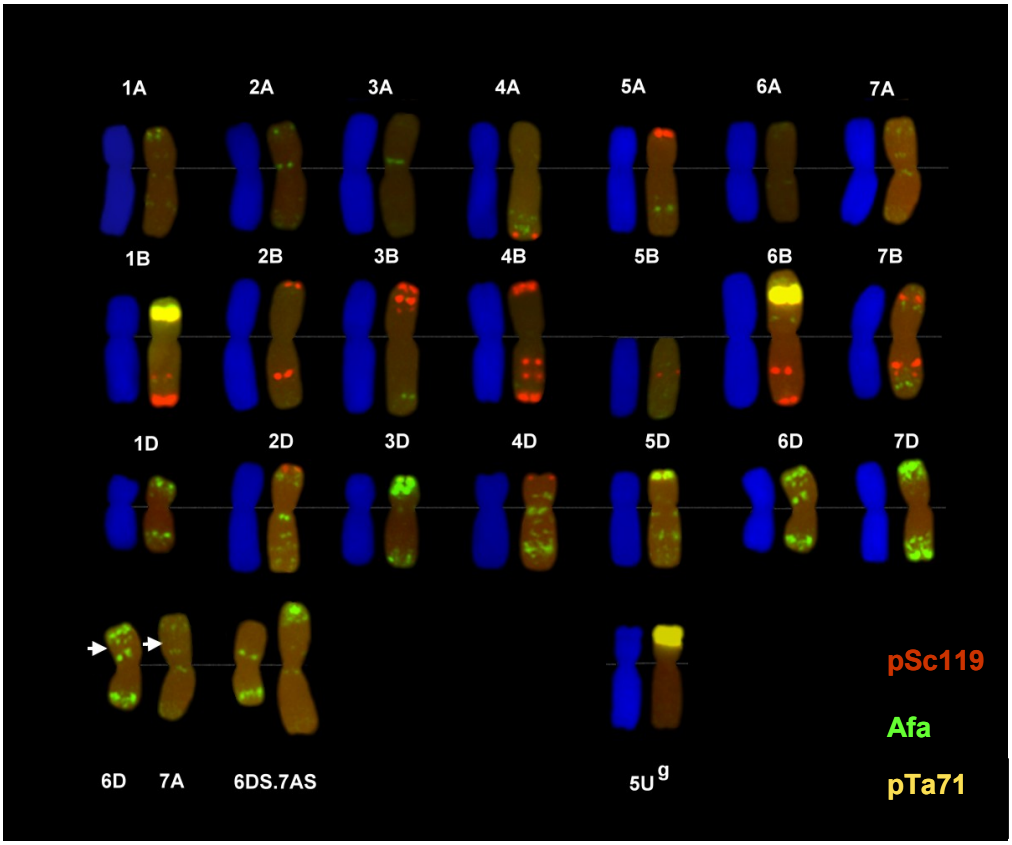



Fig. 1: FISH karyograms of wheat-*Aegilops geniculata* chromosome substitution lines a) $2M^g(2A)$; b) $5U^g(5B)$. For details, see the text

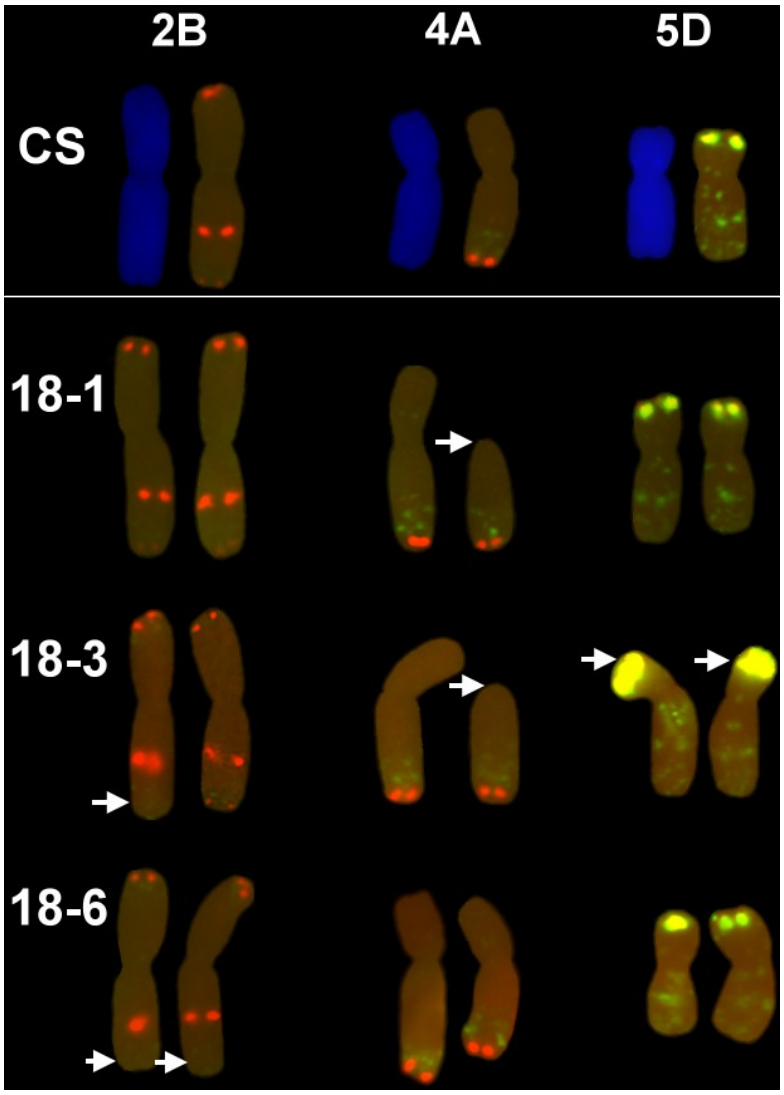



Fig. 2: Comparison of FISH patterns of wheat chromosomes 2B, 4A and 5D in cv. 'Chinese Spring' (CS) and three wheat CS - *Ae-geniculata* hybrid lines, derived from a $3U^g$(3D) substitution line. Arrows point to the differences from CS

The analysis of the electrolyte leakage kinetics showed lower values of relative conductivity under stress in the substitution line and signified the minor effect of PEG treatment on cell membranes in this line. A new parameter, 'period *T* of the prompt phase', estimated from the kinetics of electrolyte leakage has been recently introduced (Kocheva et al. 2005). It describes the contribution of the apoplast to the process of ion efflux from tissues. The faster ion release from the apoplast, indicated by lower *T* values, could be regarded as a result of greater membrane damage and/or altered permeability. In the present study, the less pronounced decrease in *T* in the substitution line (relative to the control) suggests better ability to preserve cell membrane stability.

In conclusion, this study showed that the *Ae. geniculata* chromosome $2M^g$ compensates for the missing wheat chromosome 2A and contributes to the better maintenance of water status and cell membrane stability in osmotically stressed seedling leaves.

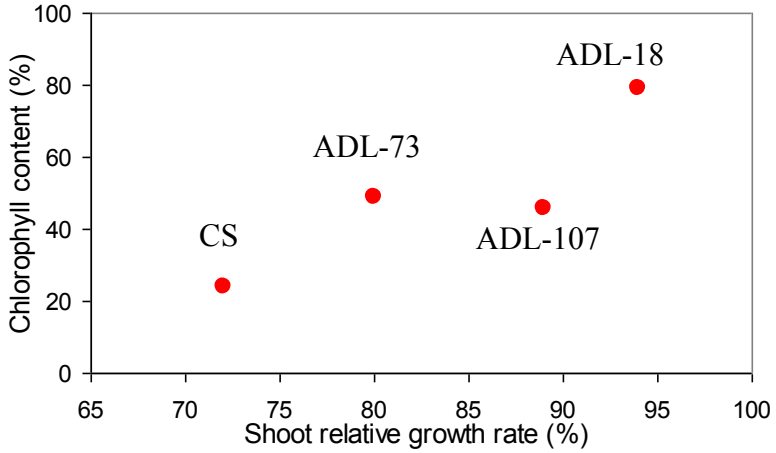
**Physiological responses to Fe deficiency**

Fe deficiency is reported mainly in calcareous soils in arid and semi-arid regions of the world as well as in some sandy soils with low organic matter and high permeability, compact soils with restricted aeration and in soils with excess contents of heavy metals and phosphorus. Chlorophyll synthesis, chloroplast development, and meristematic growth are very sensitive to Fe supply. Hence, its deficiency causes chlorosis, disturbs the normal functioning of the photosynthetic apparatus and leads to growth depression. Development of more Fe-efficient cultivars appears as a promising method to correct Fe chlorosis. Recently, some *Aegilops* species, including *Ae. geniculata* were reported to have 3-4 times higher release of phytosiderophores in Fe-deficient conditions to increase Fe uptake compared to wheat (Neelam et al. 2010) and, therefore, merit attention to be further studied and used for wheat improvement.

Seedlings of all wheat-*Ae. geniculata* substitution lines (ADL-18, ADL-73 and ADL-107), the three sub-lines of ADL-18, and CS as control were tested under Fe sufficient (100 μM of Fe, supplied as FeEDTA) and Fe deficient (1 μM of Fe) conditions. The effects of Fe supply were assessed on 10- and 17-day-old seedlings by measuring root and shoot growth and growth rate, chlorophyll content and chlorophyll fluorescence parameters to estimate the effects of stress on photosystem II (PS II) efficiency. The differences among the lines were more pronounced on day 17. The Fe deficiency caused root length reduction in all genotypes, CS being the most affected one. Shoot growth, as assessed by the changes of shoot length and shoot dry biomass, were not depressed in the substitution lines, but was significantly inhibited in CS. The relative shoot growth rate between day 10 and day 17 was decreased in the least degree in line ADL-18. The latter was the most tolerant to Fe deficiency according to the decrease of chlorophyll content of the second leaf (Fig. 3a) and the chlorophyll fluorescence measurements. This implied most preserved activity of PS II in ADL-18, whereas the PS II of CS was disturbed at highest degree. In sub-lines 18-1, 18-3 and 18-6, seedling growth under Fe deficiency was equally or even more severely depressed compared to CS. However, according to the relative shoot growth rate, degree of chlorophyll decrease and functioning of PS II in Fe-chlorotic plants, sub-line 18-3 was the least affected (Fig. 3b). The chlorophyll fluorescence parameters in Fe-chlorotic plants of sub-line 18-6 were not affected compared to the control plants suggesting preserved activity of PS II (data not shown).

The two sub-lines of better tolerance than CS as evidenced by the effects on relative shoot growth, chlorophyll content and/or chlorophyll fluorescence (18-3 and 18-6) differed from CS by a modification in the terminal bin of 2BL, where a candidate gene for ferrichrome-iron receptor was mapped (http://wheat.pw.usda.gov/pubs/2004/Genetics/Bioinfo/). This is a protein required for the transport of iron from the cell surface into the cell in some microorganisms. In addition, the most tolerant sub-line (18-3) differed from CS by modifications in the other two rearranged wheat chromosomes, 4A and 5D, and had strongly altered spike appearance. The results of this study do not associate the observed better tolerance of the wheat-*Ae. geniculata* hybrid lines to Fe-deficiency with individual alien chromosome. However, they suggest that the presence of alien chromatin in the wheat genome mediates the occurrence of rearrangements in wheat chromosomes that could possibly have effects on some biochemical and physiological processes in plants.

**a)**




**b)**

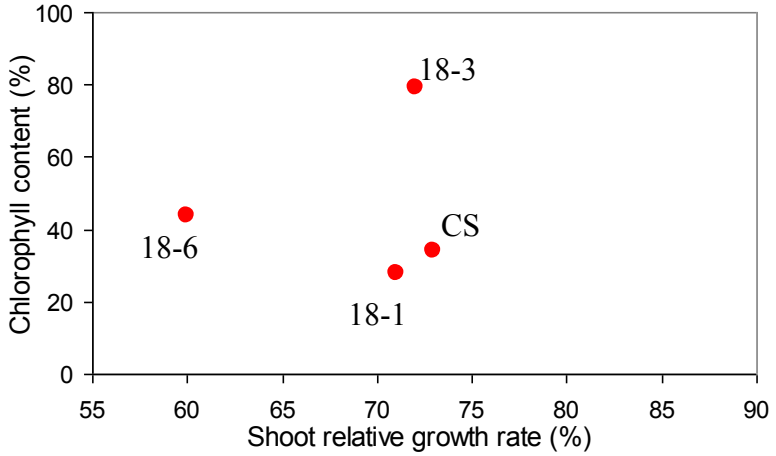



Fig. 3: Tolerance to Fe deficiency in the nutrient solution in wheat cultivar Chinese Spring (CS) and three wheat-*Aegilops geniculata* substitution lines (a), and in three ADL-18-derived sub-lines (b) expressed as the relationship between chlorophyll content in the 2$^{nd}$ leaf at day 17 and relative growth rate between days 10-17. Both indices are expressed as percentage of the values under 1 µM Fe to the values under 100 µM Fe (control)

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# Flow cytometric sorting of the U- and M-genome chromosomes facilitates physical mapping in *Aegilops* species

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Species of genus *Aegilops* represent a valuable reservoir of useful alleles of genes that would be desirable to transfer into wheat by interspecific hybridization. The allopolyploid *Ae. biuncialis* Vis. (2n = 4x = 28, $U^bU^bM^bM^b$) and *Ae. geniculata* Roth. (2n = 4x = 28, $U^gU^gM^gM^g$) originated from the hybridization of diploid *Ae. comosa* Sm. in Sibth. & Sm. (2n = 2x = 14, MM) and *Ae. umbellulata* Zhuk. (2n = 2x = 14, UU). Several resistance genes to rusts and powdery mildew (*Lr9, Lr57, Sr34, Yr8, Yr40, Pm 29*) have been transferred into wheat from these species (Friebe et al. 1996; Schneider et al. 2008) and tolerance to salt, drought, frost and heat stress was also observed in their genotypes (Waines et al. 1993; Colmer et al. 2006; Molnár et al. 2004, Dulai et al. 2005).

The introgression of favourable agronomic traits from *Aegilops* to wheat remains difficult because the characterization of *T. aestivum* - *Aegilops* introgression lines by molecular cytogenetic methods is time consuming. A small number of available U and M genome-specific molecular markers (Peil et al. 1998; Nagy et al. 2006; Schneider et al. 2010) limits the potential of marker-assisted selection of *T. aestivum* - *Aegilops* introgression lines.

Species within families have a common set of orthologous genes. Comparative genomics and phylogenetic studies across *Triticeae* and *Aegilops* taxa identified a set of genes conserved throughout evolution in both sequence and copy number. This set of >1000 conserved genes, which are referred to as conserved ortholog set (COS) markers, could be used efficiently for anchoring the *Aegilops* chromosomes and wheat genetic maps to each other as well as to the rice and *Brachypodium* maps and sequences.

Dissecting nuclear genomes into particular chromosomes by flow-cytometric sorting facilitates physical mapping, targeted development of molecular markers and integration of genetic and physical maps (Doležel et al. 2007). As the technology would greatly aid in transferring genes from wild relatives to cultivated wheat, we set out to explore a possibility of isolating by flow sorting individual chromosomes from *Ae. umbellulata* and *Ae. comosa* and from their natural allotetraploid hybrids *Ae. biuncialis* and *Ae. geniculata*. Chromosomes were sorted from individual peaks of flow karyotypes and were identified by FISH with a set of repetitive DNA probes. DNA amplified from purified chromosomes and isolated from different wheat-*Aegilops* addition lines was used as a template for PCR with previously developed COS markers with the aim to identify their genomic location in the *Aegilops* species.

## Materials and Methods

Flow cytometric analysis and chromosome sorting from each peaks of the flow karyotypes were carried out from *Ae. umbellulata* (MvGB470), *Ae. comosa* (MvGB1039), *Ae. biuncialis* (MvGB382) and *Ae. geniculata* (AE1311/00) as described by Molnár et al. (2011). The chromosome content of the peaks was accurately determined by molecular cytogenetic methods (FISH).

The following wheat-*Aegilops* genetic stocks along with their parental genotypes were used. Wheat (Chinese Spring) / *Ae. umbellulata* (JIC2010001) addition lines 1U, 2U, 4U, 5U, 6U, 7U and the wheat (Chinese Spring) / *Ae. comosa* (JIC2110001) addition lines 2M, 3M, 4M, 5M, 6M, 7M, and the substitution 6M(6A) were supplied from the John Innes Centre germplasm collection, Norwich, UK. Wheat(Mv9kr1) / *Ae.biuncialis* (MvGB642) addition lines $1U^b$, $1U^b6U^b$, $3U^b$, $2M^b$, $3M^b$, $7M^b$, the substitution $3M^b$(4B) and the centric fusion $3M^b$.4BS (Schneider et al. 2005). Wheat (Chinese Spring) - *Ae. geniculata* (TA2899) addition lines $1U^g$, $2U^g$, $3U^g$, $4U^g$, $5U^g$, $6U^g$, $7U^g$, $1M^g$, $2M^g$, $3M^g$, $5M^g$, $6M^g$, $7M^g$ (Friebe et al. 1999).

DNA preparation from the wheat - *Aegilops* genetic stocks and the parental genotypes and genotyping were carried out as described by Howard et al. (2011). Multiple displacement amplification of DNA of chromosomes sorted from individual peaks on flow karyotypes was carried out according to Šimková et al. (2008). Markers covering the wheat homeologous group I - VII and the rice chromosomes 1, 3 - 11 were chosen from two publically available COS marker collections, the Wheat Genetic Improvement Network (WGIN) (http://www.wgin.org.uk/resources/Markers/ TAmarkers.php) and Tools and Resources (TR) collections (http://www.modelcrop.org/cgi-bin/gbrowse/brachyv1/).

## Chromosome isolation by flow sorting in *Aegilops* species

Flow karyotypes obtained after the analysis of DAPI-stained chromosomes were characterized and content of chromosome peaks was determined (Fig. 1). In *Ae. umbellulata*, peak I corresponded to chromosome 1U (>95%). Peaks II and III predominantly contained chromosomes 6U (74.1%) and 3U (86.4%), respectively. As expected, the composite peak IV represented chromosomes 2U, 4U, 5U and 7U. In *Ae. comosa*, peak I contained chromosomes 1M and 4M as the main fractions, while the peaks II and III, which were closely spaced, represented mainly chromosomes 6M and 5M, respectively. Chromosome 2M was present in fractions sorted from both composite peaks (II and III). Finally, peak IV contained chromosomes 3M and 7M.

Significant differences were found between the flow karyotypes of allotetraploid *Ae. biuncialis* ($U^bU^bM^bM^b$) and *Ae. geniculata* ($U^gU^gM^gM^g$). In *Ae. biuncialis*, chromosome $1U^b$ could be sorted at high purities (>95%) as it was represented by well separated peak I. Peak II contained chromosomes $2M^b$, $3M^b$, $4M^b$, $6M^b$, $3U^b$, and $6U^b$, while peak III represented chromosomes $1M^b$, $3M^b$, $5M^b$, $2U^b$, $4U^b$, $5U^b$ and $7U^b$. Chromosome $3M^b$ was observed in these two peaks with similar frequency. Peak IV represented largely chromosome $7M^b$, which could be sorted at purities exceeding 80%. Peak I of *Ae. geniculata* contained chromosomes $1U^g$ and $6M^g$, while the peak II represented chromosomes $3U^g$, $4U^g$ and $6U^g$. Chromosomes $2M^g$, $4M^g$, $5M^g$, $2U^g$, $5U^g$ and $7U^g$ were found in peak III, while the largest chromosomes $1M^g$, $3M^g$ and $7M^g$ were represented by peak IV.

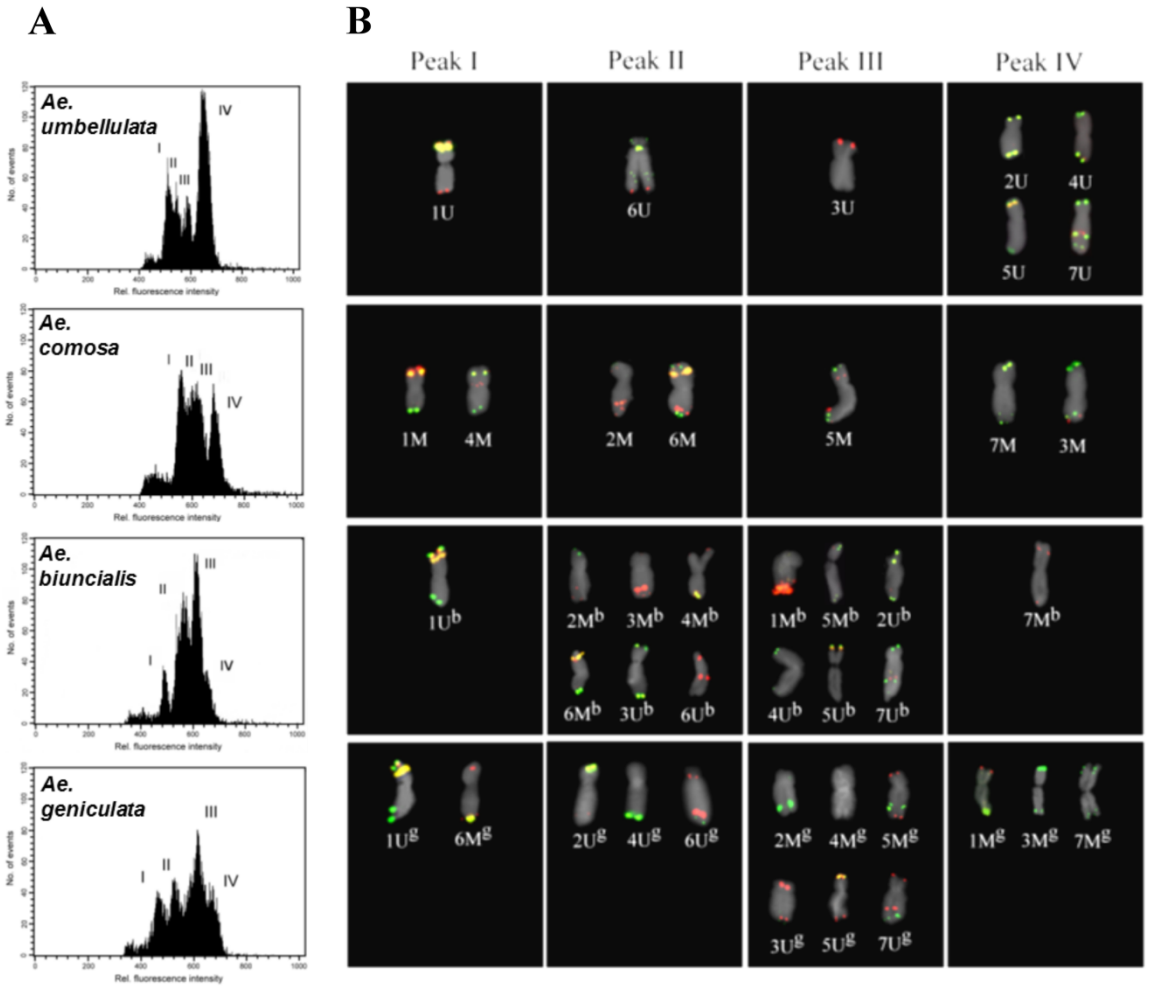


Fig. 1: Flow-karyotypes (A) and the chromosomes sorted from the peaks on flow-karyotypes of *Ae. umbellulata*, *Ae. comosa*, *Ae. biuncialis* and *Ae. geniculata*

**Chromosomal location of COS markers in the *Aegilops* species**

141 COS markers specific for different ESTs were tested. Out of the 141 markers, 100 markers could be located on at least one *Aegilops* chromosome. Most of the markers (78) could be identified on the wheat-*Aegilops* addition lines by the length polymorphism between the parental wheat and *Aegilops* genotypes. The genomic position of the markers could be identified with the help of their dominant presence in one of the peaks of the flow karyotypes. Comparing the quantity of PCR product in the peaks of the flow karyotypes, a significant number (22) of non-polymorphic markers could also be located on *Aegilops* chromosomes. The *Aegilops* chromosome-specific markers with significant level (≥2bp) of polymorfism between the parental wheat and *Aegilops* genotypes were considered as suitable for the marker assisted selection of new wheat-*Aegilops* introgression lines (Table 1). At the present there are markers specific for 12 out of the 14 chromosomes of *Ae. biuncialis* and *Ae. geniculata*.

Most of the COS markers used in this study are specific for expressed sequence tags (ESTs) previously bin-mapped on wheat chromosomes. This opens the way for the examination of large scale macrosynteny between the chromosomes of wheat and the U and M genome chromosomes of diploid and tetraploid *Aegilops* species. Figure 2 shows the chromosomal

location of COS markers on D-genome chromosomes of wheat and on the U and M chromosomes of the four *Aegilops* species. Most of the markers located on the 1D and 3D chromosomes were also detected on the group 1 and 3 chromosomes of the *Aegilops* species, indicating a close homeolougos relationship between these wheat and *Aegilops* chromosomes.

Table 1: COS markers suitable for marker assisted introgression of the U and M genome chromosomes from diploid progenitors *Ae. umbellulata* (UU) and *Ae. comosa* (MM) and from *Ae. biuncialis* and *Ae. geniculata*.

| | diploid progenitors | *Ae. biuncialis* | *Ae. geniculata* |
|---|---|---|---|
| 1U | *2B* | *1B, 2B* | *1B, 2B* |
| 2U | *2N*, 2P*, 2U*, TR146* | *2N*, TR146** | *2N, TR146, 2U, 2P* |
| 3U | *3J, TR62, TR63, TR80*, TR83*,* | *3J, TR62, TR63, TR77, TR80, TR83* | *3J, TR62, TR63, TR80, TR83* |
| 4U | *TR72, TR76, TR92, TR102, TR103* | *TR72*, TR76*, TR92*, TR102*, TR103*,* | *TR72, TR76, TR92, TR129, 5M, 6J, TR102,* |
| 5U | *5I*, 5Q*, TR128, TR131* | *5I*, 5Q*, TR128*, TR131** | *5I, 5Q, TR128, TR131* |
| 6U | *2I*, 4C, 6A, TR91* | *2U, 2I, 4C, 6A, TR91* | *4C*, 6A*, TR91** |
| 7U | *3B*, 7C*, 7I, TR4* | *3B*, 7C*, 7I*, TR4* | *6A, 3B, 7C, 7I*, TR4* |
| 1M | | | |
| 2M | *2R*, TR146, TR451* | *2R*, TR146, TR451** | *TR146, 2U, 2R, 2I, TR451* |
| 3M | *TR80* | | |
| 4M | *TR88* | *TR88*,* | *TR88*,* |
| 5M | *5Q*, TR128* | *5Q*, TR128** | *5Q, TR128, 5A* |
| 6M | *TR93, TR103, TR104* | *TR93*, TR103*, TR104** | *6J, TR93, TR103, TR104* |
| 7M | *7I* | *6A, 7C, 7I* | *5M, 6A, 7I* |

*: Specific PCR product were present, but the chromosomal location was not identifid due to the lack of addition line.

In contrast with this observation, a high number of markers specific for group 2, 4, 5, 6 and 7 chromosomes of wheat were located on different homeologous groups in the *Aegilops* species, wich means that significant genome rearrangements occurred in the U and M genomes relative to wheat since the evolutionary divergence of wheat and *Aegilops* genomes. Intragenomic chromosome rearrangements were previuosly reported in the U genome of *Ae. umbellulata* by Zhang et al. (1998) and Devos and Gale (2000), but the genome rearrangements in the U genome of polyploid *Aegilops* species and in the M genomes were reported in first time by the present study.

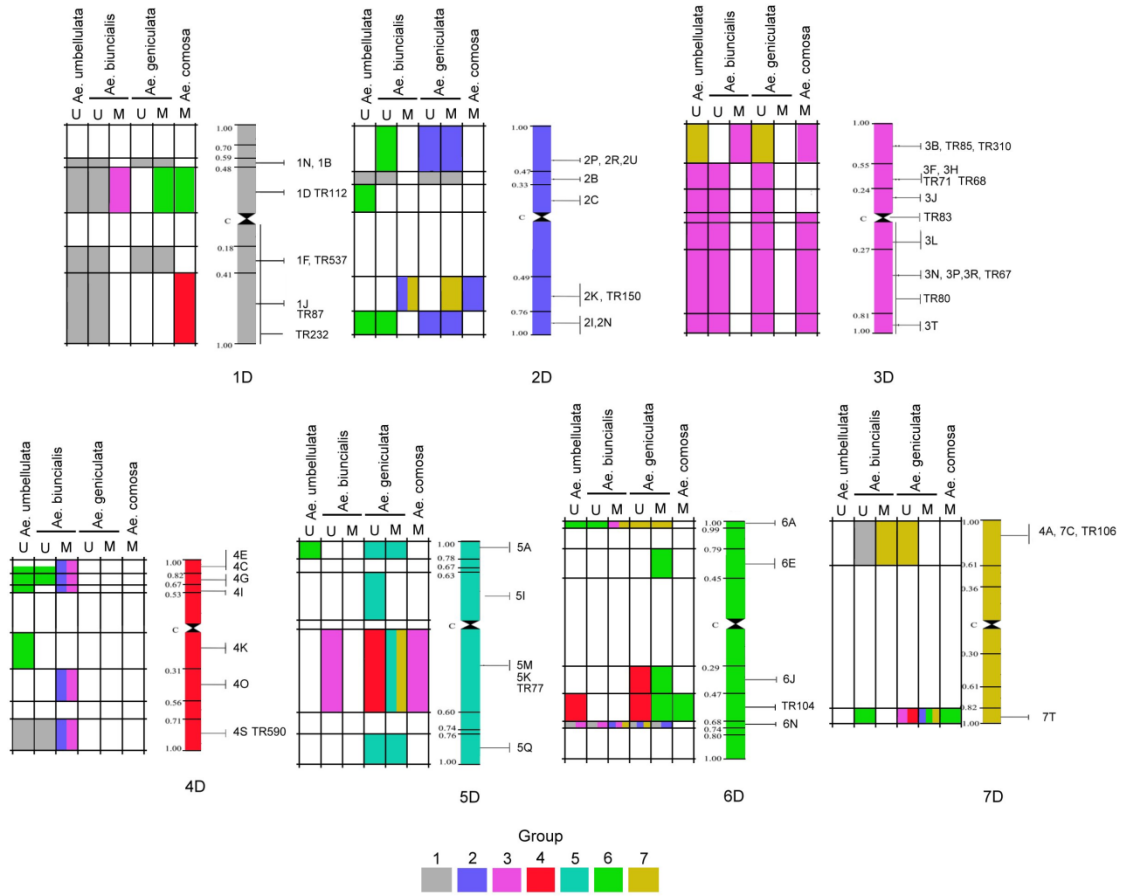


Fig. 2: Chromosomal location of bin-mapped COS markers in wheat (D genom) and in *Ae. comosa* (MM), *Ae. umbellulata* (UU), *Ae. biuncialis* ($U^bU^bM^bM^b$) and *Ae. geniculata* ($U^gU^gM^gM^g$)


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# Effects on yield related traits from introgression of *Thinopyrum ponticum* chromosomal segments onto the 7AL arm of durum wheat

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## Introduction

Nowadays, with an ever increasing human population, important challenges for sufficient food production require urgent consideration. As to wheat, which is the most cultivated cereal worldwide, an annual yield increase of 2% is the projected goal to be reached in order to feed the 9 billion people forecasted by 2050 (Braun et al., 1998; Cohen, 2001). In this view, and in a sustainable agricultural perspective, genetic interventions remain of primary importance. Wild wheat relatives, so far particularly exploited for relatively simple traits (e.g. resistance to diseases), can represent a useful source also for more complex traits, yield included.

Several species belonging to the genus *Thinopyrum* (Dewey, 1984), encompassing a wide range of ploidy levels, have significantly contributed to both bread and durum wheat improvement (e.g. Li and Wang, 2009; Ceoloni et al., 2005). One such species is the perennial tall wheatgrass, *Th. ponticum* (syn. *Lophopyron ponticum*, *Elytrigia pontica*, *Agropyron elongatum*). Its auto-allodecaploid genome (2n = 10x = 70; Chen, 2005), overall originally named as 'Ag' (e.g. Sears, 1978), has been extensively manipulated to create substitution, translocation and recombinant lines in the wheat background, aiming at the transfer of various useful traits, mainly corresponding to resistance to biotic and abiotic stresses (e.g. Li and Wang, 2009).

The existence of a QTL positively affecting yield (here called *Yld-7AgL*) in wheat-*Th. ponticum* stocks, determining an increase in grain yield (13%), biomass (10%) and grain number (15%), was originally suggested on the basis of results obtained by CIMMYT, using near-isogenic lines (NILs) of the original T4 (= Agatha) translocation (70% of 7AgL arm inserted into wheat 7DL) into various bread wheat backgrounds (Singh et al., 1998; Reynolds et al., 2001; Monneveaux et al., 2003). However, not until recently dissection of a complex trait such as yield into its various components has been undertaken in wheat. In common wheat, Quarrie et al. (2005) reported a strong yield QTL, mainly affecting grain number per ear, to be distally located along the 7AL arm. Its expression has been suggested to be associated to a gene(s) controlling flag-leaf width and chlorophyll content, indirectly affecting the amount of assimilates transferred to the spike (Quarrie et al., 2006). Interestingly enough, in a cabinet experiment, the allelic variant of the closest marker associated with higher yield in the materials analysed, seemed to be expressed mainly under stress conditions (exogenous ABA treatment, low nutrient, see Quarrie et al., 2007).

On the other hand, the successful transfer of *Th. ponticum* 7AgL chromatin segments, of different size but of the same origin as that of line T4, was accomplished with durum wheat as recipient (Ceoloni et al. 2005). A set of 10 recombinant lines was obtained, each one having a different amount of the 7AL arm replaced by 7AgL chromatin. Near-isogenic lines (NILs) of some of these recombinant lines, with wheat-alien breakpoints around the suggested location of the 7AL yield QTL (Quarrie et al., 2005, 2006), have been employed in a two-years field experiment, following treatment with exogenous ABA, to assess the expression of several yield-contributing traits and eventually associate them to defined 7AgL sub-regions, thus dissecting the possibly various effects so far generally ascribed to the presence of a sizable portion of the alien arm into genetically and physically distinct determinants.

## Materials and methods

NILs of three durum wheat recombinant lines and of the bread wheat T4 translocation line were used in all experiments (Fig 1). The durum wheat recombinant lines included the 7AL-7AgL recombinants R23-1, R112-4, R5-2-10, each carrying portions of the same 7AgL chromatin present in line T4 (40%, 28% and 23% of the distal 7AL arm, respectively). The 3 durum lines were represented by homozygous carriers (+) and non-carriers (-) of the corresponding 7AgL segment, i.e. recombinant lines selected after a number of BCs to the recurrent cv. Simeto. The "-" control for the T4 NIL was its background genotype, i.e. cv. Thatcher.

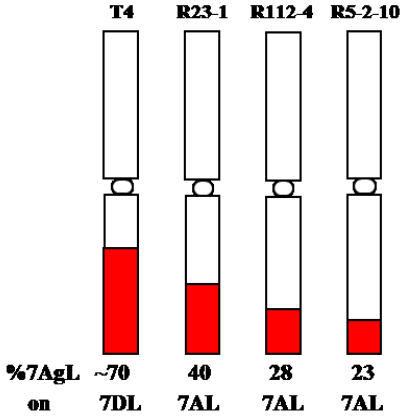


Fig. 1: Recombinant/translocation chromosomes of the genotypes used in the 2-years experiment

Trials were carried out in 2009 and 2010 growing seasons in Viterbo (Central Italy). Plants were grown in pots under greenhouse conditions and, when at the double ridge stage of ear development, treated with an ABA solution (3.8 x $10^{-4}$M, first tiller only). Pots of treated and control plants were later transferred to the field, organized in 3 randomized blocks. During vegetative growth, at maturity and at post-harvest stages, the following traits were measured: flag-leaf width (FLW), flag-leaf lenght (FLL), ear length (EL), height (TH), pikelet number per ear (SPNE), seed number per spike (SPS), days to heading (DTH), total tiller number at maturity (TTN), grain yield/plant (GY), grain yield/ear (GYE), seed number/plant (SN), seed number/ear (SNE), thousand kernel weight (TKW), and, in 2010 only, biomass/plant (B) and harvest index (HI). Data were statistically analyzed with SYSTAT12 (Systat Software Incorporated, San Jose, CA, USA), by means of General Linear Model (GLM).

**Results and discussion**

*Durum wheat genotypes:* GLM analysis applied to investigate all sources of variation for the yield-related traits recorded, revealed different involvement of factors and their interactions on the measured traits. ABA treatment (T) showed to be significant for several traits of the first tiller (FLW, EL, SPNE) and in all cases it resulted in a negative effect, decreasing values in all genotypes, independently of presence/absence of the 7AgL segment (T x 7AgL interaction not significant). Consequently, if differences in expression of the measured traits emerged, they were not ascribable to the ABA treatment, but to some other factor(s). Thus, as a whole, at least in our experimental conditions and with the present materials, the *Yld-7AgL* QTL(s) does not seem to be ABA-dependent.

On the other hand, the analysis in most cases revealed significance of genotype interaction with the presence of the 7AgL segment, indicating a relevant contribution of 7AgL segments in the control of the parameters analyzed. The segments had a prevailingly positive effect, except for a few traits in line R23-1. Then, considering the interaction of the experimental year with 7AgL presence/abscence, two groups of traits were distinguishable, those stably expressed (DTH, EL, FLL, TTN, SNE, TKW; Tab 1), and those expressed in a year-dependent manner (FLW, TH, SPNE, SPS, GYE, GY, SN), probably due to climatic differences.

Table 1. Mean values ± SE of traits stably expressed by durum wheat recombinant lines across experimental years. Letters correspond to ranking of groups after Tukey test (T): capital letter = $P<0.01$, lower case = $P<0.05$, ns = not significant difference

| Trait | R5-2-10 | | | | R112-4 | | | | R23-1 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | + | | - | | + | | - | | + | | - | |
| DTH* | 94.4 ± 0.38 | C | 93.5 ± 0.52 | C | 97.5 ± 0.42 | B | 95.1 ± 0.41 | C | 101.0 ± 0.33 | A | 100.7 ± 0.31 | A |
| EL* | 65.6 ± 0.87 | B | 61.9 ± 1.17 | B | 61.5 ± 0.95 | B | 61.1 ± 0.94 | B | 71.2 ± 0.76 | A | 74.4 ± 0.71 | A |
| FLL* | 284.3 ± 5.00 | AB | 292.5± 6.74 | AB | 276.9± 5.46 | B | 274.6± 5.31 | B | 271.7± 4.35 | B | 301.1± 4.05 | B |
| TTN | 16.9 ± 0.57 | AB | 15.5 ± 0.77 | B | 18.9 ± 0.62 | A | 15.1 ± 0.60 | B | 16.8 ± 0.49 | AB | 14.6 ± 0.46 | B |
| SNE | 41.7 ± 0.95 | ns | 39.7 ± 1.40 | ns | 38.6 ± 1.03 | ns | 41.6 ± 1.00 | ns | 40.7 ± 0.82 | ns | 39.3 ± 0.76 | ns |
| TKW | 51.7 ± 0.88 | AB | 51.5 ± 1.30 | AB | 47.8 ± 0.96 | B | 47.6 ± 0.93 | B | 41.8 ± 0.76 | C | 52.3 ± 0.71 | A |
| B** | 82.9 ± 3.80 | a | 73.7 ± 4.39 | a | 85.5 ± 4.30 | a | 66.6 ± 4.15 | b | 84.1 ± 3.36 | a | 77.5 ± 3.05 | a |
| HI** | 0.4 ± 0.01 | ns | 0.4 ± 0.01 | ns | 0.4 ± 0.01 | ns | 0.4 ± 0.01 | ns | 0.4 ± 0.01 | ns | 0.4 ± 0.01 | ns |

*traits recorded on the first tiller; ** traits recorded in 2010 only

Presence of the 7AgL segment significantly increased TTN in R112-4 and R23-1 lines by 24.8% and 15.1%, respectively. As this increase was not observed in R5-2-10 line, it can be hypothesized that the genetic determinant(s) controlling this trait is situated in the 7AgL region common to R112-4 and R23-1 and absent in R5-2-10. The higher total tiller number is evidently associated with the significant increase in biomass (28.3%) that line R112-4 also exhibited in 2010. Overall, this line ranked as the best among the three durum recombinants for most of the agronomically valuable traits tested. Interestingly, in both years FLW resulted significantly higher (+11.6%) when the R112-4 segment was present (Fig. 2). The same was not observed for the other two durum recombinants. As a matter of fact, this trait showed significantly lower values in R23-1 line, particularly in 2009. No difference in FLW was observed in "+" vs. "-" NILs of the R5-2-10 recombinant; hence, the genetic factor involved in its control might be located in the 5% 7AgL region spanning the R5-2-10 and R112-4 7AL-7AgL breakpoints.

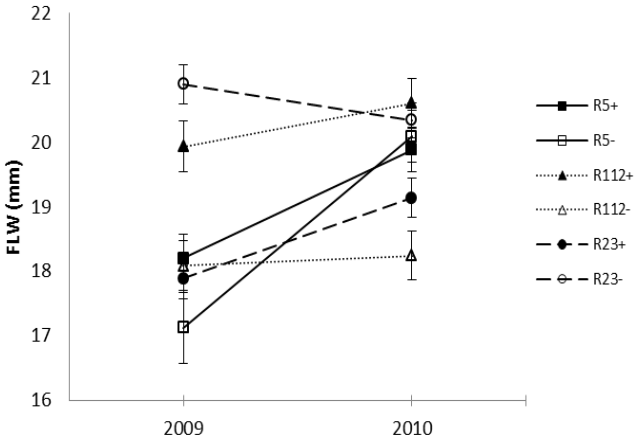


Fig. 2: Year x (Genotype/7AgL segment) interaction for flag-leaf width in durum wheat recombinants

In 2010, GY and SN were as well significantly enhanced by certain portions of the 7AgL arm. When compared to the control, R112-4 had quite higher GY (+36%) and SN (+27%), while in R23-1 the same was true only for the latter trait (+36%). Thus, a genetic determinant responsible for the increase in seed number/plant seems to be localized in the 7AgL region shared by the two recombinants (between 28 and 40% distal 7AgL arm), but absent in R5-2-10. Additionally, in 2010, B was also increased in R112-4, which correlates with the increase in TTN and FLW and indirectly with GY and SN. On the other hand, the absence of any GY increase in R23-1 is correlated with a significant reduction in TKW (~ 20%). The negative effects associated with the 40%-long 7AgL segment of line R23-1, also affecting FLW, FLL, and SPNE, as well as other aspects of the plant phenotype, are most probably related to the inclusion of a segregation distortion (*Sd*) gene(s) in the most proximal portion of the 7AgL segment present in this line (Grossi et al., 2009).

As a whole, line R112-4 turned out to be the most promising genotype in terms of overall yield potential. Compared to its 7AgL non-carrier control line, it showed a wider flag-leaf of the first tiller, a higher number of tillers and increased above-ground biomass, altogether significantly contributing to the observed higher grain yield, at least in the more favourable 2010 season. It is likely to hypothesize that more than one gene/QTL resides within the 28% 7AgL chromatin present in R112-4 and controls the expression of the various traits observed, including FLW, SN, TTN, and B. Since the same remarkable effects were not exhibited by the R5-2-10 line, it seems plausible to assume that the 5% 7AgL segment differentiating the two lines is the alien region where these genes/QTLs are located.

*Bread wheat line T4:* Differently from previous observations by CIMMYT (see Introduction), line T4 did not show a better performance for the measured traits compared to its Thatcher control. GLM showed almost no effect of the ABA treatment, resulting significant only for one trait of the first tiller (SPNE), negatively affected irrespectively of the presence of the 7AgL segment. Indeed, it was always the Thatcher genotype to exhibit better values for the measured traits as compared to the T4 line. It cannot be excluded that the climatic conditions of Central Italy have contributed to this outcome. An additional factor may have been the background effect, being different from those of CIMMYT materials (Monneveaux et al., 2003).

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## QTL mapping and mining candidate genes affecting important agronomical traits in NS wheat breeding program


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### Introduction

Plant breeders face the challenge of creating new crop varieties that will meet the increasing demand for food and feed. New varieties will be required with a set of agronomical and physiological traits suited for rapidly changing and unpredictable conditions. With increasing knowledge of genetics, genomics and molecular biology, plant breeders in this century have

accelerated the improvement of crops for enhanced yield, adaptability and quality (Suslow et al. 2002).

The latest biotechnological methods are supplementing and extending traditional breeding methods to enhance the production of food, fiber and other agricultural products. One of these technologies is marker-assisted selection (MAS), which has received increased attention during the last decades. Numbers of markers that are known to be associated with genes/QTLs for some agronomical important traits are being deployed for MAS in wheat breeding programs worldwide (Gupta et al. 2009, 2010).

The Institute of Filed and Vegetable Crops Novi Sad (IFVCNS) has 74 years long tradition in implementing the latest scientific achievements and methodologies in agricultural practice. Owing to efficient application of biotechnology, in addition to conventional methods in vegetable and field crops breeding, the Institute has become the leading institution in the area of plant breeding and seed production, not only in Serbia, but also in the South-Eastern Europe.

The following aims have been formulated from the early beginnings of the molecular work at the IFVCNS:

- Efficiency improvement of the breeding programs of the Institute by combining traditional and modern breeding tools and technologies.
- Using this approach improvement of crop's tolerance to biotic and abiotic stress factors, in conditions of undergoing climate changes.
- Application and improvement of new procedures for the identification and differentiation of the individual genotypes using DNA markers – DNA fingerprinting.
- The use of molecular tools for detecting genetic diversity and identification of new variability for agronomic traits determining adaptation and yield potential in the breeding materials and wild gene pools, speeding up the introgression of this variability into cultivated genotypes.
- Application and improvement of new molecular-genetic methods for detecting economically important characteristics localized in the crop genome.
- Acceleration and efficiency improvement of the existing breeding programs of the Institute through the development of breeding programs based on MAS.

In achieving these goals, molecular marker work in the Institute has included a few phases, from assembling of appropriate germplasm panels to selection of candidate genes/QTLs for MAS, which are reviewed in this paper.

**Assembling of appropriate germplasm panels**

The exploitation of a germplasm collection with a wide genetic basis may make a substantial contribution to the future tasks of breeders, which will involve reducing the risks faced by cereal production in changing environment. In this phase, a double haploid (DH) mapping population between high-yielding English (Savannah) and Serbian (Renesansa) cultivars have been produced and provided to us by John Innes Centre, UK. The other mapping population (International Triticeae Mapping Initiative-ITMI) was kindly provided to us by IPK Gatersleben, Germany. Beside these populations, specific germplasm collections were selected from our huge world genetic collection. All these genetic materials, suitable for

mapping genes and QTLs associated with important traits, were introduced in various field and laboratory trials.

**Phenotypic and molecular evaluations of the germplasm panels**

In the second phase phenotypic characterisation of important agronomical traits determining adaptation, quality and yield potential in the germplasm panels have been performed (Denčić et al. 2011). In the same time molecular evaluations of genetic diversity existing in the mapping populations and selected germplasm collections were performed by using a large number of SSR molecular markers (Kobiljski et al. 2002, Karsai et al. 2011, Brner et al. 2011).

**Integrating genotypic and phenotypic information in QTL identification**

Four elements are required for QTL detection: a population of plants that is genetically variable (developed in the first phase); marker systems allowing genotyping of the population (chosen and used in the second phase); reproducible quantitative phenotyping methodologies (performed also in the second phase); and appropriate experimental and statistical methods for detecting and locating QTL (Cavanagh 2008, Gupta 2010). In the past, QTL interval mapping were conducted using be-parental mapping populations. However, in recent years, association mapping based on linkage disequilibrium (LD) was used as an alternative approach. In our studies both methods for QTL detection have been used (Neuman et al. 2010, Rehman Arif et al. 2011).

**Validation of identified QTLs across a wide range of growing conditions**

QTLs identified by marker-trait association analyses need to be validate in a relevant breeding material, as well as in a different environments, because they are usually not stable across wide range of growing conditions. In cooperation with our colleagues from other Serbian, as well as internationally recognised Institutions (John Innes Centre, Norwich, UK; IPK Gatersleben, Germany; Agricultural Research Institute of the Hungarian Academy of Sciences Martonwasar, Hungary etc.) we performed trials with our material in different growing conditions, in order to validate QTLs identified in Serbian agro-ecological conditions (Kobiljski et al. 2009, Dodig et al. 2010).

**Selection of candidate genes/QTLs for MAS**

Based on the results of all our mentioned studies, we selected a list of QTLs for important agronomical (ear length, spikelet number, grain number, grain weight, grain yield, heading and flowering time) and quality traits (protein content, sedimentation) which could be potentially useful for MAS in NS wheat breeding program.

Modern plant breeding programs need the strategies and innovations that come through the application of molecular technologies. This could be achived by linking breeders and molecular biologists, with the goal of defining targets and identifying key germplasm for analysis, as well as developing more efficient breeding tools and approaches.

**Summary**

Recent advances in molecular markers technology have led to the development of a number of approaches that offer the possibility for efficiency improvement of breeding programs worldwide. This paper provides an overview of the current status of molecular breeding in NS wheat breeding program. The molecular marker work in the Institute has included the following phases: 1) assembling of appropriate germplasm panels (including mapping populations, and specific collections of varieties/breeding lines) representing known genetic variation for agronomic traits; 2) phenotyping and genotyping of important agronomical traits determining adaptation, quality and yield potential in the germplasm panels; 3) integrating genotypic and phenotypic information in QTL identification, and testing potentially useful QTLs in realistic breeding conditions; 4) exploring the actual value of particular alleles (affecting important agronomical traits) across a wide range of growing conditions. We applied both a standard quantitative trait locus analysis based on segregation from biparental crosses, and an association analysis using a germplasm panel to detect marker trait associations. Using these approaches, we were able to determine potentially desirable segments of the wheat genome responsible for increased yield and quality in target environment(s). The results will permit the greater utilisation of resources for variety development, gene discovery and marker-assisted selection in Serbia.

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# Genome wide association mapping of agronomic traits in bread wheat

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As the main crop in the world bread wheat has a major importance for matching the future demand of feeding mankind, especially in the context of a growing world population in accordance with ongoing climate change. The better our understanding of the genetics of the quantitative agronomic important traits and their interaction is, the more it is possible to increase yield and yield stability with the help of molecular markers in breeding. With an association mapping approach one can directly use historical data from breeding trials to gain valuable inside into complex quantitative traits.

## Material and methods

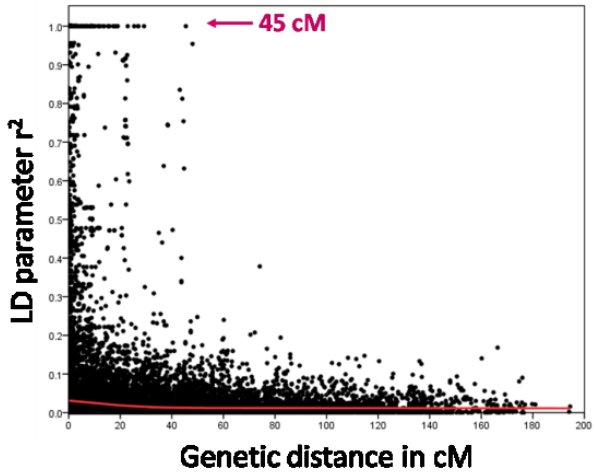
The Institute of Field and Vegetable Crops in Novi Sad, Serbia, created a diverse wheat collection based on contrasting phenotypes for main breeding traits. We used a subset of 96 genotypes that were evaluated in the field in Novi Sad for twenty agronomic important traits in up to eight growing seasons. Next to phenotypic diversity the genotypes are also genetically broad, originating from 21 different countries across five continents. More details see Neumann et al. (2011).

Genoytping was done by Triticarte Pty. Ltd (Canberra, Australia; http://www.triticarte.com.au/). It revealed over 800 polymorphic Diversity Array Technology markers (DArTs). For 525 a map position was available based on Crossa et al. (2007). A Q-Matrix was constructed using STRUCTURE (Pritchard et al. 2000), resulting in two subpopulations according to origin and pedigrees. Tassel (Bradbury et al. 2007) was used to calculate a kinship matrix and the extent of linkage disequilibrium (LD) and for the analysis of associations between markers and traits. For all traits two models were used: a GLM with Q-Matrix only and a MLM with Q- and Kinship-Matrix. Significance criterion was a p-value $< 0.05$ in both models and that in certain years of investigation, e.g. in six out of the eight years for heading and height.

## Results and discussion

LD extent in the 96 panel decays to a mean $r^2$ of 0.1 within 4.5 cM. Nevertheless, some marker pairs are in complete LD up to a distance of 45 cM (Fig. 1a) still that is less than in the larger population of Crossa et al. (2007). The total mean of $r^2$ in all intrachromosmal pairs is 0.069, with 14.5% of pairs in significant LD. LD between chromosomes exists as well, but here only 3.2% of all pairs are in LD with a mean $r^2$ of only 0.019. In association analysis 115 of all mapped markers were found to be associated with at least one trait. Often they have association to more traits and very close markers with similar marker-trait-associations (MTAs) can be regarded as one locus. The highest number of associated markers is carried by the group 7 chromosomes, ten MTAs on 7A, twelve on 7B and one on 7D (Fig. 1b).

a)




b)

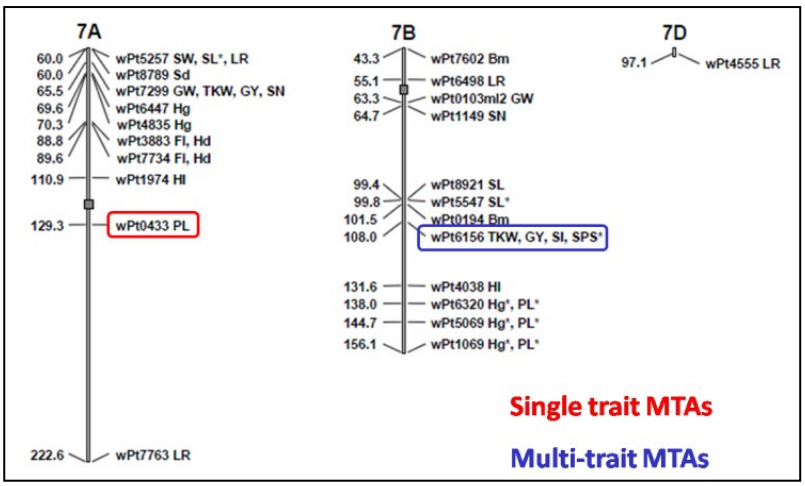



Fig. 1: a) Intrachromosmal LD-plot. Longest distance for a total LD was 45 cM. The red bottom line is a loess-curve fitted to the data. b) Group 7 chromosomes with all associated markers

For flowering time 12 different loci were detected via association mapping (Fig. 2). Only one consists of two close markers in complete LD, all other are single-marker loci. The Loci are located on 1BL, 1D, 2BL, 2DS, 4BL, 5BL, 5DS, 6AS, 6BS and 7AS. Flowering and heading date are depending on genes for vernalization response, photoperiodic response and earliness per se (*Eps*). The loci on 1BL and 1DS most likely refer to variation in *Eps* genes. The photoperiod response genes (*Ppd-1*) map to the short arms of the group 2 chromosomes (McIntosh et al. 2008) and in our study one marker associated with heading and flowering was detected on 2DS. Considering the GLM only, there were also MTAs on the short arms of 2A and 2B but with MLM they were not significant (but close by). Another QTL associated with photoperiod response and described by Kuchel et al. (2006) is located in the centromeric region of 7AS. This locus may very well correspond to a major locus detected on 7AS in the present study. All the entries in the germplasm were pure or facultative winter forms, so no allelic variation at the *Vrn-1* genes was expected, and indeed there was no overlap of detected MTAs and the regions on the group 5 chromosomes where these genes reside (McIntosh et al.

2008). One MTA was identified on 5BL, but in a position only ~ 10 cM away from the centromere (whereas *Vrn-B1* is genetically independent of centromere: Leonova et al. 2003). Altogether seven loci for flowering do not correspond to a known gene or QTL, therefore they could represent a new source for candidate genes and in breeding.

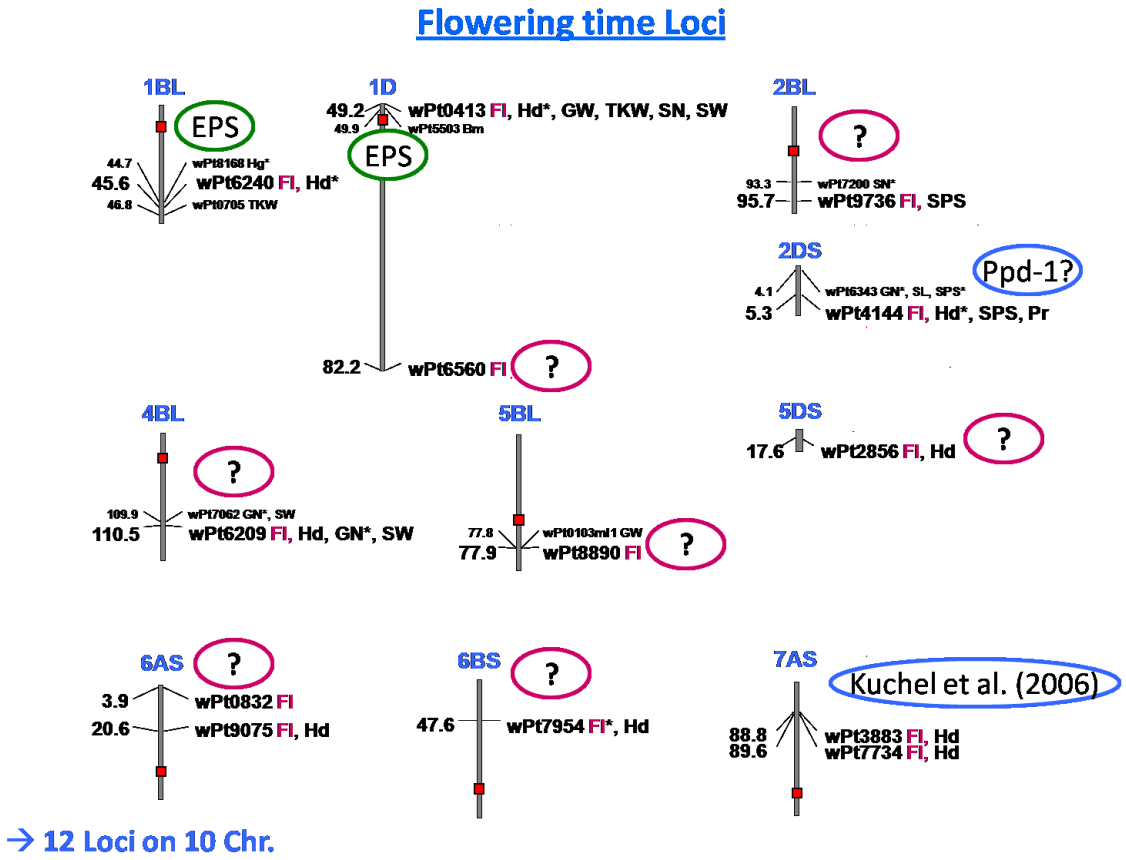



Fig. 2: Loci for flowering time (Fl) detected by association mapping and their possible match with known genes/QTLs

To validate the association mapping approach via bi-parental mapping two of the main loci for flowering time were selected, each explaining ~ 15% of phenotypic variance. For the first locus on 1D at 49.3 cM (one marker; *eps* as candidate gene) two crosses of contrasting parents, for the second on 7A at 89 cM (two markers in complete LD; QTL by Kuchel et al. (2006)) three crosses were developed. Parents were selected on their genotypic allelic composition, in all flowering loci they differed only in the alleles of the locus of interest, for all other flowering loci they had the same alleles. The parents for the two crosses on 1D differed in their flowering time in 11 and 5 days, respectively. The parents of the three mapping populations for 7A differed 14, 13 and 10 days in flowering, respectively. In 2011 the parents and the $F_2$ were sown in pots in IPK, Germany. In four of the five crossings the differences between the early and late flowering parent remained high and significant, only for one cross with a former difference of only 5 days no parental difference was observed (Fig. 3). Nevertheless, also in that population large differences in the genotypes of the $F_2$ were visible (110 – 127 days) and that was true also for the four other populations. The strategy now is to genotype the $F_2$-populations with CAPS-marker (Cleaved Amplified Polymorphic Sites) developed from DArT-sequences published online on the Triticarte website. $F_3$ will

phenotyped in the field for flowering time. Moreover, other loci validation regarding grain yield loci that were common to Crossa et al. (2007) is planned as a next step.

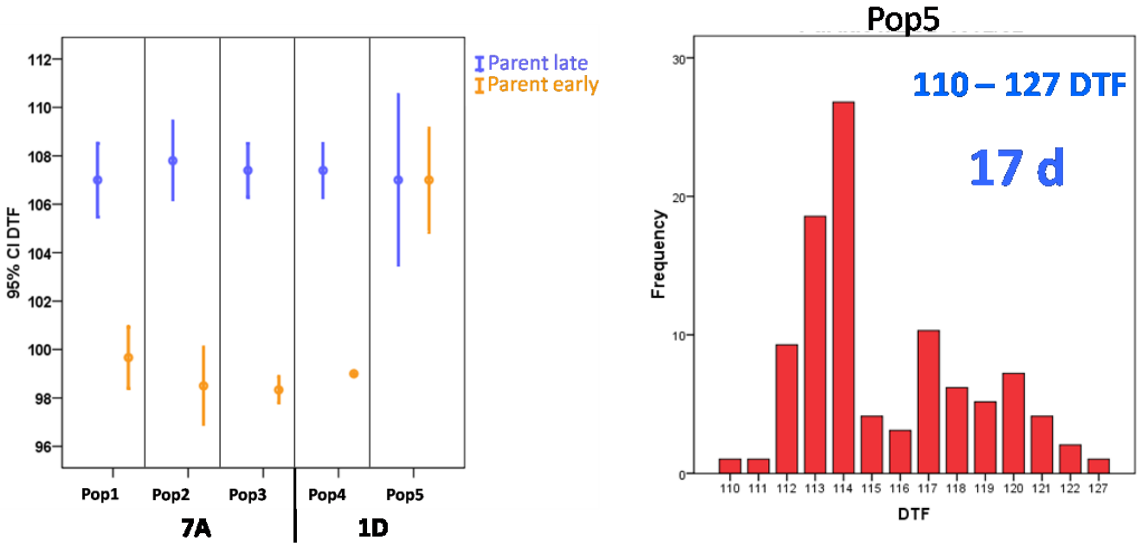



Fig. 3: Errorbars of the 95% Confidence interval (CI) of days to flowering (DTF) of the parents of the five crossings (left) and histogram of the individuals of the $F_2$ of pop 5 (right).

## Conclusions

Our investigations proved the success of the approach to use existing field data in breeding germplasm for association mapping. Even if the sample size with 96 genotypes is rather small for such an approach, the diversity in phenotypes made it possible to detect known and potential new loci. Further


## Acknowledgements

We thank all people involved in the work of phenotyping the 96 wheat lines, knowing this was the hardest part of all. Furthermore we thank Dr. Inge Matthies for their help in association analysis and Annette Marlow, who did the crossings for loci validation.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# Using of DNA markers for selection of common wheat in Polish breeding programmes

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## Introduction

Fungal diseases cause significant losses in grain yield of wheat. The best way to limit the impact of fungal diseases is breeding of cultivars containing resistance genes that are effective against the population of pathogens existing in certain area. Diseases that occur annually and cause significant crop losses in many countries, including Poland, are powdery mildew and brown rust. The main ways of crop protection against these diseases are chemical fungicides utilization or cultivation of resistant cultivars. Introgression of resistance genes has been the method of choice for controlling mildew and rust diseases in wheat, moreover cultivation of resistance plants is the most economic and environmentally favorable (Kowalczyk 2004).

Only for a little number of the resistance genes identified so far virulence of the fungus does not occur or is insignificant. Within these genes are *Lr9* and *Lr19*, which are the most effective resistance genes in Europe. In the case of *Lr19* isolates of *P. recondita* f. sp *tritici* virulent to lines containing this resistance gene were not observed in Poland (Huerta-Espino et al. 2011, Strzembicka pers. comm.). In wheat breeding in Europe, the most used 10 powdery mildew resistance genes are: *Pm1a, Pm1e (Pm22), Pm2, Pm3a, Pm3d, Pm4b, Pm5, Pm6, Pm8, Pm9* (Zeller, Hsam 1998). In Poland, wheat cultivars usually contain a combination of *Pm2* and *Pm6* genes, moreover there are also genes: *Pm3d*, *Pm5*, *Pm8*, *Pm4b* (Kowalczyk et al. 1998; Kowalczyk et al. 2006; Wiśniewska, Kowalczyk 2005).

Pyramiding of major resistance genes is one of two strategies used in resistance breeding of plants. It gives full resistance. The second way is the accumulation of partial resistance genes, which give plants a quantitative resistance. Introgression of *Lr* and *Pm* genes in the wheat genome is a procedure that requires much effort, and the immunity conferred by a single gene is usually overcoming rapidly with the emergence of virulent races of the pathogen population (Kowalczyk 2004; Huerta-Espino et al. 2011).

The use of DNA markers, enables and facilitates the selection of desirable genotypes, influences the efficiency and speed up the selection process, what is especially important in the genes pyramiding. Marker assisted selection (MAS) is a combined approach joining traditional genetics and molecular biology. Within last couple of years combination of molecular markers and traditional selection method has become a valuable tools widely used for support of plant breeding processes (Francia et al. 2005).

The aim of presented studies was identification of powdery mildew resistance genes; *Pm4b* and *Pm6*, as well as leaf rust resistance genes: *Lr19*, *Lr35* and *Lr47* in new wheat breeding lines obtained from Polish breeding companies by means of molecular markers.

## Material and methods

The objects of the study were advanced breeding lines of common wheat developed by Smolice, DANKO and Strzelce Breeding Companies, moreover by Poznan Plant Breeding

and Plant Breeding Malopolska. DNA was isolated from the leaves of ten-day-old seedlings according to the CTAB method (Doyle, Doyle 1987). After drying, the precipitate was diluted in 50 µl of deionized water. DNA concentration was determined by electrophoresis on 1.5% agarose gel by comparison with standard molecular weight marker (MassRuler ™ DNA Ladder Mix, Fermentas, Lithuania). The samples were then adjusted to equal DNA concentration of 20 ng/µl. PCRs were performed in a Biometra T Professional thermocycler. Sequences of primers are summarized in Table 1.

Table 1: Primers names, sequences and references

| Gene | Name | Sequnence | References |
|---|---|---|---|
| *Pm4b* | Pm4F<br>Pm4R | 5'-TCATTCTTGTTTTACTTCCTTCAGT-3'<br>5'-GTCTCGTCTTCAGCATCCTATACA-3' | Yi et al. 2008 |
| *Pm6* | $NAU/STS_{BCD135-2L}$<br>$NAU/STS_{BCD135-2R}$ | 5'-GCTCCCAACCAAGAGAAGAA-3'<br>5'-TCTGTCGGTCCTCTGATGTG-3' | Ji et al. 2008 |
| *Lr19* | SCS253-F<br>SCS253-R | 5'-CATCCTTGGGGACCTC- 3'<br>5'-CCAGCTCGCCATCCA-3' | Gupta et al. 2006 |
| *Lr35* | Lr35-F<br>Lr35-R | 5'-AGAGAGAGTAGAAGAGCTGC-3'<br>5'-AGAGAGAGAGCATCCACC-3' | Gold et al. 1999 |
| *Lr47* | PS10R<br>PS10L | 5'-GCTGATGACCCTGACCGGT-3'<br>5'-TCTTCATGCCCGGTCGGGT-3' | Helguera et al. 2000 |

**Results and discussion**

After including in the PCR primers SCS253-F and SCS253-R in many lines 736 bp DNA fragment was observed. Obtained results indicate that these lines did not contain *Lr19* gene. The lack of this product indicate at the presence of *Lr19* gene in 20 breeding lines of wheat from Strzelce Company and in five breeding lines of wheat from Smolice Company. After including in the PCR primers Lr35-F and Lr35-R the 900 bp amplification product was observed in 18 lines. Within these lines 8 derived from Smolice Company, 7 from Strzelce Company and 3 from Poznan Plant Breeding. This product is linked to *Lr35*, but for undoubted identification of this gene confirmation based on pedigree of these lines is needed. The *Lr47* leaf rust resistance gene was absent in all tested lines. In earlier studies *Lr19* and *Lr35* genes were not found in Polish common wheat cultivars (Kowalczyk et al. 2000; Wiśniewska et al. 2003).

On the basis of the experiments results it was shown that *Pm4b* gene is widespread and widely used in Polish breeding programmes of common wheat. As a result of DNA amplification using STS-PCR markers, the 241 bp PCR product was found in 162 lines of common wheat. The presence of this product indicates at presence of *Pm4b* gene in these lines. This gene was found in 47 breeding lines developed in DANKO, 44 from Strzelce Breeding Company, 32 from the Poznan Plant Breeding, 23 from Plant Breeding Malopolska and 16 from Smolice Breeding Company (Table 2).

After including in PCR $NAU/STS_{BCD135-2L}$ and $NAU/STS_{BCD135-2R}$ primers, for many lines amplification of the 230 bp DNA fragment was observed. This PCR product is linked to *Pm6*

gene. This gene was found in 119 breeding lines developed in Smolice Breeding Company, 98 from Strzelce Breeding Company, 51 from the Poznan Plant Breeding and 24 from DANKO (Table 2). Kowalczyk et al. (2006) showed that the *Pm6* gene occurs in many Polish wheat cultivars in combination with *Pm2* gene, but many cultivars and lines grown in 2000, is heterogeneous in terms of these genes.

Combination of *Pm4b* + *Pm6* genes is still effective against *Blumeria graminis* f. sp. *tritici* population exist in Poland. Combination of these genes were found in 43 breeding lines developed in Strzelce Breeding Company, 16 from Smolice Breeding Company and 10 from Poznan Plant Breeding.

Table 2: Number of lines with *Pm4b* and *Pm6* genes developed by Polish breeding companies

| Breeding company | Number of analyzed lines | Number of lines with *Pm* genes | | |
|---|---|---|---|---|
| | | *Pm4b* | *Pm6* | *Pm4b* + *Pm6* |
| Smolice Breeding Company | 200 | 16 | 119 | 16 |
| Strzelce Breeding Company | 200 | 44 | 98 | 43 |
| DANKO | 100 | 47 | 24 | 0 |
| Plant Breeding Malopolska | 100 | 23 | 0 | 0 |
| Poznan Plant Breeding | 100 | 32 | 51 | 10 |

It was shown that the use of molecular markers assisted selection (MAS) is a straightforward and comprehensive tool for fast selection of pathogen resistant forms within wheat lines during breeding programmes, without necessity of additional time consuming physiological tests performance. To sum up, it should be noted that the strategy of common wheat resistance breeding, especially for brown rust, is correct. It is based on the introduction of effective genes for resistance to brown rust, such as *Lr19*. Powdery mildew resistance breeding of common wheat in Poland based primarily on the use of *Pm4b* and *Pm6* genes. *Pm6* gene is not effective against *Blumeria graminis* f. sp. *tritici* population exist in Poland, but combination of this gene with *Pm4b* increases resistance to this pathogen. However, there is a need of introduction new effective *Pm* genes into Polish wheat breeding programmes.

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# The *Rht* and *Ppd-D1* genes in Ukrainian winter bread wheats: effects and distribution

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Wheat grain yields have increased following the wide spread incorporation of reducing height (*Rht*) and photoperiod insensitive (*Ppd*) alleles in wheat breeding programs to produce semi-dwarf (Chapman et al., 2007), early flowering wheats.

The GA-responsive *Rht8c* (2D) and GA-insensitive *Rht-B1b* (4B) and *Rht-D1b* (4D) dwarfing genes are used to overcome lodging without significant reduction of final crop biomass. Mutant plants with *Rht8* respond to the exogenous application of GAs, but its role, if any, in GA metabolism or signalling remains unknown (Gasperini et al., 2009). *Ppd-D1a* (2D) is closely linked to the *Rht8* (20.9 cM) gene and reduces height through conferring photoperiod insensitivity and hence time to jointing in short days (Addisu et al., 2009) due to a 2.089 bp deletion upstream of the coding region leading to mis-expression of the 2D pseudo-response regulator gene (Worland et al., 1998 a, b; Beales et al., 2007; McIntosh et al., 2008). Owing to its mode of action the use of *Rht8c/Ppd-D1a* prevails in the spring wheats introduced by CIMMYT and in varieties from South and South-Eastern Europe and also from Southern Ukraine and Russia.

The *Rht-B1b* and *Rht-D1b* alleles encode transcription factors which belong to the DELLA proteins that act as plant growth repressors insensitive to GA (Peng et. al., 1999). The point mutations of *Rht-B1b* and *Rht-D1b* lead to the introduction of a stop codon into a conserved region known as the DELLA domain, which is predicted to be in the N-terminus of the protein. Peng et al. (1999) proposed that translation might restart after the introduced stop codon, resulting in shortened proteins which are resistant to GA-induced degradation. Accumulation of the mutant DELLA protein causes continuous growth inhibition and, accordingly, leads to agronomically advantageous dwarfed plant height and improved straw strength by inhibition of stem cell elongation (Dalrymple, 1986; Flintham et al., 1997 a; Peng et al., 1999).

Presence of *Rht8*, *Rht-B1*, *Rht-D1* dwarfing and *Ppd-D1* photoperiod insensitive alleles have been actively investigated (Ganeva et al., 2006; Knopf et al., 2008; Gulyas et al., 2011 and others). According to different data (Worland et al., 1998 a,b; Rebetzke et al., 2001; Ellis et

al., 2004; Fayt, Mokanu, 2008; Gasperini et al., 2009; Addisu et al., 2009; Kolev et al., 2010; Voss, 2010 and others) the presence of the dwarfing and *Ppd-D1* genes in genotypes of wheat effects the rate of development, winter hardiness, drough tolerance, resistance to *Fusarium* head blight, nitrogen use efficiency, coleoptile length, seed viability, leaf elongation rates, yield. These investigations have shown importance of the several alleles for specific climatic conditions.

Aims of our work were to investigate effects of the alleles *Rht8, Rht-B1, Rht-D1, Ppd-D1* genes and their complexes on agronomically important traits (plant height, kernel size, WTK, coleoptile length, *in vitro* androgenesis) of winter bread wheat in conditions of South Ukraine and detect the alleles of *Rht8, Rht-B1, Rht-D1* and *Ppd-D1* genes in genotypes of Ukrainian wheat varieties.

As a material were used analogue-lines created on the genetic background of varieties wich are adapted to the steppe region such as: Kooperatorka, Odesskaya 3, Odesskaya 51, Stepnyak that differ in plant height and dwarfing genes and varieties from Ukrainian breeding centers: MIW (Myronivka); IASR (Kherson); IPPG (Kyiv); IPP (Kharkiv); DIAP (Donetsk); LIAP (Lugansk); PBGI (Odessa) and several private companies.

PCR-analysis of *Rht8* gene according to Korzun et al., (1998); *Rht-B1* and *Rht-D1* loci as recommended by Ellis et al., (2002; 2007) and *Ppd-D1* as shown by Beales et al., (2007) have been applied. Analysis of agronomical traits: plant height (PH), stem length (h), heading date (DH), flowering date (DF), 1000 kernels weight (WTK), productive tillering (PT), number of kernels from the main ear (NME), number of ears from the main ear, weight of kernels from the main ear (WME), spike density (D), number of kernels from the secondary ears (NSE), weight of kernels from the secondary ears (WSE), number of kernels from the plant (NKP), weight of kernels from the plant (WKP) have been done in the field trials (2007-2010 years). The conditions of vegetation for field trials were diverse, but generally quite favorable for winter wheat.

Statistical analysis was done with the program Statistica 8.

**Results**

The complexes of dwarfing and *Ppd-D1a* alleles reduced PH stronger than individual genes (Kertez et al., 1991). According to the data of 3 factorial ANOVA the factors “Genotype”, “Year” and their interaction have significant influence on the PH. The year conditions significantly effect PH (F=51.9; P=99.9 %; $p^{in}$=59.8 %), but genotype reaction on the various weather conditions were unidirectional (Fig. 1A), there were no triple interaction of both genetic factors with environment. Thus, the complex of alleles *Rht8c+Ppd-D1a+Rht-B1e* decreased PH – by an average 47.6%, and the complex *Rht8c+Ppd-D1a+Rht-B1b* by 39.2% compared to the high and photoperiod sensitive plants in the conditions of South Ukraine according to the data of 2008-2010 years (Fig. 1A). On the Fig. 1B is shown that analogue lines that have been created on different genetic background but have the same alleles of dwarfing genes have not significant differences at PH.

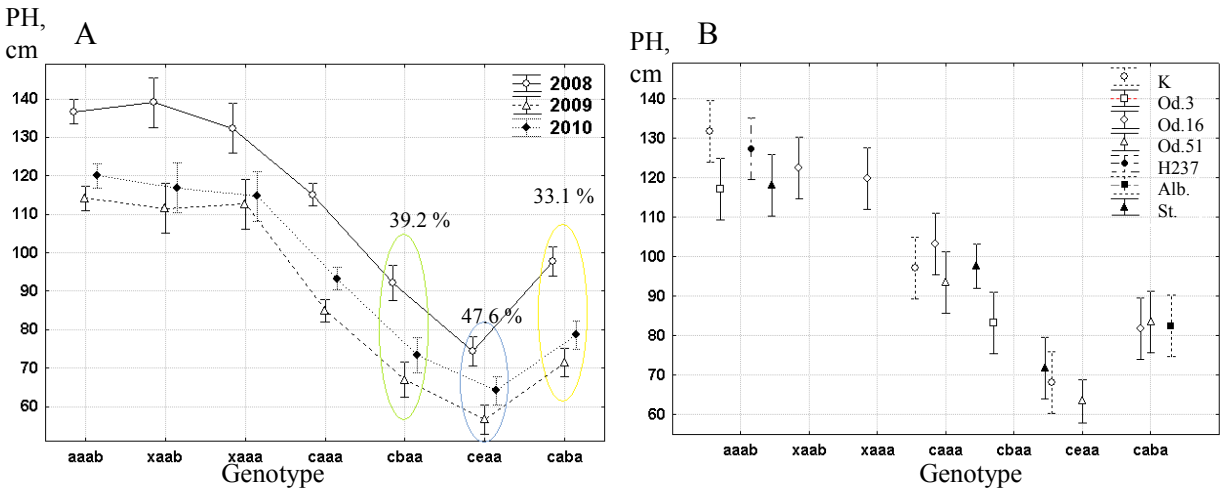


Fig. 1: A) Plant height depended on the genotype and year of investigations: aaab – *Rht8a Rht-B1a Rht-D1a Ppd-D1b*; xaab – *Rht8x Rht-B1a Rht-D1a Ppd-D1b*; xaaa – *Rht8x Rht-B1a Rht-D1a Ppd-D1a*; caaa – *Rht8c Rht-B1a Rht-D1a Ppd-D1a*; cbaa – *Rht8c Rht-B1b Rht-D1a Ppd-D1a*; ceaa – *Rht8c Rht-B1e Rht-D1a Ppd-D1a*; caba – *Rht8c Rht-B1a Rht-D1b Ppd-D1a*. B) Plant height depended on the genotype and genetic background: К – Kooperatorka; Od.3 – Odesskaya 3; Od.16 – Odesskaya 16; Od.51 – Odesskaya 51; H237 – Hostianum 237; Alb. – Albatros odesskiy; St. – Stepnyak

On the genetic background of variety Odesskaya 16 (*Rht8x Rht-B1a Rht-D1a Ppd-D1b*) allele *Ppd-D1a* from Bezostaya 1 leaded to the reduction of height for 0-3 % in dependence on the year conditions. On the background of variety Stepnyak allelic differences in *Ppd-D1* gene does not significantly influence on PH. Lines with *Ppd-D1a* allele were heading earlier on 2.5 – 5 days in dependence of year conditions in comparison with lines that characterize by *Ppd-D1b* allele.

The presence of dwarfing and photoperiod insensitive alleles in the analogue lines separately or in different combinations: *Rht8c+Ppd-D1a* (P=0.01), *Rht-B1b*, *Rht8c+Ppd-D1a+RhtB1b* (P=0.01), *Rht-B1e* (P=0.01), *Rht8c+Ppd-D1a+Rht-B1e* (P=0.01) reduce coleoptile length in comparison with coleoptile of wild type lines (*Rht8a+Rht-B1a+Rht-D1a+Ppd-D1b*).

The complex of alleles *Rht8c+Ppd-D1a* increased WTK on 17 % in comparison with plants that characterized by *Rht8a+Ppd-D1b* alleles. On the other hand combinations *Rht8c+Ppd-D1a+RhtB1e* and *Rht8c+Rht-D1b+Ppd-D1a* reduced WTK on 15 % in comparison with the *Rht8a+Ppd-D1b* plants. *Rht8c+Ppd-D1a* alleles increased kernel size on 11.5 % in comparison with genotype without mutant alleles, but complex of *Rht8c+Rht-D1b+Ppd-D1a* reduce it on 6,3 % and *Rht8c+Ppd-D1a+RhtB1e* on 13.4 % in comparison with *Rht8c+Ppd-D1a*.

According to the data of *in vitro* androgenesis investigations the dwarfing analogue-lines have been characterized by higher level (P≤0.05) of callus induction (15.8 %) and regeneration of plants (1.2 %) in comparison with the tall varieties.

By the use of variance, discriminant and factor analysis we can distinguish (97.7% of variation) the class of genotypes with *Xgwm261* – 192bp (blue circle on the Fig. 2) from plants with other alleles of locus *Xqwm261* by traits that characterize plant (Root 2: DF, PT, DH, WTK) and main ear development (Root 1: *h*, NME, WME, *D*). But this model is useless for highly significant distinguishment of *Xgwm261* – 164 and 214 bp alleles. We need to note that 192 bp allele as have been shown by Korzun et al., (1998) are diagnostic for *Rht8c* gene

and in genotypes of Ukrainian winter bread wheat varieties this allele linkage inherited with *Rht8c* allele which mostly added by *Ppd-D1a* allele and by one of dwarfing *Rht-D1b/Rht-B1b* genes.

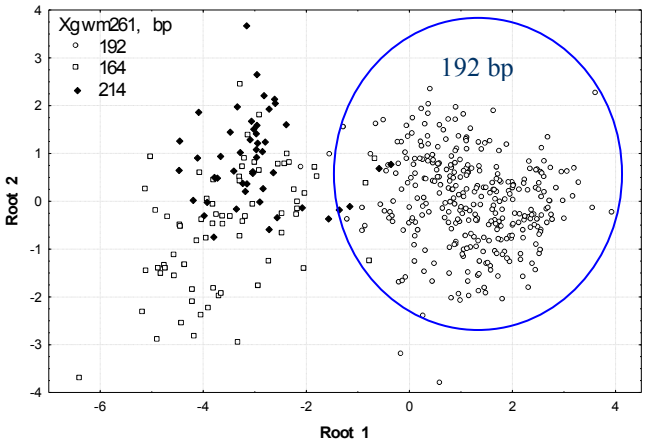


Fig. 2: Diagramm of in two roots of discriminant function of plants differring by the alleles of *Xgwm261* locus

According to the data of 2010 year the semi-dwarf and tall varieties does not differ significantly by number of ears from the main ear but the length of the main ear was bigger among tall plants. That means that compactisation of the main ear is observed in semi-dwarf plants (*Rht8c* allele). Coefficient of spike density increased from *D*=16.7-29.2 among tall plants to *D*=17.6-35.5 among dwarf plants.

The distribution of *Rht* and *Ppd-D1* genes among Ukrainian winter bread wheat cultivars is well investigated. About 30 % of varieties that are registered in Ukraine are produced by Plant Breeding and Genetics Institute (Odessa). In general, the breeding program in the South Ukraine steppe region has resulted in the *Rht8c* and *Rht-D1b/Rht-B1b* dwarfing (*Rht-B1b* more rarely present in genotypes of Ukrainian wheat varieties then *Rht-D1b*) and the *Ppd-D1a* genes in the most modern winter wheat varieties. However the same distribution was no observed on the other territory of Ukraine. The *Rht-B1b* allele is somewhat less distributed than allele *Rht-D1b*. Among the varieties produced in the East part of Ukraine the frequency of the *Rht-D1b* is 54.5 %. Winter wheat varieties from the central part of Ukraine are characterized predominantly by the alleles of wild type *Rht-B1* and *Rht-D1* genes. In spring wheat varieties an other distribution is observed. Only 22 % of spring wheat varieties are characterized by *Ppd-D1a* and 78 % by *Ppd-D1b.* Dwarfing alleles in spring wheat varieties is also rarely used than in winter wheat varieties. We have not detected complex of *Rht-B1b* and *Rht-D1b* alleles in one genotype in spring cultivars produced by Ukrainian breeders. Among 23 investigated varieties only 13 % have the allele *Rht-D1b* and the same amount of the varieties were characterized by the *Rht-B1b* allele.

## Conclusions

In general, the breeding program in the South Ukraine steppe region has resulted in the *Rht8c* and *Rht-D1b/Rht-B1b* dwarfing and *Ppd-D1a* genes in the most modern winter wheat varieties. In the breeding programs of spring wheat the dwarfing alleles are more rarely used.

Besides plant height the dwarfing and *Ppd-D1* genes lead to the reduction of: life cycle (heading earlier); coleoptile length; WTK (except *Rht8c+Ppd-D1a*); kernel size (except *Rht8c+Ppd-D1a*); length of the main ear (compactisation) and to the induction of: haploproduction potential and number and weight of kernels from the secondary ears.

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# Identification of leaf rust resistance gene *Lr19* in wheat genetic stocks from South Eastern Europe

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Leaf rust caused by *Puccinia triticina*, is considered to be one of the most significant fungal diseases of bread wheat (*Triticum aestivum* L.) in Europe and worldwide (Kolmer et al. 2007; Lind and Gultyaeva 2007; McCallum et al. 2007).

Resistance to the leaf rust pathogen has major importance in many wheat breeding programmes. Breeding wheat cultivars with resistance to leaf rust is the most effective, economical and environmentally friendly method of disease control. One of the most important gene determining resistance to *Puccinia triticina* is *Lr19*, introgressed into common wheat from *Thinopyrum* sp. This alien gene provides valuable resistance to leaf rust in many parts of the world and can be utilized in combination with other *Lr* genes to provide long lasting resistance against leaf rust (Roelfs 1988; Prins 1997; Tomar and Menon 1998; Pink 2002; Mesterhazy 2000).

Traditional method for identifying *Lr* genes are labor- and time-consuming. At present, individual *Lr* genes can be identified with the use of molecular markers (Feuillet et al. 1995; Schachermayr et al. 1997; Cherukuri et al. 2005). They enable identification of desirable genes in a simple way and, consequently, allow for a fast and effective selection of interesting forms. Moreover, expression of molecular markers is not affected by environmental conditions, and they can be detected at all stages plant growth. In the past few years STS sequence –target size as well as SCAR sequence-characterized amplified regions markers were developed for fast and certain identification of *Lr19* gene presence in wheat genetic material (Prins et al. 2001; Gupta et al. 2006).

The aim of presented studies was identification of *Lr19* gene in wheat accessions from South Eastern Europe.

## Material and methods

Analyzed material consisted 96 common wheat cultivars from 7 countries: Albania, Austria, Belarus, Bulgaria, Croatia, Czech Republic and Estonia. As a positive and negative control we used Thatcher near-isogenic line with *Lr19* gene and without leaf rust resistance genes.

Genomic DNA was extracted from 5-day-old ethiolated seedlings using CTAB method. Examinations were based on SCAR marker specific for *Lr19* gene designed by Gupta et al. (2006). The primers sequences were: 5'-GCTGGTTCCACAAAGCAAA-3' and 5'-GGCTGGTTCCTTAGATAGGTG-3'. Conditions for PCR were as follows: 1 x buffer PCR (10x: 100 mM Tris-HCl pH 8,8 w 25 C; 500 mM KCl, 0,8% Nonidet P40); 2 mM $MgCl_2$; 200 μM of each dNTP; 250 nM of each primer; 0,75 U DNA *Taq* polymerase (Fermentas); 20 ng of genomic DNA. Thermocycling condition were: initial denaturation at 95 °C for 2 minutes; fallowed by 35 cycles of 94 °C for 1 minute, 60 °C for 1 minute, 72 °C for 2 minutes, and final extension step of 7 minutes at 72 °C. PCR was performed in T1 Biometra thermocycler.

PCR products were separated in 1,5% agarose gel containing 0.1% EtBr. Gene Ruler$^{TM}$100bp DNA Ladder Plus was used to establish molecular weight of the amplification products. Fragments were visualized under a UV transiluminator and photographed using the PolyDoc system.

## Results and discussion

Till date leaf rust resistance gene *Lr19* has been identified using many molecular markers. Autrique et al. (1995) identified RFLP marker for *Lr19* resistance gene. Prins et al. (2001) found AFLP marker co-segregated with *Lr19* gene and converted this marker into SCAR. Tyryshkin et al.(2006) check utility of this primers on 55 common wheat accessions from Vavilov Institute collection. In results authors did not observed the 130bp product size in some accessions possessing *Lr19* gene. Obtained results suggested that SCAR marker described by Prnis et al (2001) is not completely linked with *Lr19* gene. Gupta et al. (2006) found RAPD markers co-segregated with *Lr19* gene. They converted obtained markers into SCAR. Results shown that marker $SCS253_{736}$ was closely linked to analyzed gene. The validation of this marker in common leaf rust susceptible cultivars confirmed utility of this marker for identification of *Lr19* gene in marker-assisted selection.

In presented paper to identify *Lr19* gene in wheat genetic stocks we use pair of specific primers designed by Gupta et al (2006). We check utility of this primers on Thatcher near isogenic line with *Lr19* gene. We receive 736 base pair product size in susceptible individuals. Presence of this band indicate on absence of *Lr19* gene in wheat genotypes. Absence of 736bp amplification product indicate on presence of *Lr19* gene in analyzed material.

Among 96 analyzed wheat cultivars from 7 different countries we identified only two cultivars with *Lr19* leaf rust resistance gene were identified in Bulgarian winter cultivar Mir and in spring cultivar Planinka which come from Croatia (Tab.1).

## Conclusions

This result suggests that *Lr19* gene is not very popular source of wheat resistance to leaf rust in breeding programmes in South Eastern Europe countries. Moreover, because of rare occurrence of *Lr19* gene in European wheat genetic stock, two selected forms can be considered as a new donors for its introduction into wheat genetic background in breeding programmes.

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Table 1: The presence of *LR19* gene in wheat genetic stock from South Eastern Europe

| L.p | Cultivar | Country of origin | Presence of Lr19 gene | L.p | Cultivar | Country of origin | Presence of Lr19 gene |
|---|---|---|---|---|---|---|---|
| 1 | Itrashe | Albania | - | 49 | Vrazhdebka 4-4 | Bulgaria | - |
| 2 | Adam | Austria | - | 50 | Zenit | Bulgaria | - |
| 3 | Angerner | Austria | - | 51 | Zlatostruy | Bulgaria | - |
| 4 | Bonitus | Austria | - | 52 | Demetra | Croatia | - |
| 5 | Brillant | Austria | - | 53 | Golubica | Croatia | - |
| 6 | Cadenza | Austria | - | 54 | Goranka | Croatia | - |
| 7 | Equisit | Austria | - | 55 | Kata | Croatia | - |
| 8 | Farmer | Austria | - | 56 | Livanjka | Croatia | - |
| 9 | Granat | Austria | - | 57 | Mirjana | Croatia | - |
| 10 | Kaerntner | Austria | - | 58 | Planinka | Croatia | X |
| 11 | Kalif | Austria | - | 59 | Vidovica | Croatia | - |
| 12 | Loosdorfer | Austria | - | 60 | Barroko | Czech Republic | - |
| 13 | Multibraun | Austria | - | 61 | Ceska presivka | Czech Republic | - |
| 14 | Primus | Austria | - | 62 | Detenichka vouska | Czech Republic | - |
| 15 | Rubin | Austria | - | 63 | Dobrochna | Czech Republic | - |
| 16 | Saturnus | Austria | - | 64 | Dregerova cheska vouska | Czech Republic | - |
| 17 | Spartakus | Austria | - | 65 | Dvorskeho zora | Czech Republic | - |
| 18 | Tschermaks Blaukoerniger | Austria | - | 66 | Granny | Czech Republic | - |
| 19 | Weipro | Austria | - | 67 | Hanacka belka | Czech Republic | - |
| 20 | Belorusskaya 15 | Belarus | - | 68 | Hodoninska bezosinna | Czech Republic | - |
| 21 | Berezina | Belarus | - | 69 | Leguan | Czech Republic | - |
| 22 | Garmoniya | Belarus | - | 70 | Meritto | Czech Republic | - |
| 23 | Kandidatka | Belarus | - | 71 | Nela | Czech Republic | - |
| 24 | Kolhoznaya | Belarus | - | 72 | Sarka | Czech Republic | - |
| 25 | Lada | Belarus | - | 73 | Slovenska skora | Czech Republic | - |
| 26 | Minskaja | Belarus | - | 74 | Viglasska | Czech Republic | - |
| 27 | Minskaya Bolotnaya | Belarus | - | 75 | Wagin | Czech Republic | - |
| 28 | Plamya | Belarus | - | 76 | Yezka | Czech Republic | - |
| 29 | Poshuk | Belarus | - | 77 | Zdar | Czech Republic | - |
| 30 | Soyuz 50 | Belarus | - | 78 | Zidlochvicka lada | Czech Republic | - |
| 31 | Turavchanka | Belarus | - | 79 | Zuzana | Czech Republic | - |
| 32 | Akerman 17 | Bulgaria | - | 80 | Eka | Estonia | - |
| 33 | Bononija | Bulgaria | - | 81 | Helle | Estonia | - |
| 34 | Bulharska | Bulgaria | - | 82 | Iygeva kauka | Estonia | - |
| 35 | Burgas | Bulgaria | - | 83 | Jogeva 22 | Estonia | - |
| 36 | Carodejka | Bulgaria | - | 84 | Joni | Estonia | - |
| 37 | Dimitrovka 5-12 | Bulgaria | - | 85 | Kalvi | Estonia | - |
| 38 | Kaliakra | Bulgaria | - | 86 | Kuusiku | Estonia | - |
| 39 | Kardam | Bulgaria | - | 87 | Liniya 36 | Estonia | - |
| 40 | Karnobatskaya Rannespelaya | Bulgaria | - | 88 | Luunja | Estonia | - |
| 41 | Mir | Bulgaria | X | 89 | Luunya uluchshennaya | Estonia | - |
| 42 | Momtchil | Bulgaria | - | 90 | Meri | Estonia | - |
| 43 | Oenus | Bulgaria | - | 91 | Pikker | Estonia | - |
| 44 | Pliska | Bulgaria | - | 92 | Puuk | Estonia | - |
| 45 | Rusalka | Bulgaria | - | 93 | Sabu | Estonia | - |
| 46 | Stolec | Bulgaria | - | 94 | Sani | Estonia | - |
| 47 | Trapezica | Bulgaria | - | 95 | Umarik | Estonia | - |
| 48 | Vratza | Bulgaria | - | 96 | Universaal | Estonia | - |

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# A new *Vrn-B1* allele of wheat, *T.aestivum*: gene structure, transcription and geographical distribution

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Vernalization, or induction of flowering by exposing plants to low temperatures (Porter and Gawith 1999) is one of the important genetic factors affecting time to flowering and maturity in wheat (*Triticum aestivum* L.). Vernalization sensitivity in wheat is basically controlled by alleles at the major vernalization loci, *Vrn-A1*, *Vrn-B1*, *Vrn-D1*. Winter wheat possesses recessive alleles at all loci, whereas spring wheat has dominant alleles at one or more of these loci (Pugsley 1971). In recent years, the *Vrn-1* alleles were cloned and characterized at the DNA sequence level. It has been demonstrated that the dominant *Vrn-1* alleles for spring growth habits are associated with mutations in the promoter region as well as with large deletions or insertions in the first ($1^{st}$) intron of the corresponding gene (Yan et al. 2004, Fu et al. 2005). Stelmakh (1998) reported that the *Vrn* genes have different effects on heading time. The variation in common wheat heading time is also affected by multiple alleles of the dominant *Vrn-1* loci (Roberts and McDonald 1984; Koval and Goncharov 1998).

Previously, the NILs of winter wheat cv. Bezostaya 1 carrying the dominant alleles $Vrn\text{-}B1^{S}$ and $Vrn\text{-}B1^{Dm}$ (iBz/S29 and iBz/Dm, respectively) were obtained (Efremova et al. 2011). It was found that the time of ear emergence in iBz/S29 began 10 days earlier than in the iBz/Dm line.

Here, we report on the detailed molecular characterization of the $Vrn\text{-}B1^{S}$ and $Vrn\text{-}B1^{Dm}$ alleles and the analysis of their distribution among different Russian spring wheat cultivars.

## Materials and methods

To search for the molecular differences between $Vrn\text{-}B1^{S}$ and $Vrn\text{-}B1^{Dm}$ alleles using PCR, we chose the promoter and $1^{st}$ intron regions that contain the most mutations associated with the dominant alleles of *Vrn-1* genes for spring growth habit (Yan et al. 2004, Fu et al. 2005). For both regions, specific primers were designed based on genome-specific polymorphisms deduced from a database search and the comparison of different *Vrn-1* sequences from the

wheat genomes A, B and D (Fig. 1). The structure of primers, PCR conditions, cloning and sequencing of PCR products are described in Shcherban et al (2011). For the analysis of *Vrn-B1* transcription, we isolated RNA from plants at the different stages before heading: third-leaf , fourth-leaf and fifth-leaf stages. There were two independent experiments. Using reverse transcription, cDNA was obtained for each sample. Then we conducted PCR with specific primers allowing to amplify only the *Vrn-B1* gene sequences.

For the analysis of *Vrn-B1* polymorphism we selected 42 spring wheat cultivars from the collections in the Institute of Cytology and Genetics (Novosibirsk, Russia) and the Vavilov All-Russian Institute of Plant Industry RAN (St Petersburg, Russia). PCR primers reported in Fu et al. (2005) and Shcherban et al. (2011) were used to detect the presence of dominant or recessive alleles of *Vrn-B1* gene.

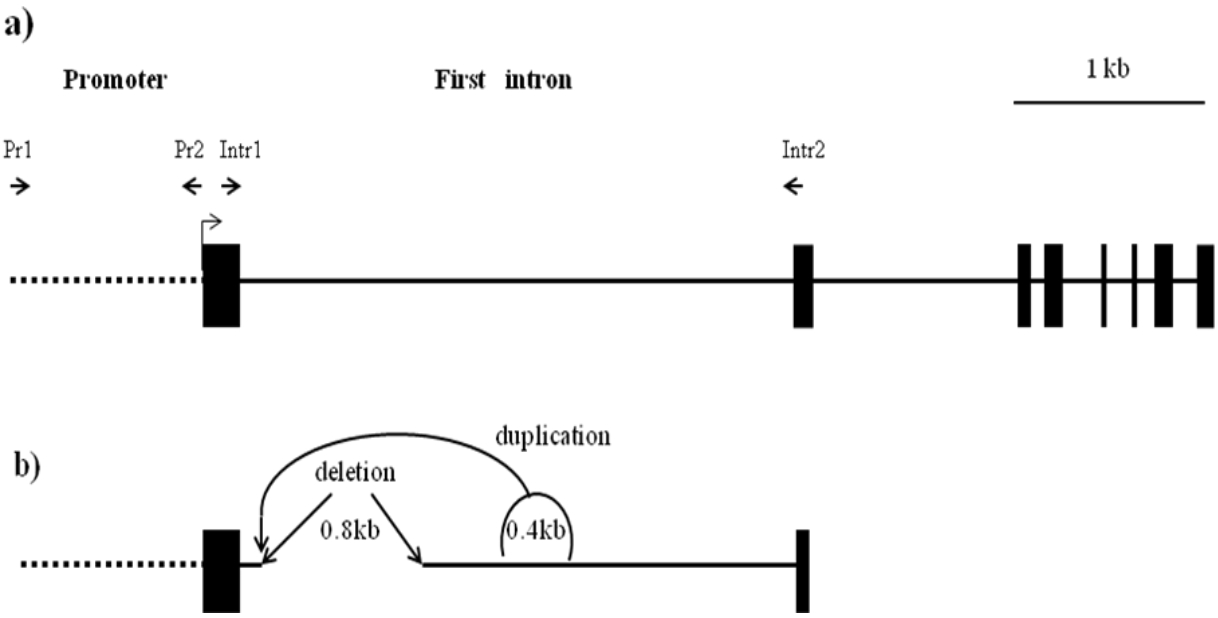


Fig. 1: Schematic representation of the *Vrn-B1* alleles: a) *Vrn-B1a* (AY747603); b) part of *Vrn-B1*$^{S}$ (Saratovskaya 29). Arrows above the scheme indicate the position of specific primers. The promoter region is indicated by dotted lines. The transcription initiation start is shown by an arrow. Exons are represented as short vertical black lines. Alterations within the *Vrn-B1*$^{S}$ allele relative to *Vrn-B1a*/*Vrn-B1*$^{Dm}$ are shown

**Results and discussion**

We carried out PCR analysis of iBz/S29 and iBz/Dm NILs and their respective donor cultivars Saratovskaya 29 and Diamant 2. In the case of the promoter region all PCR products were identical in size, whereas in the case of the 1$^{st}$ intron we observed a difference, namely, the NIL iBz/S29 and corresponding cultivar have a shorter product than the NIL iBz/Dm and cv. Diamant 2. The sequencing of the isolated PCR products revealed the complete identity of the promoter sequences of *Vrn-B1*$^{S}$, *Vrn-B1*$^{Dm}$ and previously studied *Vrn-B1* alleles, as dominant and recessive. The 1$^{st}$ intron sequences of cv. Diamant 2 and derivative NIL were almost identical to the previously studied sequence of *Vrn-B1a* allele from Triple Dirk B (Fu et al. 2005). We found significant differences in the structure of the 1$^{st}$ intron of the *Vrn-B1*$^{S}$ allele when compared to *Vrn-B1*$^{Dm}$ (*Vrn-B1a*), specifically, the deletion of 0.8 kb coupled

with the duplication of 0.4 kb (Fig.). We designated the new *Vrn-B1*$^{S}$ allele as *Vrn-B1c* to distinguish it from the previously studied *Vrn-B1* alleles.

We showed that transcription of the *Vrn-B1c* allele starts earlier than transcription of the *Vrn-B1a* allele, leading to earlier initiation of reproductive phase in corresponding NIL. Thus, we suggested that the observed changes within the 1$^{st}$ intron of *Vrn-B1c* allele leads to more rapid accumulation of the *Vrn-B1* product and earlier start of the reproductive phase in NIL iBz/S29.

Molecular characterization of *Vrn-1* genes in spring wheat germplasm from Russia and adjacent regions showed that 48% of cultivars have the *Vrn-B1a* allele, whereas 40% of them contain the *Vrn-B1c* allele. Identification of a novel *Vrn-B1c* allele will further aid in modifying flowering time, especially if breeders wish to employ the vernalization-sensitive dominant *Vrn* alleles in their breeding programs.

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## Relationship between anthocyanin biosynthesis and abiotic stress in wheat


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It is known that the scavenging of reactive oxygen under stressed environmental conditions is one of the suggested roles of plant anthocyanins. The level of the anthocyanins tends to increase under different stresses. For example, drought, cold, toxic metals in soils, UV-B irradiation, and pathogen attack induce biosynthesis of anthocyanins in different plants species (Treuter 2006). In bread wheat (*Triticum aestivum* L.), anthocyanin pigments were found in culm, leaf, auricle, pericarp, coleoptile and anther. However, protective role of anthocyanin pigmentation of wheat plant organs has not been widely studied yet. In the current study, relationship between anthocyanin biosynthesis in wheat seedlings and different types of abiotic stress (salinity, drought and heavy metal toxicity) was investigated.

## Materials and methods

*Plant material:* The following wheat genotypes with contrast grain pericarp and intensity of coleoptile colorations were used in the study: cultivar 'Saratovskaya 29' (S29) carrying *Rc* (red coleoptile) gene determining weak pigmentation and near-isogenic lines (NILs) i:S29Pp1Pp2$^{PF}$ and i:S29Pp1Pp3$^{P}$, developed on S29 background (Arbuzova et al. 1998), but having additional *Rc* gene conferring strong coleoptile pigmentation, along with 2 complementary *Pp* (purple pericarp) genes for anthocyanin pigmentation of pericarp, obtained from cultivar 'Purple Feed' and 'Purple', respectively (Fig.1a).

*Experimental procedure:* The germinated one-day old seedlings, growing on filter paper at 20°C under 12 hours daily cycle in climatic chamber «Rubarth Apparate» (RUMED GmbH), have been exposed to 0 (control), 100 mM and 200 mM NaCl, 25 µM and 50 µM $CdCl_2$ and 15% polyethylene glycol (PEG 6000) simulating salinity, heavy metal toxicity and drought stress, respectively. The experiments were conducted in three replicates (10-16 plant per replicate) for each concentration and each genotype. At the 3-7$^{th}$ day after germination, anthocyanins were extracted from the coleoptile in 1%-HCl/methanol according Khlestkina et al. (2011). The relative anthocyanin content was evaluated by spectrophotometry at 530 nm wavelength. To evaluate the role of anthocyanins in stress response at the seedling stage the length of shoots and roots was measured and compared among weak colored S29 and strong colored NILs under control and stress conditions. The differences among genotypes were tested by Mann-Whitney U-test, taking $p \leq 0.05$ as significant.

## Results and discussion

When grown on distilled water, S29 contained 3-4 times less anthocyanins in the coleoptile in comparison with the NILs (Fig.1b). Under salinity and drought stress conditions, the anthocyanin content in the coleoptile of S29, i:S29Pp1Pp2$^{PF}$ and i:S29Pp1Pp3$^{P}$ increased significantly 1.2-1.9-fold in comparison with untreated control, whereas under heavy metal stress, anthocyanin content increased (1.2–fold) in the coleoptile of S29 only (Table 1).

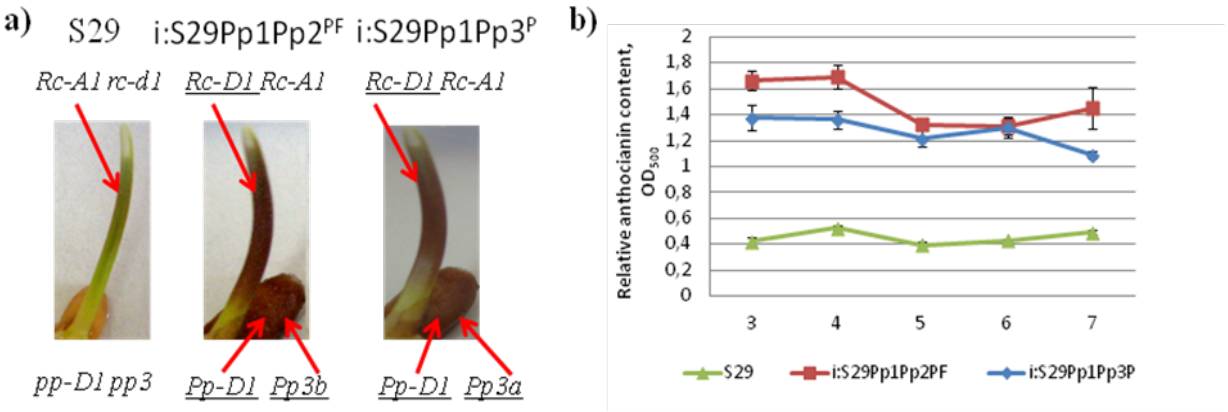


Fig.1: (a) Seedlings of S29, i:S29Pp1Pp2$^{PF}$ and i:S29Pp1Pp3$^{P}$ and (b) relative anthocyanin content estimated by spectrophotometry in coleoptiles of 3-7 day-old seedlings grown on distilled water, m±SE, n=3

To evaluate the role of anthocyanins for stress resistance at the seedling stage we estimated growth ability of S29 and NILs treated with NaCl, PEG and $CdCl_2$. The most sensitive

morphological parameter of the seedling under the abiotic stress conditions is roots length (Wilkins 1957, 1978). Relative root lengths of S29, i:S29Pp1Pp2$^{PF}$ and i:S29Pp1Pp3$^{P}$ are presented in the Table 2. Under drought conditions, root (Table 2) and shoot lengths (the data are not presented) of both NILs were significantly higher than those of S29, suggesting higher drought stress tolerance of the intensively colored NILs in comparison with S29. There was some tendency of a better growth ability of the NILs in comparison with S29 under medium salinity (100 mM NaCl; i:S29Pp1Pp2$^{PF}$ only) and heavy metal (25 µM $CdCl_2$; both NILs) stress, but not under high concentrations (200 mM NaCl and 50 µM $CdCl_2$; Table 2). When our data was ranged from 'strong' to 'weak' stress according to root growth suppression it was found that anthocyanins had adaptive effect under weak abiotic stress, when root length was 77–92 % relative to control. Under strong and medium stress, when root length varied from 8-60 % relative to control, the presence of the anthocyanin was not efficient. Based on this it may be suggested that antocyanins have adaptive role under weak abiotic stress only.

Table 1: Changes in relative anthocyanin content under salinity, drought and heavy metal stress conditions in coleoptiles of S29, i:S29Pp1Pp2$^{PF}$ and i:S29Pp1Pp3$^{P}$ in comparison with untreated control. ↑ - fold increase

| Stress factor | Intensity | S29 | i:S29Pp1Pp2$^{PF}$ | i:S29Pp1Pp3$^{P}$ |
|---|---|---|---|---|
| Salinity (NaCl) | 100 mM | ↑ 1.8 | ↑ 1.7 | ↑ 1.9 |
| | 200 mM | ↑ 1.6 | ↑ 1.4 | ↑ 1.3 |
| Drought (PEG) | 15% | ↑ 1.7 | ↑ 1.2 | ↑ 1.3 |
| Heavy metal ($CdCl_2$) | 25 µM | ↑ 1.2 | no increase | no increase |
| | 50 µM | ↑ 1.2 | no increase | no increase |

It is known, that under different types of stress reactive oxygen species (ROSs) are generated in plant cells (Miller et al. 2009; Michalak 2006). ROSs damage cellular membranes, DNA, proteins, lipids and chlorophyll. Anthocyanins can help plant to withstand ROSs because of their antioxidant activity (Wang et al. 1997). Also it is known that anthocyanin compounds can act as osmoregulators (Chalker-Scott 1999), what is very important under osmotic stress induced by high salinity and drought, and can chelate and sequestrate heavy metal ions (Elhabiri et al. 1997; Hale et al. 2001). This may explain (1) the increase of anthocyanin content in the wheat coleoptile we observed in different genetic models under salinity, drought and heavy metal toxicity (Table 1) and (2) the correlation between growth ability of wheat seedlings under abiotic stress conditions and genetic differences in the loci determining anthocyanin pigmentation of the grain pericarp and the coleoptile (Table 2).

**Conclusion**

Thus, using different genetic models significant intensification of anthocyanin biosynthesis in wheat coleoptiles under salinity, heavy metal and drought stress was shown. Though different effect of the anthocyanin pigmentation on the growth ability under different stress condition was observed, nevertheless there was the tendency of the lines having more intensive anthocyanin pigmentation to have better growth ability under stress condition. Taken together the results obtained it may be suggested that anthocyanin production in wheat seedlings is

tightly related with the response to abiotic stress such as drought, salinity and heavy metals toxicity.

Table 2: Root length of S29, i:S29Pp1Pp2$^{PF}$ and i:S29Pp1Pp3$^{P}$ relative untreated control under salinity, drought and heavy metal stress conditions during 5 day of experiment, divided to that in S29. * - significant between S29 and the near-isogenic lines at $p \leq 0.05$ (U-test).

| Stress factor | Intensity | S29 | i:S29Pp1Pp2$^{PF}$ | i:S29Pp1Pp3$^{P}$ |
|---|---|---|---|---|
| Salinity (NaCl)/ exp1 | 100 mM | 1.0 | 1.1 | 0.9 |
| | 200 mM | 1.0 | 1.0 | 1.0 |
| Drought (PEG) | 15% | 1.0 | 1.1* | 1.1* |
| Heavy metal ($CdCl_2$) | 25 μM | 1.0 | 1.1 | 1.1 |
| | 50 μM | 1.0 | 1.0 | 1.0 |

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# Development of a high-density consensus map in durum wheat

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Although durum wheat accounts for about 10% of the total wheat production, it is particularly important for its end products, which are mainly pasta, couscous and burghul. An intense breeding activity has been carried out over the last century to improve durum wheat varieties in terms of their grain yield and quality, disease resistance, and drought tolerance (De Vita et al. 2007). The phenotypic variations of many complex traits of agricultural or evolutionary importance are influenced by multiple quantitative trait loci (QTLs), their interactions, the environment, and the interactions between QTLs and the environment (Gupta et al. 2008).

Linkage mapping has been largely adopted in wheat to identify genomic regions involved in the control of complex traits (Gupta et al. 2008; Breseghello and Sorrells 2006; Kuchel et al. 2007), and many genetic maps of durum wheat have been published (Blanco et al. 1998; Nachit et al. 2001; Elouafi et al. 2004; Zhang et al. 2008; Peleg et al. 2008; Mantovani et al. 2008; Gadaleta et al. 2009).

The construction of integrated maps provides the opportunity to overcome the limitation in the number of markers that can be added to a biparental map due to the low level of polymorphism between durum wheat cultivars (Trebbi et al. 2011). The importance of the construction of consensus maps relies on the development of genetic tools that provide an essential basis for further genomic research.

The main aim of this study was to develop a high-density durum wheat consensus map that was derived from the integration of six individual maps, as a reference resource for durum wheat scientists in molecular breeding programs, as well as for comparative genomics within grass species.

## Materials and methods

A total of six mapping populations were developed to serve the specific needs for the qualitative and quantitative trait analyses, and they were integrated into a single consensus map (Table 1).

All of the listed parental genotypes are durum wheat varieties, except MG4343, which is an accession of *Triticum turgidum* (L.) sub-species *dicoccoides*. The individual maps carried between 290 (CN) and 830 (CS) loci. The marker density was from 2.1 (CS) to 6.6 (MM) cM/marker. Several sources of markers were considered.

The JoinMap 4.0 software was used to reproduce the six durum wheat genetic maps and to generate the consensus map. The significance of deviations of the observed allelic frequencies from the expected ratio (1:1 or 1:2:1) ($P < 0.01$) was tested by Chi-squared analysis. The segregation datasets of each mapping population were first analyzed chromosome by chromosome, using a minimum LOD score of 4. The Kosambi mapping function and the "fixed order" of marker loci were used to reproduce linkage groups corresponding to the

single maps developed previously. Subsequently, the linkage groups for each chromosome derived from the six mapping populations were joined using the "combine groups for map integration" function within the JoinMap software.

Table 1: Summary of the six mapping populations used to construct the consensus map of durum wheat

| Parents | Population size | Total markers | Map length (cM) | Marker density (cM/marker) | Reference |
|---|---|---|---|---|---|
| 'Creso' × 'Pedroso' | 123 | 575 | 2221.3 | 3.8 | Marone et al. 2009 |
| 'Ofanto' × 'Cappelli' | 161 | 618 | 1649.4 | 2.6 | Verlotta et al. 2010 |
| 'Cirillo' × 'Neodur' | 178 | 290 | 1568.5 | 5.4 | Russo et al. 2012 |
| 'Ciccio' × 'Svevo' | 120 | 830 | 1765.8 | 2.1 | Gadaleta et al. 2009 |
| 'Latino' × 'Primadur' | 121 | 440 | 1066.2 | 2.4 | Blanco et al. 2011 |
| 'Messapia' × 'MG4343' | 65 | 440 | 2913.2 | 6.6 | Blanco et al. 2004 |

Once developed, the consensus map was compared with the six individual maps, and with bread and durum wheat maps available in the literature, in terms of the marker order and the genetic distances between markers.

**Results**

The six mapping populations were used to integrate nearly 2,000 unique loci into a single consensus map. A total of 650 mapped markers (216 PCR-based, and 434 DArT) were common among at least two of the mapping populations, while a total of 1,397 markers unique to a specific mapping population.

The loci integrated into the durum wheat consensus linkage map were arranged into 27 linkage groups. The total length of the integrated map was 3,058.6 cM. The mean length of the 27 linkage groups was 218.4 cM. The distance between loci was between 0.9 (4A and 3B) and 3.9 cM (5B), with an average density of one marker per 1.6 cM.

Segregation distortion was found across many of the durum wheat chromosomes, with a higher frequency for the B genome. This high-density consensus map allowed the scanning of the genome for chromosomal mutations. Translocations and inversions that were already known in the literature were confirmed, and new putative rearrangements have been identified.

This consensus map constitutes the most detailed genetic map for durum wheat to date. Furthermore, since different classes of molecular markers are well represented in the consensus map (from RFLP to DArT), this represents an optimal tool to collect QTL data from most of the published studies. The QTL information available in the literature can be

referred to precise chromosome regions on the consensus map by means of meta-QTL analysis. Integrated maps are also essential for the evaluation of linkage disequilibrium in germplasm collections and for performing association mapping analysis.

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# Linkage disequilibrium and population structure in tetraploid wheat

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*Triticum turgidum* is an annual, predominantly self-pollinating species. The taxonomic classification of *T. turgidum* (L.) (2n=4x=28; AABB genome) includes five sub-species (ssp.): *carthlicum* (Nevski), *dicoccoides* (Körn), *dicoccum* (Schrank), *turgidum* (L.), and *paleocolchicum* (Men.). The ssp. *turgidum* comprises four convarieties (convar.): *durum* (Desf.), *turanicum* (Jakub.), *polonicum* (L.) and *turgidum* (L.) (Mac Key 1975). Durum wheat (*T. turgidum* ssp. *turgidum* convar. *durum*) is the main sub-species, and it is cultivated on approximately 17 million hectares worldwide. For this species the combined effect of domestication and modern plant breeding have contributed to greatly reduce the genetic diversity in comparison to the wild forms (Fu et al. 2005, 2006; Haudry et al. 2007; Thulliet et al. 2005). In this context, landraces, wild forms (*T. turgidum* ssp.) and other related wild species have important roles in breeding programs (Moragues et al. 2006, 2007; Peleg et al. 2008; Valkoun 2001).

Genetic diversity can be estimated by different approaches, which include pedigree analysis, genetic markers (morphological traits, biochemical and molecular markers), and quantitative traits. In particular, pedigree and marker information are crucial to correctly describe the population structure and level of linkage disequilibrium (LD) that characterize a species, and the population used for association analysis.

In this context, the objectives of our study were to: (a) examine the population structure and genetic diversity in a collection of tetraploid wheat germplasm, based on SSR and DArT markers; and (b) to estimate the level of LD.

## Materials and methods

Plant material: The tetraploid wheat (*T. turgidum* L., 2n=4x=28; AABB genome) collection, classified according to Mac Key (1975) and listed in Table 1, consists of 19 accessions of *T. turgidum* ssp. *dicoccum*, 12 accessions of *T. turgidum* ssp. *carthlicum*, 12 wild accessions of *T. turgidum* ssp. *dicoccoides*, and 187 accessions of *T. turgidum* ssp. *turgidum*. This last comprised 128 varieties of ssp. *turgidum* convar. *durum*, 20 accessions of ssp. *turgidum* convar. *turanicum*, 20 accessions of ssp. *turgidum* convar. *polonicum*, and 19 accessions of ssp. *turgidum* convar. *turgidum*.

These durum wheat varieties represent a comprehensive selection of Italian accessions (mainly elite varieties), designed to be representative of Italian durum wheat breeding programs over the last 100 years, with a subset of varieties from the most important durum wheat production areas (96 are a comprehensive selection of Italian accessions, while 32 are from other countries). These were collected by the Cereal Research Centre (CRA-CER, Foggia, Italy) and the Department of Environmental and Agro-Forestry Biology and

Chemistry of the University Aldo Moro (Bari, Italy). The remaining wild and domesticated wheat accessions were kindly provided by the National Small Grain Collection (Aberdeen, USA), John Innes Centre (Norwich, UK), Institute of Crop Production, Genebank Department (Czech Republic) and CNR Institute of Plant Genetics (Bari, Italy). Twenty plants of each accession were sown in Valenzano (Bari, Italy), and a single plant that represented the prevalent biotype of each accession was selected and grown to maturity, to produce self seed.

Table 1: Tetraploid wheat collection

| Taxomonic classification *Triticum* (L.) | Number accessions |
|---|---|
| *T. turgidum* ssp. *turgidum* var. *durum* | 128 |
| *T. turgidum* ssp. *turgidum* var. *turanicum* | 20 |
| *T. turgidum* ssp. *turgidum* var. *polonicum* | 20 |
| *T. turgidum* ssp. *turgidum* var. *turgidum* | 19 |
| *T. turgidum* ssp. *carthlicum* | 12 |
| *T. turgidum* ssp. *dicoccum* | 19 |
| *T. turgidum* ssp. *dicoccoides* | 12 |
| Total | 230 |

*Morphological and biochemical analysis:* The accessions were evaluated for six morphological traits that were recorded according to the descriptors defined by the International Plant Genetic Resources Institute (http://www.cgiar.org/ipgri). In particular, during the growing season of 2008-2009, the traits evaluated were: glumes color (for the outer glumes), awns color, awned and awnless form, spike and culm glaucousness. At harvest time, the trait of naked/ hulled kernel was evaluated.

The collection was investigated for glutenin alleles located at five loci (Glu-A1, Glu-B1, for high molecular weight subunits, HMW-GS; GluA3, Glu-B3, Glu-B2, for low molecular weight subunits, LMW-GS), and for gliadin alleles at four loci (Gli-A1, Gli-B1, Gli-A2, Gli-B2).

*DNA extraction and SSR and DArT analysis:* The leaf tissue of the representative plants of the prevalent biotypes of the accessions were used for DNA extraction, according to the protocol described by Sharp et al. (1988). The wheat collection was genotyped with 26 simple sequence repeat (SSR) markers selected from durum wheat maps developed by CRA-CER (Foggia, Italy) and Department of Environmental and Agro-Forestry Biology and Chemistry of the University Aldo Moro (Bari, Italy). The selection of the SSR markers was based on the following criteria: locus-specific amplification, low complexity, robust amplification, good genome coverage (one maker for each chromosome arm).

Genotyping of the DArT was performed by Triticarte Pty. Ltd. (Canberra, Australia; http://www.triticarte.com.au), a whole-genome profiling service laboratory, as described by Akbari et al. (2006). The locus designations used by Triticarte Pty. Ltd. are adopted in this

study, and these DArT markers are named using the prefix 'wPt-'. Alleles occurring at a low frequency ($p < 0.05$) were excluded from the analysis.

## Results

*Genetic diversity for morphological traits and seed storage protein loci:* The wheat collection was evaluated for six morphological traits and nine seed storage protein loci. The durum wheat group together with *T. turgidum* ssp. *turgidum* convar. *turgidum*, ssp. *dicoccum* and ssp. *dicoccoides* showed higher allele numbers than those detected in *T. turgidum* ssp. *turgidum* convar. *turanicum*, convar. *polonicum* and ssp. *carthlicum*.

*Genetic diversity for SSR and DArT markers:* A set of 26 SSRs were tested on the wheat collection. Eleven markers were localized on genome A and fifteen on genome B. A total of 436 alleles were amplified over all of the accessions and the allele numbers ranged from 133 (ssp. *carthlicum*) to 211 (ssp. *turgidum* convar. *durum*). Differences were less evident for the genetic diversity ($H_E$) among the sub-species of these tetraploid wheats. For the dataset of 970 DArT markers, there was a loss of genetic diversity, which confirms that the landraces and wild accessions have higher allelic diversity than the modern durum wheat varieties, for both genomes and for all chromosomes, in terms of total numbers of alleles and allelic richness.

*Structure and linkage disequilibrium of the tetraploid wheat collection:* The substructure within this collection of 230 tetraploid wheat accession was investigated, using 26 unlinked SSR markers distributed across the genomes (one marker for each chromosome arm), by Bayesian analysis using the software package STRUCTURE 2.1 (http://pritch.bsd.uchicago.edu/structure.html). According to the method of Evanno et al. (2005), $\Delta K$ was plotted against the number of sub-group K. The maximum value of $\Delta K$ occurred at K=2 and K=7.

The LD between DArT markers was assessed by calculation of $r^2$ between markers, using TASSEL 2.1 (http://www.maizegenetics.net). For genome-wide association mapping, more LD can be an advantage if the marker density is low. For analyzing LD decay, the genetic distances for 592 DArT markers were obtained from a consensus linkage map constructed by the integration of six durum wheat mapping populations developed to serve specific needs in qualitative and quantitative trait analysis. The predicted $r^2$ value declined to 0.1 within 15 cM. The wild accessions were characterized by very low levels of LD, while the subgroup containing the durum wheat genotypes had a higher LD. Overall, our data indicate that the wild and domesticated accessions studied represent a rich source of useful alleles for plant breeding, and a powerful tool to detect and identify genes and QTLs using association mapping.

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# MBR1012 x Scarlett: A new DH population for genetic dissection of resistance to different pathogens in barley


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Leaf rust and net blotch, caused by the fungi *Puccinia hordei* and *Pyrenophora teres*, respectively, are important diseases of barley (*Hordeum vulgare* L.) worldwide. The appearance of new virulent races of these pathogens and depletion of the gene pool of cultivated barley for major resistance genes demands a continuous supply of new sources of disease resistance. Although the diversity present in barley landraces represents a good base for search and transfer of important traits into elite cultivars, they are scarcely used in breeding programs. A barley landrace collection from Serbia was surveyed for novel resistances to *P. hordei* and *P. teres.* The landrace 'MBR1012', exhibited a hypersensitive resistance reaction to *P. hordei* isolates, and displayed a high level of resistance to three *P. teres* isolates in leaf segment tests. In order to map resistance genes, a population that consists of ninety-one doubled haploid (DH) lines derived from a cross to the German malting barley cultivar Scarlett, which is susceptible to all tested isolates, was constructed and lines were evaluated for resistance to both pathogens. In parallel, the population was genotyped on the

newly developed Illumina iSelect custom 9K bead chip, resulting in the construction of a high density SNP map and the identification of closely linked markers for resistances studied. Our results revealed the presence of novel fungal resistance genes and in parallel provide the tools for their efficient deployment in a barley breeding program.

## Introduction

Leaf rust, caused by *Puccinia hordei* Otth, and the net type of net blotch caused by the fungal pathogen *Pyrenophora teres* f. *teres* (Died.) are important diseases of barley (*Hordeum vulgare* L.) worldwide. Under experimental conditions yield losses caused by leaf rust can be up to 60% in susceptible barley cultivars (Das et al. 2007), while *P, teres* usually causes yield losses up to 40% (Liu et al. 2011). In barley (*Hordeum vulgare)* 20 major leaf rust resistance genes (*Rph1* to *Rph20*) have been described (Hickey et al. 2011). These major genes are effective against different pathotypes of *P. hordei*, but when deployed singly have often been overcome by new pathotypes. Also, *Pyrenophora teres f. teres* is a highly variable pathogen. This variability combined with the adoption of reduced or zero tillage practices has increased the incidence of the net type of net blotch significantly in recent years (Lehmensiek et al 2007). In order to prevent yield losses caused by these pathogens resistance is the most cost-effective and environmentally friendly approach. Although, many resistance genes and quantitative trait loci against the main diseases affecting barley are already known (Ordon 2009), most of these resistances, have been overcome by virulent races raising the need for new and durable sources of resistance. The long term use of genetic resistances in breeding is hampered by the rapid breakdown of these resistances due to the appearance of new strains of respective pathogens (Dreiseitl 1990; Fetch et al. 1998, Steffenson et al. 1993, Silvar et al. 2010). Therefore, breeders need a continuous supply of new sources of disease resistance and tools for an efficient incorporation into elite lines to ensure a sustainable barley production.

To identify new sources of rust and net blotch resistance, barley germplasm from Serbia were evaluated (Perovic et al. 2001). A landrace ('MBR1012') exhibited a qualitative resistance reaction to isolate I-80 *of P. hordei* and to three *P. teres f. teres* monoconidial isolates (BST-AR, - F74 and – d8_4) in leaf segment tests. Thus, studies were undertaken to (i) investigate the genetics of leaf rust and net blotch resistance in 'MBR1012', (ii) develop molecular markers for these resistance genes facilitating efficient marker based selection procedures, (iii) saturate resistance loci through the use of the newly developed Illumina iSelect custom 9K bead chip, and (iv) develop a high density barley map.

## Material and methods

*Plant material*: An $F_1$-derived doubled haploid (DH) population produced via anther culture comprising 91 lines of a cross between the landrace 'MBR1012', that is resistant to leaf rust and net blotch, and the German cultivar 'Scarlett', which is susceptible to corresponding isolates of both pathogens was used for mapping.

*Leaf rust resistance tests:* Five to ten plants of each DH line and the parents were inoculated with the leaf rust isolate I-80 in the greenhouse. Plants were inoculated at the seedling stage, according to Ivandic et al. (1998). Infection types were recorded between 10 and 12 days after inoculation according to the 0 – 4 scale of Levine and Cherewick (1952). Plants exhibiting infection types from 0, $0_{nc}$ (hypersensitive reactions with necrotic/chlorotic 'flecks'), 1, 2-, or

0-2- were considered as resistant, while those exhibiting infection types 2+, 3, 3-4 and 4 were considered as susceptible. The chi-square test was used to assess segregation ratios for goodness of fit to expected ratios.

*Net blotch resistance tests:* Regarding net type of net blotch, *P. teres* inoculum was prepared and inoculation and disease rating conducted according to Tekauz (1990). Inoculum of three monoconidial isolates (BST- AR, - F74 and – d8_4) was newly produced prior to each test from sporulating leaf tissue pieces cultivated in moist chambers. Resistance tests were performed on five individual plants of each DH-line. In parallel, in order to get data on field resistance, the DH lines and parents were grown at three locations (Quedlinburg, Thüle and Leopoldshöhe) in Germany in a randomized block design with two replications during 2009 and 2010 in so called summer hill trials. Artificial infection was achieved by infested straw debris before sowing (early August) and the area under the disease progress curve (AUDPC) was calculated using the RESI software (Moll et al. 1996).

*DNA extraction and marker analyse:* Genomic DNA of DH-lines was extracted from leaves of 14-day-old plants according to Stein et al. (2001). The concentration and quality of DNA of DH lines was determined using the NanoDrop ND-100 spectrophotometer (PeQLab, Erlangen, Germany) and gel electrophoresis. All DNA of DH lines were adjusted to a final concentration of 20ng/µl. For bulk segregant analysis (BSA) (Michelmore et al. 1991), equal aliquots (10µl) of DNA from nine leaf rust resistant and nine susceptible DH lines were pooled. DNA for the iSelect analysis was adjusted to a final concentration of 50ng/µl. Initially, parental lines were screened for polymorphism with 175 SSR and 73 CAPSs markers. The sequences of the SSR primer pairs and amplification protocols were obtained from Struss and Plieske (1998), Ramsay et al. (2000), Macaulay et al. (2001), Thiel et al. (2003), and Varshney et al. (2007), while the sequences of the SNP primer pairs and the respective amplification protocol and restriction sites were obtained from Kota et al. (2008) and Perovic et al. (2004). PCR, capillary electrophoresis and CAPS analysis were performed according to Perovic et al. (2004).

*iSelect genotyping and linkage analysis:* In a next step 91 DH lines were additionally genotyped on the newly developed 9K Infinium iSELECT high density custom genotyping bead chip (Comadran et al. in prep). The genetic map of DH lines was constructed using JoinMap 4.0 (van Ooijen 2006) applying the Kosambi function (Kosambi 1944), while the map of the iSelect SNPs was constructed by using a maximum likelihood algorithm of Joinmap 4. Only markers with a LOD score of 3 were integrated into the map.

**Results and discussion**

Leaf rust phenotypic analysis with isolate I-80 revealed that the infection type of the susceptible parent Scarlett was 2-3, whereas the corresponding one of the resistant parent ‘MBR1012’ was 0-2-. The 91 DH lines derived from the cross of the resistant landrace ‘MBR1012’ and the susceptible German cultivar ‘Scarlett’ segregated 48 resistant: to 43 susceptible ($\chi^2 = 0.29$). This segregation pattern fits well to the expected 1 resistant : 1 susceptible ratio, indicating that leaf rust resistance against isolate I-80 in ‘MBR1012’ is inherited in a monogenic manner (König et al. under review). Initial screening of parental lines with a set of 248 co-dominant molecular markers revealed that 89 SSR (51%) and 21

SNP markers (29%) were polymorphic. Polymorphisms between the resistant and susceptible bulk were detected on chromosome 1H (König et al. under review). Overall, out of the 32 SSR and 11 SNP markers localized on chromosome 1H, 14 and 3 were polymorphic, respectively. Based on these markers, a final genetic map of 119 cM of chromosome 1H was constructed and the leaf rust resistance in landrace 'MBR1012' was mapped on the distal portion of the short arm of chromosome 1H.

Net blotch infection type of the resistant landrace 'MBR1012'to monoconidial isolates varied from 1 to 3, whereas the corresponding ones of the susceptible cultivar 'Scarlett'varied from 4 to 8 (data not shown). According to this result, the different scores of DH lines were grouped as follows:1 - 3 resistant and 4 - 9 susceptible. The 91 DH lines segregated ((BST – AR; 50r : 41s ($\chi^2 = 0.91$); BST - d8-4; 45r : 44s ($\chi^2 = 0.02$); and BST - F74; 38r : 53s ($\chi^2 = 2.51$). Respective resistance genes were mapped to barley chromosomes 2H, 3H and 4H. At the same time, analysis of field experiment showed quantitative variation of infection fitting to a Gaussian distribution. Phenotypic results revealed a good correlation between locations (Pearson r>0.8) and years (Pearson r>0.7). Heritability for the net blotch resistance was calculated at $h^2 = 0.57$. Quantitative trait loci (QTL) for resistance were detected on barley chromosomes 1H, 3H, 5H and 7H accounting from 6.2% to 14.3% of the phenotypic variance.

The 91 DH-lines of the cross between the landrace 'MBR1012' and the German cultivar 'Scarlett' including parental lines were genotyped on the newly developed Illumina iSelect custom 9K bead chip (Comadran et al. in prep). Based on these results a set of 2806 SNP markers has been placed onto the 'MBR1012' x 'Scarlett' map, which after including 111 SSRs and SNPs consists of 2917 markers covering 2326 cM. Therefore, this map represents currently one of the most dens barley maps. At the $Rph_{MBR1012}$ locus, 32 new SNP markers were placed between flanking markers allowing fine mapping and marker saturation of this resistance locus (data not shown). Genetic map of the 'MBR1012'x 'Scarlett' population also enable mapping of three monoconidial resistances and QTL detection of field resistances to net blotch.

## Acknowledgements

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# Genome wide association analysis for cold resistance in barley (*Hordeum vulgare* L.)

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## Introduction

Plant responses to abiotic stresses such as drought, cold (chilling and freezing) and soil salinity are major factors limiting crop genetic yield potential, thus regulating their geographical distribution and has critical implications for agriculture. Understanding how genotypes interact with the photo-thermal environmental queues driving crop adaptation is a difficult task. The main reasons being the unpredictability, in terms of timing, intensity and duration of abiotic stresses, the highly polygenic nature of the traits - controlled by many

genes with small additive effects - and strong genotype by environment interaction (Rizza et al. 2011). Winter-hardiness refers to the ability of plants to tolerate and withstand chilling and freezing temperatures that occur during the winter season, and can be defined as the final manifestation of many component traits and is induced by exposition to low non-freezing temperatures, a process known as *hardening* (Hayes et al., 1997; Flower et al., 1999; Giorni et al., 1999). The degree of winter-hardiness depends on the differential response of genotypes to the duration and intensity of the hardening period prior to freezing temperatures and the duration, intensity of the freezing events, which can alternate with thaw periods. One of key traits is the capacity of vegetative tissues to survive freeze-induced desiccation (Steponkus et al., 1993) often called frost resistance. Major efforts to dissect the genetic basis of winter-hardiness in the recent past, have been based on the use of classical bi-parental cross – mapping populations. Bi-parental populations can be used to estimate QTL genetic map positions and their associated genetic effects. However, in last ten years a limited number of major and minor QTL have been reported for cold resistance. The usually small population sizes used coupled with the presence of several major genes segregating within the mapping population (most bi-parental populations used to study winter-hardiness involve spring x winter crosses) limit our power to detect smaller genetic effects and important gene x gene interactions, and may obscure the interpretation of the data. Recently, thanks to the reduction in genotyping costs, there is currently great interest in so-called genome wide association studies (GWAS) where a collection of genetically diverse accessions, instead of bi-parental populations, are used to fine-map traits of interest.

To identify barley varieties with superior cold tolerance and advance our understanding of the genetics of low temperature tolerance, we assembled barley accessions sampling the cultivated diversity across the Mediterranean basin, genotyped with 1536 SNPs. Analysis of the phenotypic data for cold resistance collected during an extraordinary cold season in Spain during year 2005 revealed 8 genomic regions involved with cold resistance. Some of the associations involved SNPs tightly linked to known major genes and *loci* determining cold resistance in barley. Association analysis of this data shows the genetic basis underlying cold tolerance in autumn sown winter growing conditions is genetically richer than a priori thought.

## Materials and methods

*Phenotypic and genotypic data:* The assembled 185 barley dataset for the study consisted of MABDE (Comadran et al. 2009) accessions selected to represent the past and present of the cultivated barley genetic diversity around the Mediterranean basin. Plant material utilized in this study represents a geographically diverse range of spring, winter and facultative cultivated barley forms that have been described in more detail in a previous manuscript (Comadran et al. 2009). The 185 accessions were genotyped using bOPA1 Illumina GoldenGate oligonucleotide pool assay which contains probe sets for 1,536 SNPs. Details about the array and genetic map position of the SNP markers are described in detail in a previous study (Close et al. 2009). DARwin v.5.0 (Perrier and Jacquemoud-Collet 2006) was used to construct a Neighbor joining tree of the 184 barley cultivars from simple matching distances of 1,307 SNPs with MAF > 10%. The set of selected accessions was sown in November 2004 in Foradada (Spain, 41º39'N, 01º29'E), The trial was grown as an augmented cyclical design using an incomplete block size of 60 including 4 checks, each repeated three times in each block laid out in a diagonal fashion at fixed distance. Agronomic practices, including sowing rate weed control and fertility management, were conducted in accordance with local practice.

Minimum and Maximum temperatures were recorded during the growing season (Fig. 1). Winter survival was evaluated at the end of March 2005. Cold injury was visually estimated on a 0-5 scale as described by Akar et al. 2009. The exceptional environmental conditions experienced, during winter 2005 in Spain made possible to get valuable information on cold tolerance under natural field Autumn-sown conditions in the association panel. Minimum and maximum temperatures recorded in Foradada (Spain) shows the severity of frost episode experienced by the GWA population with long and severe freezing periods, where minimum temperature between -5ºC and -10ºC were observed (Fig. 1).

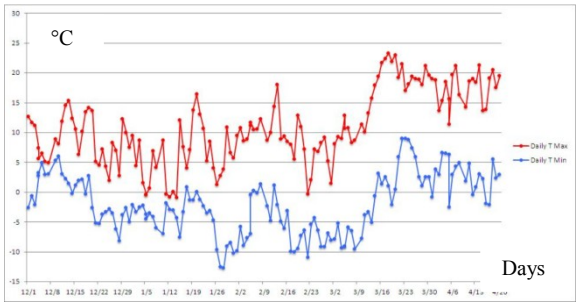


Fig. 1: Daily T Max and T Min recorded in Foradada (Spain) during winter 2005

*Statistical analysis:* Repeatability ($H^2$) of cold tolerance measures was high ($H^2 = 0.84$). Best linear unbiased predictions (BLUPs) of line performance for cold resistance were calculated using Restricted Maximum Likelihood (*REML*) directive in Genstat v.14 (VSN International). In the model, checks were used as a fixed effect, and columns, rows and test entries were used as random effects. Check varieties and partial replicates provided the overlap needed so that line BLUPs could be adjusted for trial spatial effects. ANOVA for the phenotypic data in relationship to geographic origin, growth habit and ear morphology (main drivers of germplasm genetic divergence in barley) was done with Genstat v.14 (VSN International) statistical package (Table 1). GWAs were carried out by using a mixed linear regression model (Yu et al. 2006), which accounts for multiple levels of genetic relatedness due to historical population substructure and kinship. To correct for population substructure, we used both Eigenstrat relationship model with PCA scores as random terms (Price et al. 2006 and kinship (Yu et al. 2006) mixed-model association approaches as implemented in Genstat v.14 (VSN International). TASSEL v. 3.0 was used to estimate the kinship matrix (K) from a subset of random markers covering the whole genome so that we did not over-estimate sub-population divergence.

**Results and Discussion**

Barley accessions used in this study provide a comprehensive coverage of past and present barley breeding. The severity of the frost episode experienced by plants and the broad genetic diversity present in the dataset, provided valuable information on cold resistance in barley under natural field Autumn-sown conditions. Substantial variation in cold tolerance was observed. As expected, because of historical local and / or seasonal environmental adaptation, preliminary analysis of variance of cold tolerance with regard to germplasm type (landraces, old cultivars and elite cultivars), the geographic origin and seasonal growth habit of the germplasm showed strong significance (Table 1).

Table 1. ANOVA for the phenotypic data in relation to the main drivers of germplasm genetic divergence in barley: geographic origin of the germplasm, growth habit (spring/ winter / facultative) and ear morphology (two-rowed / six-rowed types) and germplasm type (landraces / old cultivars / modern cultivars

| ANOVA fixed term | n.d.f. | Wald statistic | F pr |
|---|---|---|---|
| Germplasm type | 2 | 30.83 | <0.001 |
| Growth habit | 2 | 76.45 | <0.001 |
| Region of origin | 4 | 43.56 | <0.001 |
| Ear type | 1 | 7.85 | 0.006 |

We evaluated the genetic relationships among the accessions by generating a neighbour-joining population tree based on simple matching of allelic distances), which produced clear separated branches corresponding to each of our germplasm clusters: Turkish, Syrian and Jordan, Northern European winters, Northern European springs and South-West Mediterranean accessions. Analysis of the phenotypic data for cold resistance revealed 8 genomic regions involved with cold resistance. Some of the associations involved SNPs tightly linked to known major genes and *loci* involved in cold resistance in barley (Figure 2).

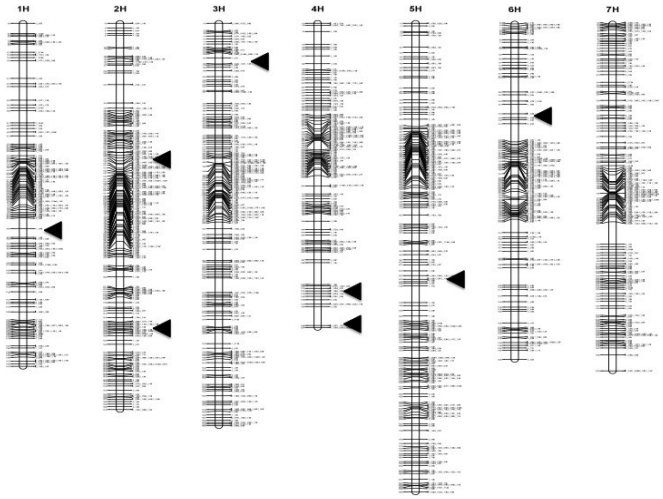


Fig. 2. Position of detected QTL on barley bOPA consensus map

The QTL on the long arm of chromosome 5H (108 cM) is located within a physically linked cluster of at least 11 family members of *C-repeat binding factor* (*CBF*) genes, also known as *DRE binding protein 1* (*DREB1*) which correspond to the cold tolerance *'Fr-2H'* locus (Francia et al. 2007). The QTL on the long arm of chromosome 2H (128 cM) mapped on the GWA panel is collinear with the recently fine-mapped '*FLT-2L'* flowering locus (Chen et al. 2009). Interestingly, the 'FLT-2L' locus had been previously reported to be closely linked to a cold QTL (Reinheimer et al. 2004), thus the associated SNP may provide us with a high map resolution anchor for further studies. A significant association identified on the chromosome 4H-123 cM which map position is coincident with results of another GWA study of cold tolerance in barley (von Zitzewitz et al. 2011), which reported a significant low

temperature tolerance QTL on top of SNP 12_30824 which targets the same barley unigene (von Zitzewitz et al. 2011). The unigene has been mapped ~4 cM from the barley vernalization gene '*VRN-H2'*. Absence of significant associations around the '*Fr-H1'* locus on the 5H-132 cM were unexpected. However, a rapid exploration of the null analysis GWA - without population structure correction within the mixed model – reports a top "uncorrected" association hit within the vicinities (~5 cM) of a gene candidate for '*Fr-H1',* vernalization gene *'VRN-H1'* on 5H cM 137 which possible role on low temperature tolerance has already been reported in the literature (Francia et al. 2004). The rest of QTL loci map to novel genome regions respectively on chromosomes 1H, 2H, 3H, 4H and 6H.

Genome wide association analysis may be a very important tool to dissect quantitative traits such as cold resistance. Recently various works have been published assessing the feasibility of this tool to dissect complex traits into the genetic components controlling them. Benefits for breeding community arising from application of GWAS analysis to cold resistance may be: (i) the QTLs detected will provide high map resolution anchors for further studies aiming to identify the genetic determinants under association hits; and (ii) the development of new molecular markers associated to cold resistance to be used in marker assisted selection.

'Turkish' entries showed the higher cold resistance scores, while 'Syrian and Jordan' spring entries surprisingly showed a higher degree of cold tolerance respect to all others spring cultivars comparable with 'North Mediterranean Winter' and 'South West Mediterranean' lines. Checking the allele frequencies of the QTL SNPs is evident that most QTL are genetically fixed / nearly fixed within the winter germplasm and though they are freely segregating in the spring germplasm. Thus, as most spring x winter crosses are going to sample a small portion of the genetic diversity present in the cultivated genepool - mostly dependant on the genetic make-up of the spring line - seems feasible to suggestworking on un-adapted spring and facultative germplasm to identify new genomic regions involved in cold resistance is an interesting alternative.

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# QTL mapping of powdery mildew resistance in oats using DArT markers

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## Introduction

Powdery mildew, caused by the biotrophic fungal species *Blumeria graminins* f. sp. *avenae*, is a common disease on oats (*Avena sativa*), particularly in the temperate areas of north west Europe including countries such as Germany, France and the UK. Disease-caused annual productivity losses of 5-10% due to reduced yield are common in the UK (Lawes et al 1983 Clifford 1995), and yield losses up to 39% have been reported (Lawes and Hayes 1965). Growing resistant cultivars is likely to be the economically and environmentally most sustainable means of combatting the disease, and the development of resistant varieties is therefore at the focus of many oat breeding programmes, including the spring oat breeding program at Lantmännen SW Seed in Svalöv, Sweden. To this end, the availability of molecular markers linked to resistance loci would significantly facilitate the breeding of mildew resistance. However, while a number of sources of powdery mildew resistance have been found in *Avena* spp. (Herrman and Roderick 1996), so far only there is only a single report of a molecular marker linked to a powdery mildew resistance locus in *Avena sativa* (Yu and Herrmann 2006). Here, we use the newly developed oat DArT markers to map a powdery mildew resistance QTL in the cross 'Firth' x 'SW Betania' (FxB).

## Materials and Methods

Genotypic and phenotypic data were collected from 184 individuals of an $F_8$ recombinant inbred line (RIL) offspring population derived from a cross between the powdery mildew resistant variety 'Firth' (Lochow-Petkus GmbH, Germany) and the susceptible variety 'SW

Betania' (Svalöf-Weibull AB, Sweden). Genotypic data comprised 451 polymorphic DArT markers (Tinker et al. 2009). After excluding markers for which parental genotypes could not be unambiguously determined, 366 markers that could be mapped in the current cross remained. To construct the genetic map, we used a combination of a heuristic search algorithm and maximum-likelihood methods, implemented in the software AntMap (Iwata and Ninomiya 2006) and MapMaker/EXP (Lander et al. 1987), respectively. We used the Kosambi mapping function, a LOD-score threshold of 6.0 and a maximum recombination distance of 40 cM. In some cases, Marker orders were occasionally manually edited using the existing 'Kanota' x 'Ogle' (KxO) DArT map (Tinker et al. 2009) as a reference.

Phenotypic infection level data was collected under natural infection pressure from a field nursery planted in 2007 at IBERS, Aberystwyth, Wales, UK, and under artificial infection in a greenhouse at Lantmännen SW Seed, Svalöv, Sweden in 2010. Field infection data were collected from adult plants, whereas greenhouse data were collected from young seedlings. Both experiments were planted in randomized block designs with three replicates. Infection levels were scored using a scale from 0 – 100 with 5 unit intervals.

Powdery mildew resistance under natural and aritifical infection were treated as two distinct traits in QTL mapping. Interval mapping was used to find QTL for mildew resistance using the R/qtl software (Broman et al. 2003). A genome-wide LOD-score significance threshold for individual QTL was determined by a permutation procedure (Churchill and Doerge 1994) using 1000 permutations. The proportion of the phenotypic variance explained by each QTL was determined from a linear model analysis of variance.

## Results and Discussion

The FxB genetic map comprised 39 linkage groups covering a total of 678 cM. The number of markers per linkage group varied between 2 and 47, and linkage group map length ranged from 0 cM (2 or more markers mapped to the same position) to 118 cM with a median of 8.4 cM. Median inter-marker distance was 1.5 cM. The FxB map thus has lower coverage than the KxO map, which spans 2028 cM (Tinker et al. 2009). Nevertheless, 123 marker were shared between the FxB and KxO maps, allowing 189 markers from the FxB map to be assigned to KxO linkage groups (see example in fig 1). Significant segregation distortion were observed for 19 marker loci, of which 15 favoured the allele inherited from the paternal parent ('SW Betania'). Several of these markers mapped to an FxB linkage group corresponding to the KxO linkage group 11_41_20_45 where clusters of markers showing segregation distortion have been observed in previous studies (Tinker et al. 2009).

Phenotypic measures of infection levels among the 184 RILs showed a greater variability and lower mean under natural (mean = 52.6, s.d. = 18.2) compared to artificial (mean = 72.7, s.d. = 7.0) infection (fig 2). Infection level differences between the parents were also much less pronounced in the greenhouse experiment (not shown). There was a weakly positive, but non-significant, correlation between infection levels in the field and greenhouse experiments (Pearson's $r = 0.0497$, $p = 0.5336$). Overall, higher levels of resistance were found in the field experiment than in the greenhouse experiment (fig 2) in which practically all RILs showed susceptibility to mildew. This pattern is typical of adult plant resistance (APR) which is a type of resistance that is expressed mainly in adult plants but not in seedlings (Jones and Hayes 1971).

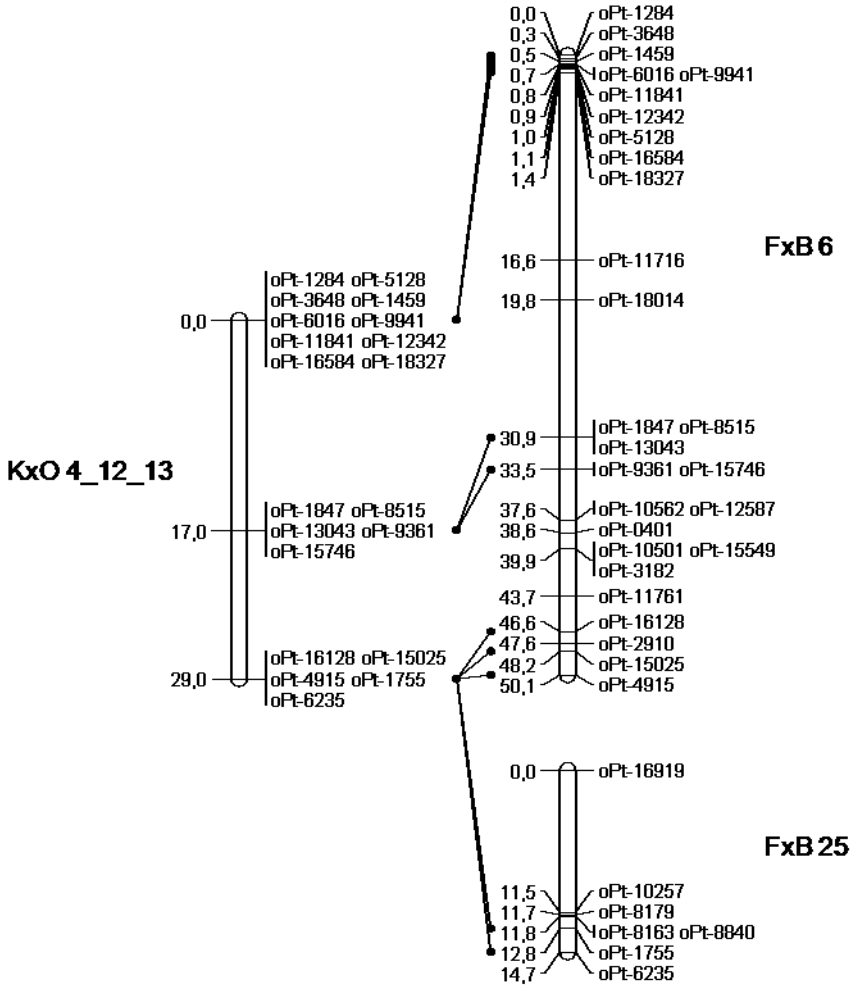


Fig. 1: Example of shared markers from the KxO and FxB genetic maps, allowing for assignment of FxB markers to KxO linkage groups

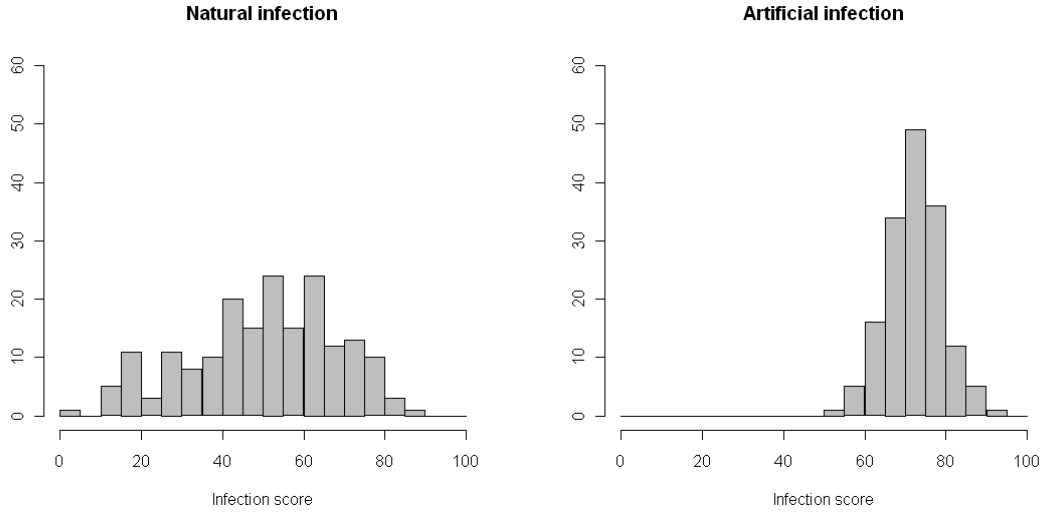


Fig. 2: Frequency distributions of powdery mildew infection scores under natural in the field (left) and under artificial infection in the greenhouse (right)

QTL analysis revealed a singe QTL for mildew resistance under field conditions, whereas no QTL were found for seedling resistance. The QTL had a LOD score of 15.1 which was significantly above the genome-wide significance threshold of 3.4. The QTL was located at the DArT marker oPt-6125, but as this marker remained unlinked to any of the other DArT markers, it was not possible to determine more precisely the position of the QTL relative to oPt-6125. However, this marker mapped to linkage group 5_30 on the KxO map. The QTL explained 31.9% of the phenotypic variation in field resistance and had an estimated effect of 20.1 units on the scale of infection level measurement. In contrast to race-specific resistance, APR is generally believed to be under polygenic control and therefore more durable (Jones 1986; Liu et al. 2000). Even though there may be additional undetected minor QTL, APR in the current cross seems to be goverened by a single major QTL, despite the continuous variation in the level of infection (fig 2). Furthermore, in a BLAST search against non-human, non-mouse EST sequences, a match with high similarity ($E<5\cdot10^{-41}$) to a wheat mRNA expressed 1 hr after inoculation with powdery mildew was found. This observation seems to indicate that the DArT marker oPt-6125 is indeed derived from a region in the oat genome containing gene(s) involved in mildew APR. Thre results of the present study agree with previous reports of lack of consistency or correlation between greenhouse and field test for mildew resistance in oats (e.g. Herrmann and Roderick 1996), but also suggest that molecular markers can be used for early selection of breeding lines with high levels of APR.

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# Genetic variation, population structure and linkage disequilibrium in a global sample of cultivated oats (*Avena sativa*) using DArT markers

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## Project outline

The aim of this project is to do an association mapping study on oat (*Avena sativa*).We have collected approximately 600 different oat lines from all over the world (fig. 1), with the majority coming from Europe. The lines are not only of different origin but also of different types like black oats, naked oats, dwarf oats and so on. The collection will be genotyped with Diversity Array Technology (DArT) markers and SNP markers. Phenotyping will be done both in the field and in the greenhouse for disease resistance (crown rust, smut, powdery mildew and Fusarium), agronomic traits (e.g. yield, straw strength, straw length, maturity) and quality traits ( protein content, beta glucan content and fat content).

## Current results

So far 493 oat lines have been genotyped with 497 polymorphic DArT markers.

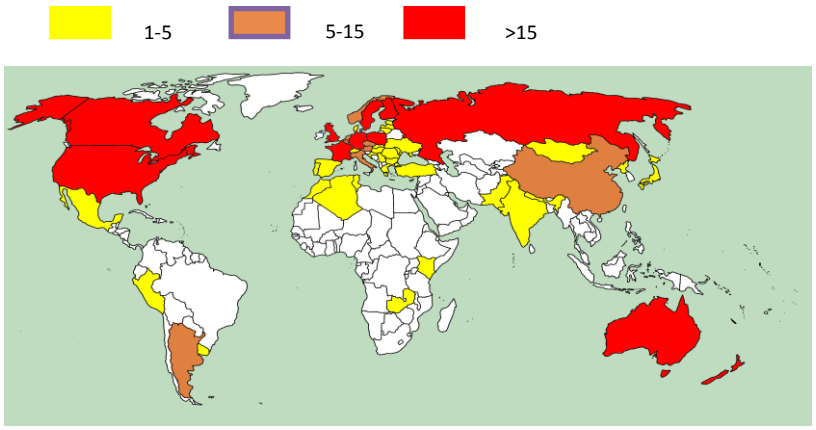


Fig. 1: Geographic distribution of the collected oat lines. Areas in red are represented by more than 15 lines, brown areas are represented by 5-15 lines and yellow areas by 1-5 lines

The set was divided into nine different geographic regions. For each region, expected heterozygosity and percentage polymorphic loci were calculated (Table 1).

Table 1: Expected heterozygosity (H) and percentage polymorphic loci (P) for the nine different regions

| | Northern Europe | Central Europe | Southern Europe | Eastern Europe | North America | South America | East Asia | Africa | Australia |
|---|---|---|---|---|---|---|---|---|---|
| # Ind | 196 | 120 | 18 | 38 | 55 | 9 | 15 | 5 | 25 |
| # loci | 497 | 497 | 497 | 497 | 497 | 497 | 497 | 497 | 497 |
| H/locus | 0,23 | 0,26 | 0,35 | 0,26 | 0,34 | 0,36 | 0,30 | 0,33 | 0,37 |
| # P | 490 | 481 | 481 | 471 | 495 | 463 | 419 | 397 | 489 |
| % P | 98,6 | 96,8 | 96,8 | 94,8 | 99,6 | 93,2 | 84,3 | 79,9 | 98,4 |

For the total set the expected heterozygosity was 0.29. A principal component analysis was also performed on the dataset (fig. 2). The PCA shows that the oat germplasm can be divided into two groups: one group with mainly European accessions and a second group with accessions of mixed, but mostly non-European, origin. However, there is broad overlap between the two groups.

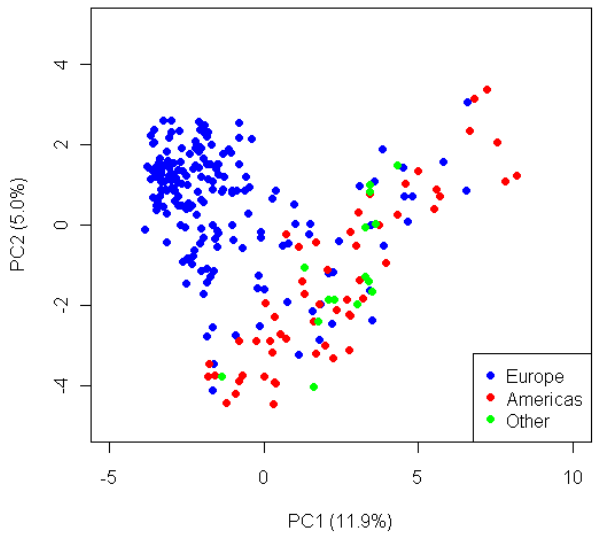


Fig. 2: PCA of 493 oat lines. Each dot represents one individual accession. Dots are colored according to geographic origin

We also studied linkage disequilibrium (LD) decay over genetic distance. Pairwise genetic distances were derive from three different maps based on 207(Tinker et al 2009), 68 (Oliver et al 2011), and 135 ('Firth' x 'SW Betania' cross; Ceplitis, unpubl.) DArT markers, respectively. There were some differences in the pattern of LD decay over distance among the three data sets, with LD extending over larger distances when using marker/distance data from the 'Firth' x 'SW Betania' cross (fig. 3). A closer look at LD decay over shorter distances shows a similar result (fig. 4).

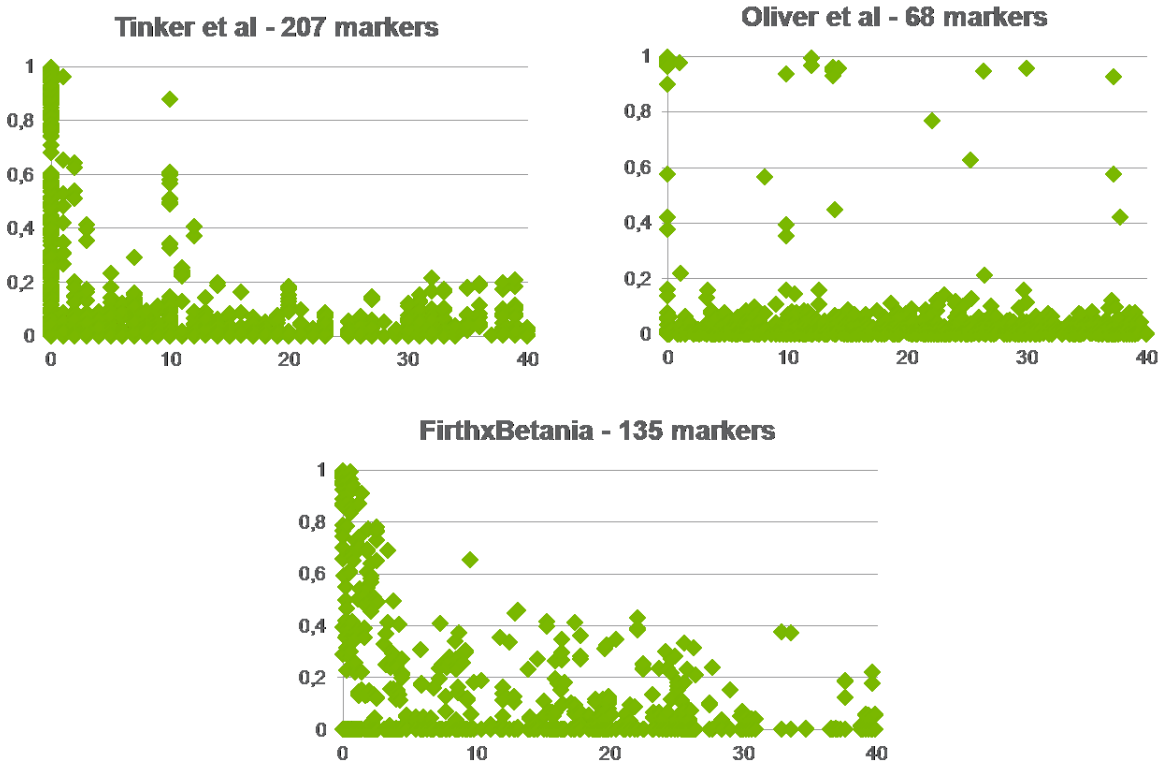


Fig. 3: LD decay over genetic distance. X-axis shows LD and Y-axis genetic distance in cM

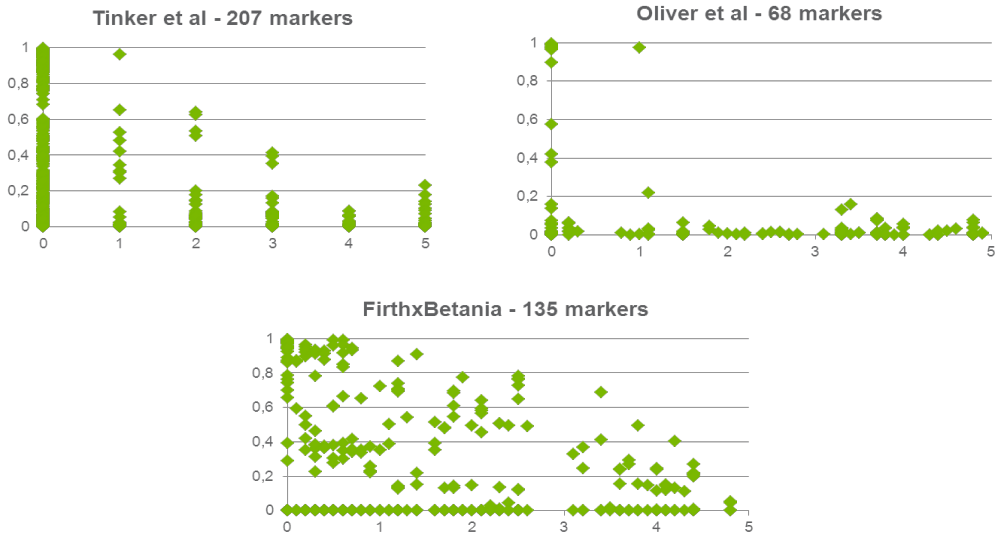


Fig. 4: LD decay over genetic distance up to 5 cM

Because of the slightly different appearances of the graphs a small study on overlap was done. Shared markers between maps were analyzed to see if different maps gave different results. The three maps had together nine shared markers and thus 81 comparisons could be made. In 52 out of the 81(=64%) comparisons all three maps agreed. It can be concluded that map quality and layout can influence results when analyzing data sets with different maps.

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## Identification of key parameters of barley root growth under drought stress

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Recent research has shown that the contribution of the root system architecture to enhance yield has been underestimated (Hammer et al. 2009). Root traits become more important against the background of an increasing world's population and an increasing demand on cereal production under unfavorable climatic conditions (Herder et al. 2010, De Smet et al. 2012).However, roots are hidden within the soil and, thus, are difficult to access for analysis. Due to these difficulties, the plasticity of the root growth still remains unclear and in contrast to shoot traits, the root system architecture of crops has been exploited only to a little extent within crop breeding programs (Gonzalez et al. 2009, Xing and Zhang 2010). Especially dynamic changes of the root growth and architecture, as they occur under stress conditions, are difficult to characterise. Measuring roots is a time-consuming business that can only deliver appropriate sample numbers if done automated.

Thus, to exploit root traits in breeding programs for adapting crops to drought and other abiotic stresses, automated root phenotyping is necessary to identify genes of the underlying root system architecture (cf. article of C.Dimaki in this publication).

### Project outline

The CROP.SENSe.network (http://www.cropsense.uni-bonn.de/) was founded in 2009 in order to build a close cooperation between researchers, breeders and companies in Germany

in the field of plant phenotyping. Cooperation partners are working together to analyse and screen plant phenotype throughout plants' lifecycles. One of the major aims of the network is a faster assessment of crop traits in lab and field experiments in order to support breeding. The development of sensors for the analysis of complex traits in crops will be highly focused by this network.

The architecture of barley under stress conditions will be used as a model for other monocotyledonous cereal species. Within the network several partners will analyse the shoot phenotype of barley under stress conditions (see article of K. Neumann in this publication). In addition to this, our institute is contributing to the analysis of the root system architecture under drought stress. Non-invasive optical sensor systems have been developed to measure the dynamic changes of root and shoot growth and to determine photosynthesis under stress in an integrated system. For this purpose a drought stress protocol for barley plants, grown in rhizotrones filled with soil, is established and the image analysis technology comprising the associated analysis software has been adapted to barley. As well as developing techniques, a major aim is to gain a better understanding of the relation between root system architecture, shoot growth, photosynthesis and drought stress. For this purpose it is a prerequisite to identify significant parameters which characterise increasing adaption of varieties to drought stress conditions.

## Material and Methods

For the purpose of establishing a drought stress protocol in rhiztotrones, pre-experiments with smaller, easier to handle rhizotrones were conducted. The pre-experiments were accomplished to clarify, whether image analysis software used was able to handle the low contrast between roots and sandy soil, and to get an indication of how strong young barley plants contribute to drought stress conditions by transpiration.

In contrast to the target process using automated image acquisition with large rhizotrons, image acquisition of smaller rhizotrons was done manually. Seeds of eight different summer barley varieties were planted directly into rhizotrones (60 x 30 x 3 cm) with a volume of 5.4 litre (Figure 1). The rhizotrones were equipped with transparent front sides in order to take images. They were filled with a mixture of quartz sand and top soil (9:1 w/w). At the beginning of the experiment, rhizotrones were watered to field capacity. The experiment was conducted as a complete randomised design with four replicates and 32 rhizotrones in total. Rhizotrones were adjusted to an inclination angle of 43° and the transparent front sides were faced downwards in order to force the roots to grow along the transparent front sides. With the exception of the time of image acquisition these front sides were covered with black plastic plates to avoid an illumination of the root system during the experiment. The volumetric water content was monitored with frequency domain reflectance (FDR) sensors, which were dug into the soil. Root and shoot growth was monitored over a time period of four weeks and at the end of the experiment shoots and roots were harvested separately and fresh and dry weights were measured.

Fig. 3: Three weeks old barley plant growing in a rhizotron (60cm x 30cm x 3cm). The volumetric water content was monitored by Frequency Domain Reflectance sensors

## Results and Discussion

Concerning the methodological questions the image analysis showed that the software was able to handle the low contrast and was furthermore adapted specifically to the root system characteristics of a monocotyledonous plant during this experiment.

The time period for the analysis of this barley subset was four weeks; then plants hit the bottom of the rhizotrones. During this time water stress was not severe enough to significantly reduce growth rates, but it was still possible to detect small differences in growth rates between the varieties (see Figure 2). This effect was only quantitative, not qualitative, which means that plants react to changing environmental parameters in the same manner, but on different scales.

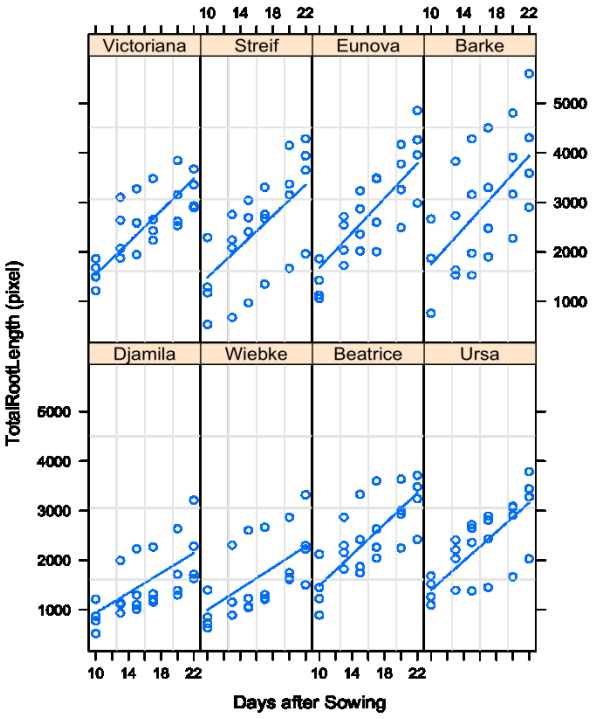


Fig. 4: Fitting a linear mixed model with random effects for both the intercept and the slope showed significant differences in total root length between the varieties

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# The International Wheat Genome Sequencing Consortium (IWGSC): Building the foundation for a paradigm shift in wheat breeding


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As the staple food for 35% of the world's population and the most widely produced crop, wheat is one of the most important crop species. Genomics offers powerful tools for

understanding the molecular basis of phenotypic variation, accelerating gene cloning and marker-assisted selection, and for improving the efficiency of exploiting genetic diversity. Genomics is leading to a new revolution in plant breeding as it enables the direct study of the relationship between the genotype and phenotype for a significant number of traits and the direct study of genes underlying those traits (Tester and Langridge, 2010; Feuillet et al., 2011). With a genome sequence in hand, breeders can have access to complete, ordered gene catalogue and an almost unlimited number of molecular markers that can be used for marker-assisted selection and precision breeding approaches (Collard and Mackill, 2008; Tester and Langridge, 2010; Prohens, 2011; Choulet et al., 2012).

Despite the socio-economic importance of bread wheat and the recognition of the power that a genome sequence brings to breeding programs, bread wheat is one of the last major crops without a high quality, reference genome sequence. Bread wheat has been slow in building genomic resources particularly because of the very nature of its genome: an allohexaploid (2n=6x=42) that is extremely large in size (17Gb, more than 6 times the size of the maize genome) and has a very high (>90%) repetitive content of the genome thereby complicating genome assembly (Paux et al., 2008; Feuillet et al., 2011). These factors have made it more difficult and more costly to sequence the bread wheat genome than any other major crop.

Recently, however, significant technological advances have occurred that have made sequencing the wheat genome tractable at a reasonable cost. After a US Department of Agriculture-National Science Foundation funded workshop that confirmed the need for sequencing the wheat genome and assessed different strategies and objectives (Gill et al., 2004), it became clear that the bread wheat genome could be sequenced as a result of these technological advancements. With a mission of rectifying the paltry state of genomic resources for sequencing the wheat genome, a group of growers, breeders, and scientists launched the International Wheat Genome Sequencing Consortium (IWGSC; www.wheatgenome.org) in 2005. The underlying goal of the consortium is to accelerate wheat improvement by obtaining a high quality, manually annotated, reference genome sequence of bread wheat that is anchored to the genetic and phenotypic maps. Using a milestone-based, adaptable strategy, the IWGSC provides breeders an increasing array of tools and resources while working towards obtaining a reference genome sequence (Feuillet and Eversole, 2007).

As an organization led by growers, breeders, and scientists rather than sequencing experts, the consortium is focused on building a foundation for wheat improvement and on facilitating rapid application of the results from IWGSC supported projects. Obtaining a "genome sequence" without any view towards the ultimate utilization of the sequence by breeders has never been a consortium goal. A major consideration in designing the strategy to obtain a reference quality sequence, thus, was to understand exactly for what the sequence would be used. We did not merely want to have a tool for comparing the wheat genome with other genomes. Instead, we wanted a genome sequence of sufficient quality to enable gene isolation, functional analyses, new allele discovery for pre-breeding, epigenetic modifications, polymorphism discovery for marker-assisted selection, and an increased understanding of the impact of transposable elements on gene regulation (Feuillet et al., 2011). To provide these capabilities, an integrated and ordered wheat genome sequence is essential.

Combined strategies are being deployed by the consortium to achieve a reference genome sequence of the hexaploid, bread wheat genome (*Triticum aestivum* L.), cultivar Chinese Spring. These include physical mapping of Chinese Spring and *Aegilops tauschii* (the D-genome progenitor of bread wheat), as well as survey sequencing and BAC-based (i.e., the minimum tiling path of the physical map) reference sequencing of Chinese Spring. The

physical map of *Aegilops tauschii* was completed in 2011 and publication of the results is expected in 2012 (*Personal communication, J. Dvorak*). The IWGSC follows a chromosome-specific approach for physical mapping, survey and high quality sequencing of Chinese Spring. The chromosome-based approach, made possible through technological advancements in flow-sorting of Chinese Spring chromosomes by the group of Jaroslav Doležel (Dolezel et al., 2007), reduces the complexity of the bread wheat genome by physical mapping and sequencing individual chromosomes or chromosome arms the size of which ranges from 224-800Mb. Further, the chromosome-based approach facilitates international collaboration and divides the costs of obtaining a reference sequence into manageable pieces. The chromosome-based approach will deliver a complete, finished reference genome sequence that, in addition to genic sequences, will provide critical information on non-coding, intergenic sequences that underlie many biological functions (Feuillet et al., 2011).

To construct the physical maps, chromosome specific BAC libraries are created for each of the 21 chromosomes of bread wheat, *cv*. Chinese Spring. As of October 2011, more than 2.2 million BAC clones had been developed for the BAC libraries of all but 4 chromosomes of bread wheat (http://olomouc.ueb.cas.cz/dna-libraries/cereals). Using these BAC-libraries, physical maps are then developed. The information produced during the construction of these maps is useful for gene cloning and marker development as well as increasing our understanding of the gene space organization and regulation. Further, the physical map itself serves as the substrate for sequencing. The completion of the physical map of the largest wheat chromosome (3B, ~ 1Gb) in 2008 confirmed the feasibility of this approach (Paux et al., 2008). Physical mapping of the remaining chromosomes is underway. By November 2011, the IWGSC reached the milestone of securing funding for the development of physical maps for all 21 bread wheat chromosomes. The next physical mapping milestone to be achieved is the completion of all of the chromosome-based physical maps. With all of the funding in place, this is expected by 2013.

To facilitate anchoring, marker development, and to gain a first insight into the gene space composition, survey sequences were completed with construction of the physical maps. As survey sequences were available for only half of the chromosomes by 2010, an internationally coordinated initiative was launched to provide survey sequences and the virtual gene order for all 21 chromosomes. By October 2011, survey sequence coverage had been achieved for all 21 chromosomes. Survey sequences of 30-50 fold coverage, based on Illumina technology, and assemblies of sequences for 29 chromosome arms were completed by the fall of 2011. Illumina survey sequences will be completed and assembled for the remaining chromosome arms by early 2012. Using the assemblies, a virtual gene order is derived by exploiting the conserved synteny among grasses through a process called the "GenomeZipper" (Mayer et al., 2009). This draft, virtual gene order in the individual chromosomes/chromosome arms is expected to be completed by early 2012. The survey sequences provide only partial information on the order and orientation of the contiguous sequences (contigs) and do not represent a complete, reference genome. Rather, they enable *in silico* mapping, facilitate annotation of genes within contigs, and support localized synteny studies. The chromosome-based survey sequence initiative is expected to be completed by mid-2012.

The next step towards obtaining a high quality reference sequence of the bread wheat genome is to sequence the minimal tiling path (MTP) of the individual chromosomes/chromosome arms. Using clones selected from a physical map containing fragments making up the genome with minimal overlap, a MTP of clones is delineated to provide the substrate for sequencing (Choulet et al., 2012). Sequencing the MTP of chromosome 3B began in 2010 and of chromosome 7B in 2011. By September 2011, the IWGSC reached two more milestones by completing the production of sequence for chromosome 3B and securing funding for the MTP

sequencing of 8 additional Chinese Spring chromosomes. The next milestone is to finalize the funding for MTP sequencing of all of the 21 bread wheat chromosomes by 2013, which, if successful, would result in the completion of sequence production by 2014.

Information on the various IWGSC projects as well as the availability of data can be found on the consortium website: http://www.wheatgenome.org. An IWGSC sequence repository has been established by INRA-URGI (http://urgi.versailles.inra.fr/Species/Wheat/Sequence-Repository). Pre-publication access to IWGSC data is publicly available for BLAST as long as the user agrees to respect the right of the IWGSC to produce the first chromosomal and global analysis of the data. Raw sequence data will be deposited in short read archive and download of sequence assemblies will be available upon publication.

The IWGSC made significant progress in 2011 and it is anticipated that even more milestones will be achieved in 2012. Membership in the consortium is open to anyone who supports the goal of obtaining a high quality, reference sequence of the bread wheat genome.

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## Genomic selection strategies for wheat improvement


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### Introduction

Plant breeding strategies are driven by new methods and technologies. Crop improvement began with the hunter-gatherers who practiced mass selection and domesticated the species

that became our crops today. Clearly they were successful with no knowledge of the underlying genetics controlling the traits they were selecting. Over time breeders, for the most part, have not abandoned old breeding methods but instead have adapted them to new technologies and integrated them into new strategies. Family selection and progeny testing methods were developed that took advantage of family relationships to increase heritabilities and quantitative genetics theory was developed. Today the focus is on tool development that taps into the power of molecular marker technologies. MAS has migrated from QTL discovery and characterization to QTL cloning, functional markers, and more recently association mapping. Complementary to MAS is a new approach advanced by animal breeders called genomic selection or genome-wide selection. Curiously, this breeding method also does not rely on our understanding of the underlying genetic control of the traits we are selecting. In fact, Fisher's 1918 theory of infinitesimal variation (Fisher 1918) is still applicable to breeding populations.

Today, knowledge of the level of genetic diversity and historical relationships among cultivated wheat germplasms can be effectively exploited for molecular breeding approaches. Those methods can be broadly classified as the assessment of genetic variation, marker-assisted selection (MAS), association breeding (Podlich et al., 2004; Bresghello and Sorrells 2006), marker-assisted recurrent selection (MARS) and genomic selection (GS; Meuwissen et al., 2001) for wheat improvement. Advancements in genotyping technologies are rapidly reducing marker costs and increasing genome coverage allowing the widespread use of molecular markers and methods in plant breeding. Marker assisted selection and recurrent selection are based on the selection of statistically significant, marker-trait associations. However, MAS strategies are not well suited for agronomically important complex traits controlled by many genes. Genomic selection incorporates genome-wide marker information in a breeding value prediction model, thereby avoiding biased marker effect estimates and capturing more of the variation due to small effect QTL (Meuwissen et al., 2001).

**Marker Assisted Selection**

The increasing cost of phenotyping and the declining cost and speed of genotyping are driving the adoption of MAS in plant breeding programs. Also, because the use of markers does not require phenotyping each generation, multiple generations per year can be realized using off season nurseries or greenhouses, dramatically increasing the efficiency of breeding programs.

The primary goals of any breeding programs are to identify superior alleles and combine them in the same genotype so as to be able to accurately select transgressive segregates that are superior for the traits of interest. Annual rate of gain is determined by heritability, selection intensity, and duration of selection cycle. We can increase heritability by reducing error or genotype by environment interaction and by increasing the genetic variation. We can increase selection intensity if we reduce the cost of selection, which in turn, allows a larger population size.

In traditional MAS programs, only significant markers with large effects on the traits interest are used for selection. These are usually qualitative traits of high heritability leading to the "catch 22" situation where the easiest traits to select using MAS are also those easiest to select based on phenotype (Holland 2004). However, MAS is still useful for costly traits or for selection in environments not conductive to phenotyping. This method is most useful for backcrossing or gene pyramiding. Backcrossing is the most conservative breeding method and by the time you have a new line with the added gene, the performance of the recurrent parent has frequently been surpassed by other advanced selections in the breeding program. Gene pyramiding is an attractive, forward breeding method that combines multiple genes in a

single superior genotype (Lee, 1995). However, each of these methods is limited in the number of genes that can be transferred simultaneously. Also, genes with small effects underlie most of the important traits and they determine the success of new varieties (Crosbie et al., 2003).

**Association Breeding**

Association mapping, also referred to as linkage disequilibrium mapping, has been widely used as an efficient genetic mapping methodology because of robust statistical approaches that increase power and reduce false positive associations. Association mapping exploits historical recombination events in a population of individuals that may be chosen to be representative of a breeding program. In association breeding, association mapping methods are superimposed on a conventional hybridization/selection/testing program for allele discovery and validation (Bresghello and Sorrells 2006). Because association mapping can be conducted on breeding lines, the results generated are directly relevant to the breeding goals. Also, the relevant genetic background effects are sampled and phenotypic variation will be observed for most traits of interest. Marker polymorphism is generally much higher than for biparental populations. Because all breeding programs have routine multi-environment variety trial evaluations, they generate high quality phenotypic data for those traits. However the most important benefit of association breeding is that novel alleles can be identified and their relative value can be assessed as often as necessary. Limitations include a higher risk of Type I error (false positives) resulting from population structure, low heritability, many small-effect QTL (heterogeneity of genetic background) and high sampling variance of rare alleles. Rare alleles that are potentially valuable are usually excluded from the analysis. Breeding programs are dynamic, complex genetic entities that require frequent evaluation of marker / QTL relationships and accurate detection and estimation of QTL effects are required to maximize gain. Most AM panels reported in crops species, contain some level of subpopulation structure and familial relatedness because of the organization and selection in breeding programs. Consequently, correction for population structure is essential for minimizing false positive associations. A unified mixed model that simultaneously considers both population structure and familial relatedness has been used widely by researchers (Yu et al. 2006). Principal component analysis has also been widely used as a way to control for population structure (Price et al. 2006). Finally, genetic variation that is correlated with population structure will not be detected. Never-the-less, even association breeding cannot deal with large numbers of small effect QTL.

**Genomic Selection**

Although marker technologies have improved dramatically, the use of MAS has not been effective for the improvement of quantitative traits (reviewed by Xu and Crouch, 2008). Bi-parental QTL mapping populations have been inefficient and the polygenic nature of important agronomic traits limit their utility in breeding programs. Genomic selection (GS) has been proposed to address these deficiencies. In GS, a training population representative of the breeding germplasm is genotyped with genome-wide markers and phenotyped in a target set of environments (Meuwissen et al., 2001). That data is used to train a prediction model that is used to estimate the breeding values of lines in a population using only the marker scores. This method assumes that there is at least one marker in LD with every QTL affecting the trait. Choosing a prediction model is critical to the success of GS. Traditional least squares regression for variable selection treats markers as fixed effects and an arbitrary threshold for significance is used to fit the markers. This usually results in overestimation of

significant effects and loss of small effects. To deal with the problem of estimating many QTL effects from a limited number of phenotypes or records we can use linear mixed models and Bayesian estimation by treating QTL effects as random effects and estimating them simultaneously. Several GS models have been developed but their comparison is beyond the scope of this paper. For a detailed comparison of different prediction models see Heslot et al. (2012). To assess model performance, GEBV accuracy is based on the Pearson correlation between the GEBV and the true breeding value (TBV). Accuracy defined in this way is directly proportional to gain from selection when selecting on the GEBV, that is, $R = ir\sigma_A$, where $R$ is the response, $i$ is the selection intensity, $r$ is the accuracy defined above, and $\sigma_A$ is the square-root of the additive genetic variance of TBV (Falconer and Mackay, 1996, p. 189).

Genomic selection models can predict performance of a wide range of traits over multiple environments. Because phenotyping is not required, generation time is reduced and annual genetic gain for GS is predicted to be greater than for a conventional phenotypic selection program, even at low accuracies. Recent reports in the literature indicate that GS accuracies of 0.50 or greater are feasible for many traits. Heffner et al. (2010) developed an analytical framework to compare gains from conventional breeding and GS for complex traits with equal budgets. They reported that for accuracies > 0.50, the expected annual gain from GS can exceed that of MAS by about 3-fold for maize and 2-fold for winter wheat (Heffner et al. 2010).

In a recent report, Heffner et al. (2011) compared GS using biparental populations to using multiple families as training populations. Training a GS model using biparental populations requires fewer markers and smaller population sizes. Also, epistasis is minimized and minor allele frequency is not an issue. However, this approach requires phenotypeing a subset of lines from each cross prior to conducting GS, thus prolonging the selection cycle. In contrast, estimating marker-effects across multiple families from multiple selection cycles allows the sampling of more environments and genetic backgrounds. This results in larger training populations and sampling more genetic diversity. In Heffner et al. (2011), phenotypic selection (PS), conventional marker-assisted selection (MAS), and GS prediction accuracy were compared for 13 agronomic traits in a population of 374 winter wheat advanced-cycle breeding lines. The average prediction accuracies using GS were 28% greater than with MAS and were 95% as accurate as PS. For net merit, the average accuracy across six selection indices for GS was 14% greater than for PS. The evidence is rapidly accumulating that this new approach to crop improvement will facilitate a better understanding of the dynamic genome processes that generate and maintain new genetic variation.

Future research should focus on improving prediction models, optimizing training populations, and methods for integrating GS into conventional breeding programs. Optimization of training populations is perhaps the most challenging because the emphasis has shifted from evaluating genotypes to evaluating alleles. Sparse testing strategies that evaluate different genotypes in different environments allow a dramatic increase in the number of genetic backgrounds sampled. Similarly, clustering environments based on marker effects rather than genotypes could reveal new ways to create mega-environments. Lastly, integrating GS into a conventional wheat breeding program requires re-structuring and coordination between a recurrent genomic selection phase and an inbreeding phase. In the recurrent selection phase, multiple generations of crossing and selection can be performed each year. From each of the cycles of selection, offspring are moved to the inbreeding phase where MAS and phenotypic selection can be applied. A final genotyping step can be used to identify the best lines to go into the training population and advanced testing. The integration of GS in a breeding program is a promising approach to increasing genetic gain per unit time.

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## Implementation of genome-wide selection in wheat


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### Introduction

To satisfy the demand of the growing world population, agriculture faces the challenge of delivering safe, high-quality, and health-promoting food and feed in an economical, environmentally sensitive, and sustainable manner while maintaining yield and stability across different environments affected by climate change. Wheat is the most widely grown crop worldwide with an average global annual harvest of 621 million tons of grains. Wheat demand is expected to increase from 621mt to 813mt in 2030 and more than 900mt in 2050 (FAO 2002). This implies annual production growth rate of about 2%, while it was limited to 0.9% from 1985 to 1995. Moreover, the rate of yield increase has slowed down from 1995 to 2005 in nearly every country (Dixon et al 2009, Reynolds and Eaton 2009), and it is close to 0 in EU, particularly in the major producing countries like France, Germany and UK. Thus, accelerating genetic progress is recognized as a priority in most countries.

In their pioneering work, Lande and Thompson (1990) proposed an extension of the index selection theory by adding a molecular score to the classical phenotypic score. They

introduced the theory for optimizing weights given to each component and demonstrated that this index is in any case at least as efficient as the phenotypic score alone. Note that this approach of marker assisted recurrent selection used only markers which have been identified as being significantly associated (linked) to QTL. The efficiency of MAS/phenotype selection is higher when the trait has a low heritability, the population size is large and the detected QTLs explain a large proportion of the trait variation. Thus further studies have shown that efficiency is improved when including QTLs with small effects, even if they are false positives, rather than being too stringent during the QTL detection step (Bernardo 2006; Moreau et al. 1998).

A further step was proposed by Whittacker (2000), who suggested including all markers in the selection index, thus skipping the QTL detection step. As the number of markers is generally higher than the number of genotypes, classical multiple regression with markers as fixed effects cannot be used. Therefore Whittacker (2000) suggested using ridge regression models to overcome this overparemetrization problem. This method is based on introducing a penalization parameter, □, which reduces the space dimensionality. Meuwissen et al (2001) applied ridge regression and several Bayesian approaches to animal populations for predicting breeding values. They proposed to use genome-wide markers to predict the genetic value of individuals. Therefore, it is appropriate to name these methods "genomic prediction". However, as genomic predictions are intended for selection purposes, the expression "genomic selection" has become common (e.g. Goddard and Hayes 2007).

The most efficient use of genomic selection is to replace costly and time consuming phenotyping by a prediction of the genetic value of the trait under selection (or any multitrait index). Thus, the main expected advantage is to shorten selection cycles. However, to benefit from shorter cycles, the genetic gain per selection cycle should be close to that expected from phenotypic or combined MAS + phenotypic selection.

The relative efficiency relies on the accuracy of prediction of the true genetic value by the marker score, which depends on the effectiveness of the markers to capture most of the information brought by QTLs. Thus marker density should be high enough, in order that every QTL be in sufficient LD with an adjacent marker. For example, this LD range is expected to be large in biparental populations, and Lorenzana et al (2009) obtained reasonably good prediction with as few as 96 markers in simulated maize progenies. But in progenies from more complex mating schemes, the required marker density will be higher ( Bernardo and Yu 2007, Heffner et al. 2009; Jannink et al. 2010). Moreover, the LD pattern changes from one generation to the next, since recombination reduces the range of LD.

For practical applications in breeding programs, one has to estimate marker effects and add them to obtain the genomic estimate of breeding value (GEBV). This estimation requires both genotypic and phenotypic information in a so-called "reference" or "training" population. Then, marker effects can be used to estimate GEBV in a "target "population with only the genotypic information, and, subsequently, selection can be made on the GEBV instead of the phenotypes. Genomic Selection (GS) can be repeated on the progeny of crosses between GEBV-selected individuals and so on. However, as the LD between markers and QTL decreases from one generation to the next, GEBV predictions are less and less accurate. Therefore, new phenotypic measurements are needed to re-estimate marker effects (see Heffner et al 2010).

In this manuscript, we report on some preliminary results about the implementation of genomic prediction of yield in the INRA wheat breeding programme. The presented results only deal with the initial prediction of target populations using marker effects estimated from training populations sampled by cross-validation.

## Material and methods

The INRA wheat breeding program is a carried out in three main research units: Clermont-Ferrand, Estrées-Mons and Rennes. Each breeder makes 100-150 crosses every year, using registered varieties (most recently in Western Europe) for 50% of parents and breeding lines from previous cycles of the program for the remaining 50%. F2 to F4 plants are conducted as bulked families with around 2000 plants per cross, then F5 grains from selected spikes are sown in single rows in a classical pedigree design. Bulked grains of F6 lines are sown is two replicate trials with randomized 6-10 m² plots in a single location, then the best F7 in 3-4 replicates, and the most advanced F8-F9 lines are evaluated in a network with 4 replicates in 8-10 locations, according to their precocity group. To have a more balanced design, we kept data from 6 locations with the higher number of common genotypes. Therefore, 30-50 most fixed "new" lines enter the most advanced evaluation network each year. Some of them are evaluated only one year, some two or three consecutive years before being presented to official registration for the best ones. As breeding lines are used as genitors only once sufficient phenotypic data are available, i.e. in F8, the duration of the selection cycle can be estimated to at least 8 years. In this study, we used those lines which have been evaluated in the complete multisite network between 2000 and 2010. After discarding some lines with too few data or to many missing markers, this gave a dataset of 318 breeding lines.

DArT markers were provided by Triticarte company (*www.triticarte.com.au/*). After cleaning markers with more than 5% missing data and minor allele frequency >5%, we obtained a dataset with 2121 polymorphic markers.

For estimating the accuracy of prediction on real data, we focused on yield, whose broad sense heritability in our design was estimated to 0.37. Because of the highly unbalanced design, we first had to correct for other factors and estimate a corrected genetic main effect. This was achieved through the use of mixed models, with environments and blocks within environments as fixed effects and genotypes as random effects, whose variance being modeled by an identity matrix to avoid confusion with further BLUP prediction using marker estimates of additive relationship matrix. Then the BLUP for each of the 318 lines were used as observed phenotypes (obsP), either in each location or over the 6 locations.

Several statistical models were compared for their prediction accuracy as measured by the correlations between GEBV and obsP.

*Four statistical methods have been used to predict GEBV:*

- The ridge regression, as described by Whittacker et al (2000) using a home written R programme (R development core team). Basically, this methods uses a mixed linear model to estimate best linear unbiased predictor (BLUP), assuming that markers have random effects with common variance. RRBLUP uses a penalty parameter, $\lambda^2$ in the estimator to shrink marker effects and to avoid over-fitting (Piepho, 2009). In this study, $\lambda^2=\sigma^2_e/\sigma^2_g$, where $\sigma^2_e$ is the residual variance and $\sigma^2_g$ is the marker effect variance – estimated from the additive genetic variance divided by the number of markers.
- The GBLUP (Costner 2010), using the pedigree library of R. The XX function solve the classical BLUP equation (Henderson 1975), using a marker-based estimate of the additive relationship matrix.
- Bayesian Ridge Regression and LASSO (De los Campos and Pérez, 2010; Pérez et al., 2010) as implemented in the BRR library of R.

**Results and discussion**

The accuracies of the four methods on the BLUP prediction of yield in each of the 6 locations and on the overall BLUP prediction are given in Table 1 and illustrated in Figure 1. The two Bayesian approaches (RRB and LASSO) clearly outperform the mixed model approaches. However the ridge regression appears to be less accurate than G-BLUP.

Prediction accuracies of the 4 methods, i.e. correlations between GEBV and obsP obtained in six different locations vary from one location to another, likely according to the within location broad sense heritability. Moreover, in some locations all 4 methods gave similar correlations, while in others there are significant differences among them. More remarkably, the ranking of the 4 methods according to their accuracy differs from the ranking observed on the obsP on all environments. It clearly appears that these BLUP estimates of yield, using single locations, differ from the overall estimate, likely due to GxE interactions.

In this preliminary attempt to predict the breeding values of elite wheat lines using genomic markers, results obtained on real data are in accordance with those obtained on simulated traits of similar heritabilities. Indeed the correlation between GEBV and either simulated or observed phenotype is around 0.5. We may assume that the correlation with TBV of real data will also be similar to that obtained on simulated data, i.e. in the range 0.6-0.7. This value is encouraging, and compared to those reported by Crossa et al (2010) who reported accuracy values ranging from 0.355 to 0.608 according to the method and the environment. Heffner et al (2010) recently reported somewhat lower correlation, but they used a more conservative approach, as the used yield in one year as training data and correlate GEBV with yield in another year. If true, an accuracy of 0.6 for TBV is encouraging, since phenotype itself cannot be viewed as a perfect predictor of TBV. Therefore selection based on GEBV may not be worse than that based on phenotypes.

However, all studies published so far have failed to obtain very high prediction accuracies. This may be due to the small size of the training population, which is most often lower than 1000. Hayes et al (2009) gave an estimate of the training population required to achieve an accuracy of 0.8, according to trait heritability. For a trait with $h^2=0.5$, the theoretical population size is about 5000, nearly twentyfold more than in the present study. Another limitation could come from sparse marker coverage. This is related to the minimal extent of LD range in the studied material, which itself depends on the number of founder lines and number of generations or the effective population size, as discussed by Heffner et al (2010). As we do not have reliable map positions for every marker, we do not present the pattern of LD in the studied material. However at first glance there are some high values of LD between markers at a few cM apart. Other parameters which affect prediction accuracy have been recently discussed (Iwata and Jannink 2011, Zhong et al 2009).

The correlation values obtained in this study appear high enough to provide prediction accuracies of TBV of the same magnitude as that provided by replicated phenotypic trials. However, compared to dairy cow, the economic advantage of replacing phenotype prediction by genomic prediction is much less obvious in wheat. Indeed, reliable phenotypic prediction of breeding value of a bull for milk production requires measuring milk production of some or hundreds of its daughters (progeny tests). This requires at least 5-6 years, and the cost is estimated to be around 40 000 € per bull (D. Boichard, pers. comm..). In wheat, 4-time replicated plots in 8-10 locations are usually considered enough to get reliable estimates of mean breeding values of a breeding line, which costs a few hundred euros. Thus the main interest of GS in wheat is shortening selection cycles to accelerate genetic gain. This should only be achieved if fast pure line fixation methods are implemented. This could be accelerated using single seed descent with off season generation in different environments (such as the

shuttle breeding used in CIMMYT's programmes), or under controlled conditions using doubled haploid methods, which allow the production of and intermating of GS-selected pure lines in only 2-3 years instead of 7-10 in classical pedigree selection.

In the framework of the French National Breedwheat programme, a fair comparison of one cycle of phenotypic selection vs two cycles of GEBV-based selection will be carried out on about 1000 DH lines from 34 breeders' crosses over a 6-year period.

Table 1: Mean (and standard deviation from 100 cross-validations) of correlations between GEBV estimated from four statistical models and yield predicted in each of the 6 locations and using all locations. RRB: Ridge regression BLUP, GB: G-BLUP, BRR: Bayesian Ridge regression, BL: Bayesian LASSO

| Site/ Model | Cf Clermont | Di Dijon | Em Estrées-Mons | Lm Le Moulon | Lu Lusignan | Re Rennes | All sites |
|---|---|---|---|---|---|---|---|
| RRB | .289(.14) | .471(.12) | .276(.15) | .332(.12) | .278(.08) | .297(.10) | .488(.12) |
| GB | .395(.10) | .447(.15) | .330(.12) | .494(.08) | .294(.11) | .344(.11) | .522(.08) |
| BRR | .329(.12) | .479(.15) | .350(.11) | .492(.08) | .276(.16) | .348(.13) | .506(.11) |
| BL | .312(.14) | .479(.13) | .333(.12) | .456(.08) | .316(.14) | .324(.12) | .504(.11) |

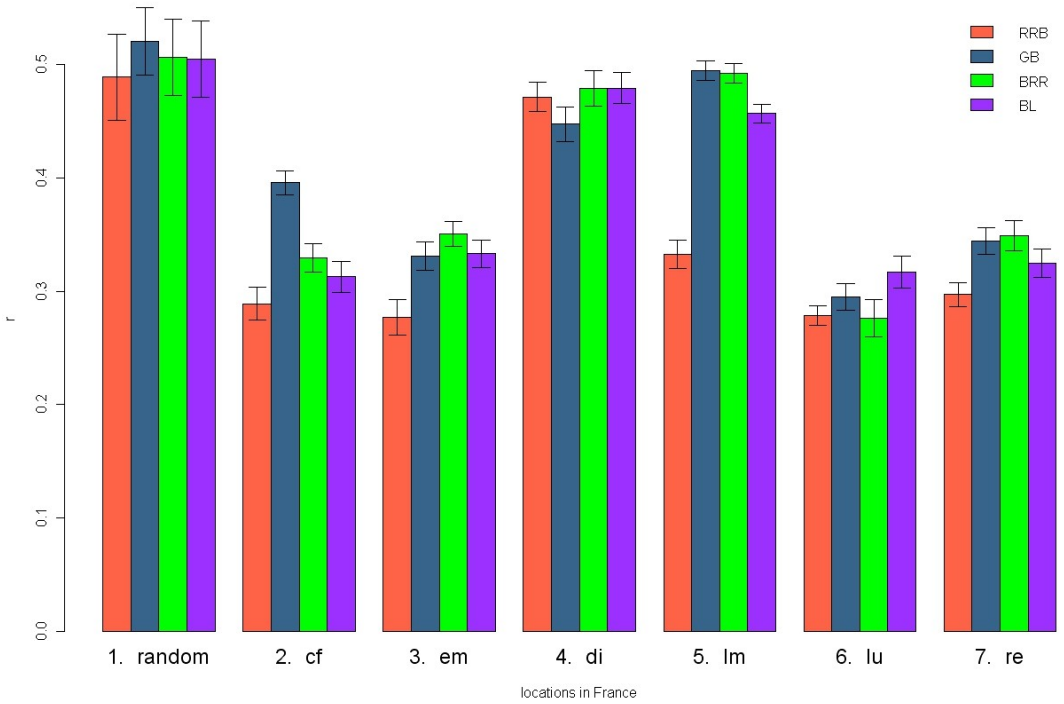


Fig. 1: Mean (and standard deviation from 100 cross-validations) of correlations between GEBV estimated from four statistical models and yield predicted in each of the 6 locations and using all locations (1. Random). RRB: Ridge regression BLUP, GB: G-BLUP, BRR: Bayesian Ridge regression, BL: Bayesian LASSO

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# Utilization of alien genetic material in spring bread wheat breeding in Western Siberia

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Western Siberia is a region of Russia with cold winter where the spring bread wheat varieties are mainly cultivated. One of the most economically important factors that stabilize wheat production in this region is selection toward adaptability to abiotic stress, especially to drought, and disease resistance. Most wheat varieties cultivated here are susceptible to leaf rust (*Puccinia triticina* f. sp. *tritici*). In addition, in recent years stem rust (*Puccinia graminis* f. sp. *tritici*) has also spread. Selection of new spring wheat varieties in Siberian Agricultural Research Institute (SARI) (Omsk, Russia) is based on the combination of high yield and resistance to biotic and abiotic factors. For this purpose breeders often involve introgressive lines obtained by hybridization of wheat with other cultivated and wild species. Our work is aimed in the detection of alien genetic material and identification of genes in new commercial wheat varieties that are produced in the SARI and in the development of promising lines from these varieties for their use in breeding.

## Materials and methods

New commercial varieties of spring wheat (Omskaya 37, Omskaya 38) and lines developed from the variety Omskaya 37 were studied. These varieties were obtained as a result of hybridization of different wheat varieties carrying alien genetic material derived from different sources. Earlier we have shown that some of the varieties contained wheat-rye translocation 1RS.1BL transferred from wheat variety Kavkaz (Trubacheeva et al., 2011). In this work these varieties and lines were studied using C-banding technique and molecular analysis. Chromosome preparation and C-banding were carried as described earlier (Badaeva et al., 1994). The SCAR marker iag95 was used to identify wheat-rye translocation containing genes *Lr26*, *Sr31*, *Yr9*, and *Pm8*, which were transferred from variety Kavkaz. Specific amplification product with the size of 130 bp was detected in varieties Omskaya 37, Omskaya 38, lines - derivatives of Omskaya 37 and using STS primers Gb developed for identification of gene *Lr19*. These methods were performed according to (Gultyaeva et al., 2009). The crop yields trials and testing of resistance to fungal pathogens (powdery mildew and leaf rust) was carried out on the fields of SARA. Benzimidazole-based method was applied to evaluate resistance to leaf rust pathogens at the emergence stage (Michailova, Kvitko, 1970). Leaves were inoculated with leaf rust populations from different region of Western Siberia. The varieties were also tested to stem rust resistance using inoculation with TTKST (Ug99+Sr24) in Kenya Agricultural Research Institute. Tests for drought resistance were carried out by *in vitro* method (Rosseev, 2007).

**Results and discussion**

The variety Omskaya 37 showed polymorphism in a number of characters, including plant height, heading time and resistance to pathogens. More than 300 lines, obtained from the variety Omskaya 37 have been examined. Evaluation and selection of these lines resulted in isolation of one line that gave rise to a new variety of spring wheat Omskaya 41. All these varieties are characterized by high yield, high grain quality and resistance to fungal pathogens. The results of *in vitro* test have shown that varieties Omskaya 37 and Omskaya 38 are highly resistant to drought. In field conditions all these varieties were moderately resistant to powdery mildew and highly resistant to leaf rust (Table 1). Laboratory tests showed their high resistance to leaf rust pathogens at the emergence stage. According to the assessment in Kenya Agricultural Research Institute (KARI) these varieties are highly resistant to harmful and dangerous race of stem rust TTKST (Ug99+Sr24).

Table 1. The testing resistance to fungal pathogens

| Variety | Resistance to | | | | Resistance genes |
|---|---|---|---|---|---|
| | Stem rust* | Powdery mildew** (%) | Leaf rust | | |
| | | | Fields test** | Laboratory test** | |
| Omskaya 37 | 20MR | 25 | 5TR | R | *Pm8*, *Lr26, Lr19, Sr31, Sr25* |
| Omskaya 38 | 10R | 40 | 10TR | R | *Pm8*, *Lr26, Lr19, Sr31, Sr25* |
| Omskaya 41 | 20MR | 20 | 5TR | R | *Pm8*, *Lr26, Lr19, Sr31, Sr25* |

Screening was done: * Kenya Agricultural Research Institute; ** Siberian Agricultural Research Institute.

C-banding revealed that the varieties Omskaya 37, Omskaya 38, Omskaya 41, in addition to wheat-rye translocation 1RS.1BL possess wheat-wheatgrass translocation 7DL-7Ai, where a segment of chromosome 7Ai of *Agropyron elongatum* (=*Thinopyrum elongatum*) is translocated to the long arm of wheat chromosome 7D. It is known that 1RS carries the genes *Lr26, Sr31, Pm8, Yr9*, while a translocation from *Thinopyrum elongatum* – genes *Lr19* and *Sr25* (McIntosh et al., 1995). Molecular analyses revealed that in the varieties Omskaya 37, Omskaya 38, Omskaya 41 resistance to leaf rust is determined by genes *Lr26* + *Lr19*, to stem rust – *Sr25*, *Sr31*. Varieties carrying *Lr19* gene were introduced into Volga region of Russia in the late 1980s. Resistance controlled by *Lr19* was defeated at the end of 1990s due to broad cultivation of varieties with this gene in Volga regions (Sibikeev et al., 2011). However, combination of genes *Lr19*+*Lr26* protects plants against leaf rust pathogen. Moreover, the gene *Sr25* ensures high level of protection against Ug99 race (Singh et al., 2008).

Genetic backgrounds of varieties studied in this work are favorable for the action of genes determining resistance to fungal pathogens and drought, as well as high yield and grain quality.

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## Alleles of *Ppd-D1* gene in *Aegilops tauschii Coss.* accessions, winter and spring wheat varieties

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### Introduction

Photoperiod has an important effect on plant growth and development. *Ppd-D1* (2DS) is the one of most potent genes affecting the photoperiod response of wheat (*Triticum aestivum* L.). *Ppd-D1* gene has been considered to be a member of the pseudo-response regulator (*PRR*) family (Laurie, 1997; Bö rner et al., 1998; Turner et al., 2005; Beales et al., 2007). A 2089 bp deletion upstream of the coding region of this gene was found only in photoperiod insensitive wheat with allele *Ppd-D1a*. This deletion leads to misexpression of the *Ppd-D1* gene and causes early flowering in both short- and long-day conditions (Beales et al. 2007). According to Fedorova (2004) the wheat photoperiodic response genes control the duration of II-V stages of organogenesis. The action of *Ppd* genes starts on the 7 days after vernalization and ends in 2-3 weeks before heading. The photoperiod-sensitive cultivars characterized by the delay in the development of II and III stages of organogenesis, that lead to increase tillering,

developing more leaves, formation a larger number of germ ears and larger spike (Fedorova, 2004).

*Aegilops tauschii* Coss. (2n=14; DD) is the donor of D genome of wheat. This species was inhabited a vast region in central Eurasia for more than 2 million years and up to now, it has a wide geographic distribution and it divided into subspecies *tauschii* and *strangulate*, which significantly differ genetically, morphologically and ecologically (Kihara et al., 1965; Yen et al., 1983; Jaaska, 1981; Van Slageren, 1994; Dudnikov, Kawahara, 2006; Dudnikov, 2011 a, b, c, d). According to our observation and data of Xiang et al. (2009), Matsuoka et al. (2008) *Ae. tauschii* shows a lot of variations in heading time.

In recent studies of Huang et al. (2011), three haplotypes were identified in *Ae. tauschii*, i.e. haplotype I without deletions of the large sequence - 2089 bp upstream of the coding region of *Ppd-D1*, haplotype II with a 24 bp deletion, and haplotype III with two deletions of 24 and 15 bp. The haplotype distribution was related to subspecies taxon. All typical ssp. *tauschii* accessions had haplotype I, whereas all accessions of ssp. *strangulata* had haplotype III. The results supported that ssp. *strangulata* or intermediate form was the D-genome donor of common wheat since only haplotype III were found in wheat. The aim of our work was to analyze genetic polymorphism of the gene *Ppd-D1* in accessions of *Ae. tauschii* which are frequently used for introgressive hybridization in Plant Breeding and Genetics Institute (Odessa, Ukraine).

## Materials and methods

The plant material consisted of 20 accessions of *Ae. tauschii* (k-55, k-76, k-108, k-178, k-216, k-358, k-362, k-396, k-415, k-602, k-608, k-624, k-667, k-677, k-678, k-994, k-1322, k-1761, k-1957, k-2363) and bread wheat cultivars from different breeding centers including both winter wheat varieties (20) and spring wheat varieties (27). As a control for photoperiod sensitive and insensitive wheat varieties we used cv. Kooperatorka (*Ppd-D1b*) and analogue line Kooperatorka K-90 (*Ppd-D1a*), respectively (Chebotar et al., 2010).

DNA was isolated by CTAB method from 5-7- day seedlings according to Sivolap et al. (1998). PCR was carried out using allele-specific primers for *Ppd-D1* gene as recommended by Beales et al. (2007). PCR products were analyzed in 6% PAAG by using ALF-express II Genetic Analyzer (Amersham Pharmacia Biotech). PCR products were cut from the gel and purified using the QIA-quick PCR purification kit (Qiagen, Germany). DNA sequencing of PCR products was carried out in both directions using the ABI PRISM Dye Terminator Cycle Sequencing ready reaction kit ("Perkin Elmer", USA), according to the manufacturer. Sequencing was performed in an ABI PRISM 310 Genetic Analyzer (Perkin Elmer). The known homologous DNA sequences were found in Genbank, EMBL and DDBJ data banks using the algorithm BLAST (Altschul et al., 1990) Alignment of DNA sequences was done using the Clustal W multiple sequence alignment program (version 1.7; Thompson et al. 1994) against the reference sequence of k-1322 accession of *Ae.tauschii*.

## Results

Using *Ppd-D1*-specific primers Beales et al. (2007) previously revealed the fragments of 414 and 453 bp, the both fragments correspond to allele *Ppd-D1b*. The differences between the two fragments are two insertions 24 bp and 15 bp separated by 105 bp upstream of the large insertion of 2089 bp (Beales et al., 2007). According to our PCR data we have divided the accessions of *Ae. tauschii* on three groups. Two groups were characterized by the previously

described PCR fragments: 414 bp (accessions k-108, k-358, k-396, k-602, k-608, k-1322 , k-1957); and 453 bp (k-76, k-178, k-415, k-667, k-677, k-2363), respectively. Accessions of the third group (k-55, k-216, k-362, k-624, k-678, k-1761) contain the amplification product of 429 bp. The accession k-994 was heterogenic and characterized by 429 and 453 bp fragments. The nucleotide sequences of representatives of each group (accessions k-1322, k-2363 and k-216 for groups 1-3, respectively) were compared (Fig. 1). The 429 bp fragment differs from 414 bp by 15 bp insertion. On the other hand in comparison with 453 bp it has deletion of 24 bp.

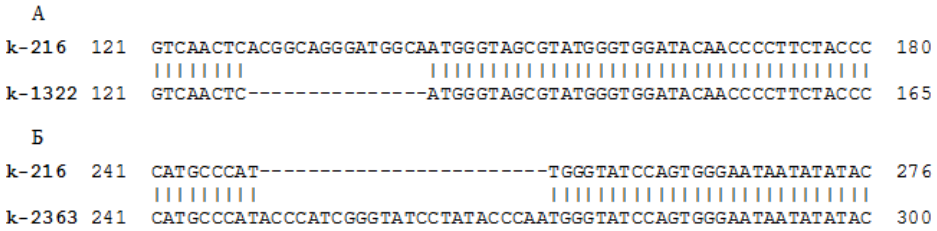


Fig. 1: Structure of fragments of the allele *Ppd-D1b*. Sequence of DNA with primers to *Ppd-D1b* allele accessions: A. k-216, k-1322 and B. k-216, k-2363

The reference 414 bp sequence of k-1322 accession was used for BLAST search to find and compare genetic similarity level of known *PRR* gene sequences from different species. This sequence was 100% homologous to the sequences of *Ae. tauschii* spp. *strangulata* isolates (AS2386, AS2387) investigated by Huang et al. (2011). These authors showed that the accessions of subspecies *strangulata* are characterized by the 414 bp fragment (haplotype III) and accessions of subspecies *tauschii* are characterized by the 453 bp fragment (haplotype I). Accessions with the fragment of 429 bp (haplotype II) were attributed to the intermediate forms between the two subspecies (Huang et al., 2011). Guo et al. (2010) showed the presence within *Ae. tauschii* of two haplotypes of gene *Ppd-D1b* which characterized by the PCR products of 414 and 453 bp size. Our results correspond to these data. Among the studied accessions of *Ae. tauschii*, we did not find *Ppd-D1a* allele, which determines insensitivity to photoperiod.

All investigated winter wheat cultivars have the dominant photoperiod insensitive allele *Ppd-D1a.* As known, photoperiod insensitive cultivars are common in regions with warm climate. According to Litvinenko, Kozlov (1986) and Fedorova (2004) winter wheat cultivars with insensitivity to photoperiod have higher potential productivity in arid desert conditions, better use spring moisture reserves, more rapidly accumulate biological yield, but have worse performance adaptability to wintering conditions. By reducing the length of growing season, due to the presence of one or several dominant alleles of *Ppd* genes, modern varieties can avoid the impact of drought and high temperatures during grain filling and brown and stem rust epiphytotics formation (Lyfenko et al, 2002;. Musich et al, 1996). On the other hand only 26 % of investigated spring wheat varieties were characterized by *Ppd-D1a*, and 67 % of spring wheat cultivars were characterized by the *Ppd-D1b* allele (414 bp) and two cultivars were heterogenic.

In compare to other geographical region, Australian and Indian varieties have dominant *Ppd* genes (Law, Scarth, 1984), also in the Middle East breeders prefer photoperiod insensitive varieties, because of their high adaptability and positive agronomic performance (Marshal et al., 1989). In the former Yugoslavia photoperiod insensitivity provides benefits in harvest more than 35 %, in Central Germany this advantage does not exceed 15%, in the UK this figure can vary from + 9 % in a warm and dry season, to -8 % at typical cool and wet summer (Worland et al., 1998).

## Conclusion

By molecular genetic analysis we revealed three haplotypes of *Ppd-D1b* gene among 20 accession of *Ae. tauschii* collection. We did not find the *Ppd-D1a* allele among the studied accessions of *Ae. tauschii.* In genotypes of modern winter wheat cultivars the allele *Ppd-D1a* is present predominantly and about 67 % of spring wheat cultivars are characterized by the *Ppd-D1b* allele.


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## Rye chromatin involved in wheat resistance to bunt

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### Introduction

Rye has already provided genes that proved useful in the wheat breeding and many cultivars that enjoyed a great success over time are carriers of translocations from rye, especially of some translocations in which the short arm of 1R chromosome is involved. The bunts are diseases with severe effects on wheat yield and quality. The wheat bunts are produsedby *Tilletia caries* (DC) Tul, *T. laevis* (Wallr) Liro., and *T. controversa* but also *T. secalis* can affect wheat. The F00628G34-M line created at NARDI Fundulea by crosses between triticale and wheat (possessing a 1AL:1RS translocation) showed good resistance to bunt in artificial infections, both in the tests from Romania done at Fundulea (Ittu et al. 2006) and Simnic (Oncică and Săulescu 2008) and in most locations of international tests from the European project "Tilletia Ring Test" (Săulescu et al. 2010).

### Material and methods

The study was done on 68 F4 lines, obtained by crossing the susceptible Litera cultivar with the F00628G34-M line.

- Phenotype observations were made in field under artificial infection;

- 21 markers specific for rye and for the 1R short arm chromosome, 22 SSR markers localized on the 1AS chromosome and 5 ISSR markers;

- Nested - PCR (F3/R3 - WMS1223);

- Because of incomplete expression of both susceptibility and resistance, characteristic for bunt, association of markers and level of attack was estimated using Chi square test. An interactive software (Preacher 2001) was used.

### Results and discussion

*Phenotypic observations:* Phenotype observations made in 2010 for the F4 generation on 68 progenies showed 18 non-bunted progenies and 50 bunted progenies, the percentage of bunted ears varied between 9 and 80 percent, whilst the susceptible parent, Litera showed a 65 percent of bunted ears. This suggests that the bunt resistance gene transmitted from F00628G34-M is recessive or partly dominant or might be repressed by certain factors from wheat genome.

*The Characterization of 1AL:1RS translocation from F000628G34-M line:*

- The molecular marker results (IAG95) have shown that the line F000628G34-M carries rye chromatin of INSAVE type, and probably has the *Pm17* powdery mildew resistance gene.
- *Sec-1* locus showed polymorphism presented by a PCR product of about 1200bp.

- The combination of molecular analysis for *Sec-1* and *Glu-A3* loci permitted to distinguish heterozygous genotypes.
- 1RS:1AL translocation present in the line F000628G34-M has the centromere from wheat genome (based on molecular markers – rems1280 and wmc278);
- Nested - PCR with markers (F3/R3 / WMS1223) suggested some rearrangements on the 1RS:1AL translocation.

*The association of bunt resistance with rye chromatin from F000628G34-M line, based on molecular markers assays*: Both the presence of the universal marker for the rye chromatin and of four other specific markers for the 1R chromosome, and the absence of 6 specific markers for the 1A chromosome proved to be significantly associated with bunt resistance inherited from the F00628G34-M line, carrier of the 1A/1R translocation. This proves that bunt resistance identified in the F00628G34-M line is associated with the presence of rye chromatin. From our knowledge this is the first time when it is proven that a bunt resistance gene is associated with the rye chromatin transfer to wheat. Molecular assays suggest a possible location of the bunt resistance gene from F00628G34-M line, on the 1RS chromosome, in the region homeologous with the *Glu-A3* locus and close to WMS1223 microsatellite locus.

Table 1: Association of molecular markers to bunt resistance

| Marker Name | Location on chromosome | Genome specificity* | Probability that the Marker is not associated with bunt resistance gene- for 68 F4 lines. |
|---|---|---|---|
| *F3 /R3* | Universal marker for rye | R | 0.00094212 |
| *SCM9* | 1R, 1B | R/W | 0.00958200 |
| *Sec-1* | 1R | R | 0.00222864 |
| *TSM106* | 1R | R | 0.00958200 |
| *TSM123* | 1R | R | 0.00958200 |
| *Xbarc263* | 1A | W | 0.00012177 |
| *Xbarc1048* | 1A | W | 0.00001093 |
| *Xgwm136* | 1A | W | 0.00002097 |
| *Xgwm (wms)1223* | 1R,1A, 1D | W/R | 0.00036605 |
| *PSP2999 (Glu-A3)* | 1A | W | 0.00006456 |
| *Xwmc818* | 1A, 1B | W | 0.00012177 |

*R-rye; W-wheat

**Conclusions**

- The bunt resistance identified in the F00628G34-M line is associated with the presence of rye chromatin;
- For the transfer of this gene in other genotypes we have proved that the marker assisted selection can be used, the most indicated markers being *Xbarc1048, Xgwm136* or *PSP2999* (*GluA3*), whose absence notifies the presence of rye chromatin in the region where the gene is localized. The *Xwms1223* marker, which produces specific products for the rye translocation, can be also used;
- The bunt resistance gene present in F00628G34-M is different from other known resistance genes.

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# Imaging based Cereals Shoot and Root Phenotyping Research at the Jülich Plant Phenotyping Centre (JPPC)


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Plant phenotyping, the quantitative analysis of plant structure and function, has become essential for coupling the advances in plant biotechnology with plant breeding science and ultimately crop production. The functional plant body (phenotype) results from the dynamic interaction of the genotype with spatially and temporally heterogeneous environment. Phenotyping research aims at identifying genetic as well as environmental factors influencing biomass yield and sustainable plant production. The Jülich Plant Phenotyping Centre (JPPC) is at the forefront of cutting-edge research in this field. We built a unique infrastructure for automated, non-invasive technologies employed to quantify relevant plant traits. These methods aim at ranking shoot and root phenotypic characteristics in controlled environments and field conditions, and enabling the transfer of promising traits from crop to crop in pre-breeding programs and agricultural applications. At the JPPC plant biologists, agronomists, physicists, engineers, bioinformaticians and modellers work together for the development of innovative phenotyping systems encompassing non-invasive optical sensors technologies applicable to cereals and several other crops for the measurement of photosynthesis and growth-related traits in both controlled environments and field scenarios (Rascher *et al.*, 2011; Fiorani *et al.*, 2012). Two of those automated systems that enable root and / or shoot phenotyping are briefly described in this manuscript. The approaches outlined here are fully applicable to measurements in 2D of root system architecture and shoot projected area, which is correlated with total aboveground biomass.

Compared with aboveground plant organs, roots are not easily accessible for morphological investigations. A better understanding of root system architecture and function is critical to improve resource use efficiency of major crops, especially under adverse conditions like water shortage, low soil fertility and increasing salinity. Research of root systems is still very challenging, based mainly on manual, destructive methods. Latest endeavours to systematically examine root systems comprise the development of techniques by which plants are cultivated in transparent soil-filled columns or rhizotrons. While for the analysis of monocot shoots several automated methods have been implemented in recent years (Berger *et al.*, 2010), automated systems for phenotyping root architecture of plants grown in transparent

soil-filled containers are generally lacking. At the JPPC we have developed the GROWSCREEN-Rhizo a robotic system, allowing simultaneous imaging of roots and shoots of plants grown in soil-filled rhizotrons (Figure 1, left). This prototype consists of 72 rhizotrons distributed into two rows. Their dimension is 90 x 70 x 5 cm, however smaller rhizotrons can also be incorporated into the system. Rhizotrons typically consist of one transparent plate, which always faces downwards with an inclination angle from 0° (vertical) to 43°. Between the two rows an imaging station carrying cameras and LED-panels is moved automatically on a linear axis to the positions of the rhizotrons that are subsequently drawn inside for image acquisition of root and shoot systems. The whole procedure is fully automated allowing imaging of 60 rhizotrons per hour. Two side-view RGB images are used to quantify the shoot projected leaf area *via* hue, saturation and value thresholds segmentation (for more details see Walter *et al.*, 2007). Moreover, a grey-value image of the whole transparent rhizotron surface is used to measure important traits of root systems, such as root system length, maximum root depth and width, root branching rates and angles, by means of a modified version of the software GROWSCREEN-Root (Mühlich *et al.*, 2008; Nagel *et al.*, 2009). Validation experiments have been performed using different plant species. This non-invasive phenotyping allows repeated measurements of the same root system at frequent time intervals permitting the study of root development with relatively high accuracy and resolution for both monocotyledonous and dicotyledonous species.

Fig. 1: The GROWSCREEN-Rhizo prototype system (left) and the Automated Shoot Phenotyping Platform (right) allow the quantitative measurement of total shoot projected area and of the visible part of the root system

A second platform at the JPPC (Figure 1, right) that combines image acquisition techniques with a robotised plant transfer system enables shoot phenotyping of pot grown plants (Visser International Trade & Engineering B.V.). Robot-based phenotyping facilitates high-throughput analysis and method standardisation, whereas manual handling would be laborious and time consuming. The system comprises: 1) A closed imaging station equipped with a rotating table, light panels and cameras for image acquisition. Two cameras are mounted at different angles in relation to the plant; that is 0° and 90° representing top and side views respectively. The rotating table at the base of the cabinet allows up to four consecutive images (with 90° angle to each other) per camera setting. 2) A mechanical gripper supported by an above-ground metal construction is committed to the transfer of plants from their growing positions to the imaging station and *vice versa*. The gripper has bi-directional motion on three axes (x, y, z) and drives to pre-defined plant positions, contrary to the belt-transfer systems. 3) Finally, an irrigation station in front of the imaging cabinet allows coupling of image acquisition with watering of individual plants up to a specific pot weight controlled by means of a scale. The coordinated pot transfer-imaging-watering procedure is fully automated and requires approx. 3 min per plant. Due to its non-invasive nature, the shoot phenotyping

platform allows screening of shoot biomass at regular time intervals throughout the plant cultivation period. Validation experiments with barley, maize, and rice have demonstrated highly significant correlations between projected leaf area and shoot biomass of monocotyledonous species ($R^2$ = 0.94 - 0.96; Figure 2).

Both JPPC prototype systems will assist the simulation of environmental conditions that may be relevant to field scenarios, investigate gene-environment interactions, identify heritable plant traits and support breeding efforts towards improved resource use efficiency (water and nutrients) and sustainable crop yields.

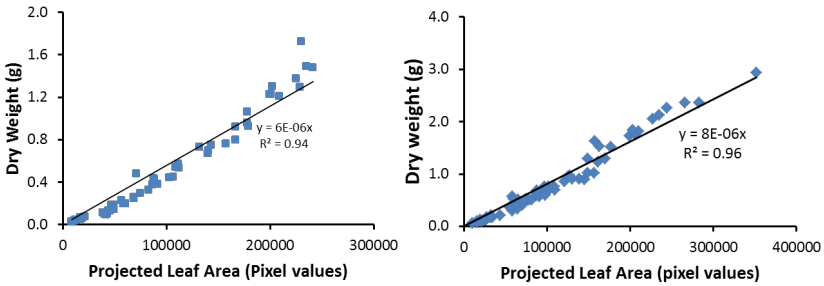


Fig. 2: Correlation between projected leaf area measured *via* RGB imaging and shoot biomass for barley (n= 63, left) and maize (n=70, right) plants. $R^2$ represents goodness of fitness of the curve

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# Substitution of homoeologous group 7 wheat chromosomes by barley *H. marinum* subsp. *gussoneanum* chromosome $7H^1L^{mar}$

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Chromosomes of related species of tribe Triticeae can be substituted within seven homeologous groups of wheat. Despite the fact that *Hordeum* L. and *Triticum* L. genera are phylogenetically divided, overcoming of incompatibility of interspecific crosses is possible in experimental conditions (Davies 1960). L.A. Pershina with co-workers obtained barley-wheat hybrids between tetraploid wild barley *H. marinum* ssp. *gussoneanum* Hudson (2*n*=28) and common wheat (Pershina et al. 1988, 1998). Using molecular-genetic methods, addition lines with a pair of telocentric barley chromosomes $7H^1L^{mar}$ were isolated from $BC_3$-plants, which resulted from backcrossing of barley-wheat hybrids by common wheat cultivars (Trubacheeva et al. 2008). It was found that genome fragments of barley are able to integrate in the genome of alloplasmic common wheat lines (Trubacheeva et al. 2003, Numerova et al. 2004). Availability of lines with addition alien chromosome enables a purposeful substitution of certain wheat chromosomes by chromosomes of related cereals, if monosomic lines of recipient cultivar also available. Methods of alien substitution lines obtaining are described in literature (Unrau 1959).

The aim of this work is a cytological analysis of meiosis in hybrids $F_1$- $F_2$, obtained from crosses of 7A, 7B and 7D monosomics of wheat cultivar Saratovskaya 29 (S29) with barley-wheat alloplasmic ditelosomic addition line with chromosome $7H^1L^{mar}$.

## Materials and Methods

The development of alien substitution lines was carried out according to known protocols (Unrau 1959). Monosomics (2*n*=41) of chromosomes 7A, 7B and 7D of cultivar Saratovskaya 29 were used as recipients. Monosomics were crossed with barley-wheat alloplasmic ditelosomic addition lines (2*n*=44) with chromosome $7H^1L^{mar}$ from wild barley *H. marinum* ssp. *gussoneanum* (Trubacheeva et al. 2008). Plants were grown in greenhouse and metaphase I (MI) at meiosis was analysed in hybrids $F_1$-$F_2$. Meiotic chromosome configuration was examined at MI in pollen mother cells (PMCs) using 2% acetocarmine smear method.

## Results and Discussion

Crossing of wheat monosomics with 44-chromosome addition line resulted in 43-chromosome plants carrying 21”+t’ chromosomes in MI and 42-chromosome double monosomics with 20”+1’+t’ chromosomes (Table 1). Telocentric barley chromosome is identified in meiosis easily and thereby facilitates the isolation of required genotype among hybrid progenies (Fig. 1).

Table 1. Metaphase I analysis of meiosis in $F_1$ hybrids (mono S29 7A, 7B, 7D × addition line, $2n$=42+2t$^{mar}$)

| Chromosome | The number of plants with the configuration of chromosomes in M1 | | |
|---|---|---|---|
| | **20"+1'+t'** | 21"+t' | Number of analyzed plants |
| 7A | **13(73)** | | 13 |
| 7B | **19(93)** | | 19 |
| 7D | **12(56)** | 1(3) | 13 |

Note: in parentheses the number of cells indicated

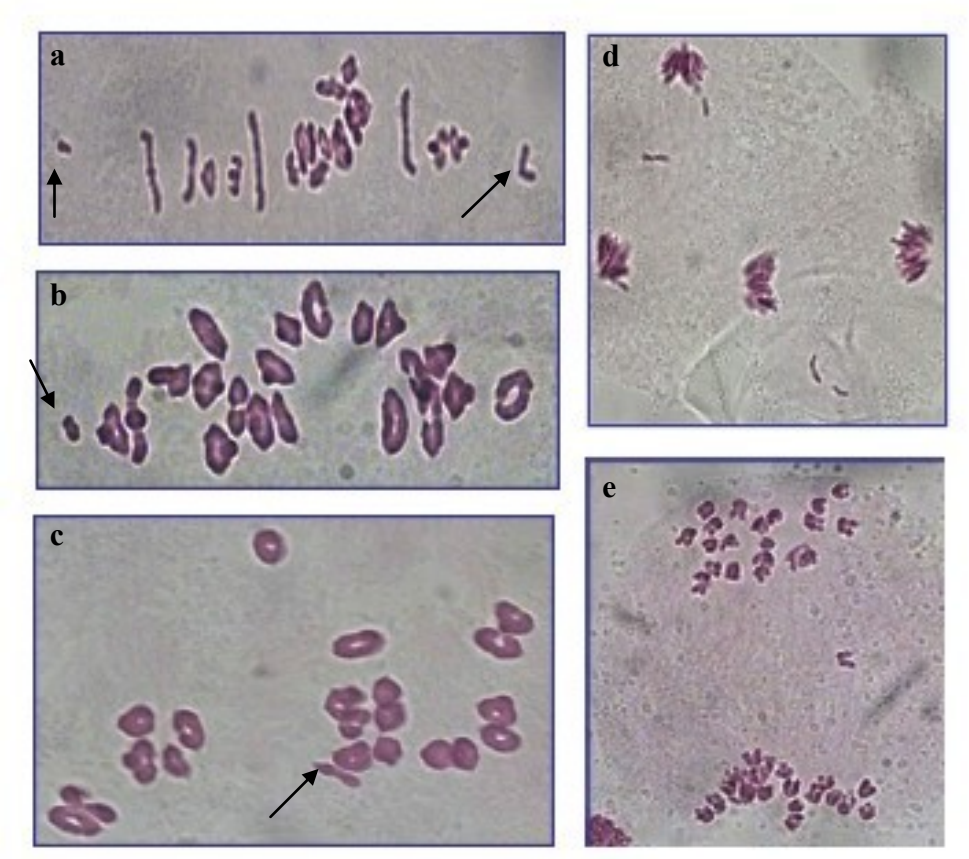


Fig. 1: Metaphase I (a) and anaphase II (d) PMCs of hybrid $F_1$ with chromosome constitution 20"+1'+t'; metaphase I (b) and anaphase I (e) of wheat monotelosomic 7H$^{I}$L$^{mar}$(7D) substitution line and metaphase I (c) chromosome pairing in wheat ditelosomic 7H$^{I}$L$^{mar}$(7D) substitution line: a) MI 20 bivalents and one telocentric barley and one univalent 7B**;** b) MI 20 bivalents and one telocentric barley; c) MI 20 bivalents and one telocentric bivalent barley d) in AII wheat univalent and telocentric barley are lagging; e) in AI barley telocentric is lagging

The 42-chromosome plants (20"+1'+t') were selected and self-pollinated (Table 2). In $F_2$ progeny six genotypes are expected: 21, 20, 20+1, 20+1+t7H, 20+t7H, 21+t7H. The analysis of M1 at meiosis in three populations studied established that the most plants contained wheat

chromosomes (67%). At that same time 33% of plants contained telocentric barley chromosome $7H^{mar}$. As a result, monotelosomic substitution plants were selected among $F_2$ hybrids. Monotelosomic $7H^1L^{mar}$(7A), $7H^1L^{mar}$(7B) and $7H^1L^{mar}$(7D) substitution lines containing 20 bivalents of wheat and 1 barley telocentric chromosome which had substituted wheat chromosomes 7A, 7B or 7D were obtained (Table 2 and Fig. 1). The frequency of barley telocentric chromosome transmission through gametes during the development of wheat-barley substitution lines has been studied. The data obtained indicate that the probability of wheat-barley substituted monotelosomics occurrence is relatively low in hybrid progeny. It has been found that paternal gametes with barley chromosome $7H^1L^{mar}$ have reduced competitiveness and the alien chromosome is transmitted better through ovules. At the same time a high competitive and compensation ability of male gametes with 21 chromosomes (20+t7H) was established during the self-pollination of monotelosomics with chromosome substitution $7H^1L^{mar}$(7D). So far euplasmic ditelosomic substitution line $7H^1L^{mar}$(7D) has been obtained (Trubacheeva et al. 2009).

Table 2. The analysis of metaphase 1 of meiosis in $F_2$ hybrids in self-pollination of plants $F_1$ with genotype $2n$=41+$t^{mar}$

| Chromosome | The number of plants with the configuration of chromosomes in M1 | | | | | | |
|---|---|---|---|---|---|---|---|
| | 20” | 21” | 20”+1’ | **20”+t’** | 20”+1’+t’ | 21”+t’ | Analyzed plants |
| 7A | 2(3) | 3(15) | 8(30) | **2(12)** | 1(3) | - | 16 |
| 7B | 3(11) | 6(27) | 17(84) | **1(4)** | 8(34) | 5(20) | 40 |
| 7D | - | 2(10) | 7(29) | **3(14)** | 2(13) | 2(9) | 16 |

Note: in parentheses the number of cells indicated

Using of cytologically marked addition line is the most precise method for obtaining of alien substitution lines. Chromosome of *H. marinum* ssp. *gussoneanum* donor is a telocentric chromosome and therefore monotelosomic ($2n$=40+t) and ditelosomic ($2n$=40+2t) alien substitution plants could be easily selected at meiosis or mitosis. The presence of barley chromosomes was detected using GISH (Fig. 2).

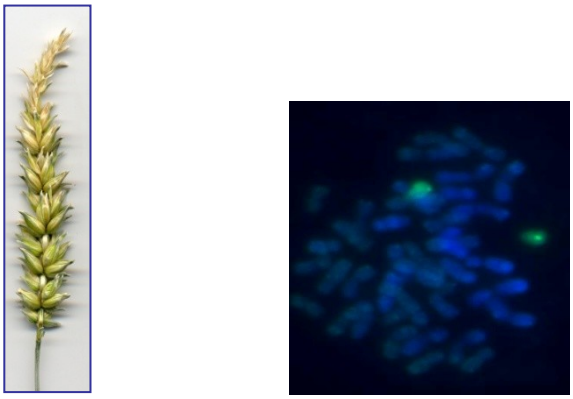

Fig. 2. Spike of ditelosomic $7H^1L^{mar}$(7D) and GISH of wheat-barley ditelosomic $7H^1L^{mar}$(7D) substitution plants.

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## The study of developmental stages of near-isogenic lines of winter wheat cultivar Bezostaya 1 with dominant genes *Vrn-A1* and *Vrn-B1*

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The timing and duration of a developmental stage of wheat may be important in its production. For example, it may allow plants to avoid unfavorable environmental conditions (Chen *et al.*, 2010). Developmental stages are under strong genetic control. Košner & Pankova (2001, 2004) have shown the significant influence of recessive and dominant *Vrn* loci on growth phases. Chen *et al*. (2010) have determined, that the *Vrn-A1* locus had the greatest impact on the development at stem elongation, the *Ppd-D1* locus – on heading date, and *Vrn-D3* locus – on physiological maturity.

Košner & Pankova (2004), when studied growth stages of substitution lines with dominant *Vrn-1* loci in the genetic backgrounds of winter cultivars, have shown, that line carrying *Vrn-A1* locus was the earliest, while line with *Vrn-B1* locus was the latest. Besides, in line with *Vrn-B1* the tillering and stem elongation were the longest phases, but the stage from the flowering to ripening was the shortest in comparison with two other lines. It confirms the data of Kuperman *et al.* (1982) that the late-ripening plants have the longer vegetative phase of development, than the early ones.

The objective of this work is to study the length of developmental stages of near-isogenic lines (NILs) of winter wheat cultivar Bezostaya 1 with dominant gene *Vrn-A1*, alleles *Vrn-B1a* and *Vrn-B1c*, and of winter cultivar Bezostaya 1.

## Material and methods

As a material of the research three NILs have served with dominant gene *Vrn-A1* and alleles *Vrn-B1a* and *Vrn-B1c* in the genetic background of winter cultivar Bezostaya 1 – i:Bez1*Vrn-A1*, i:Bez1*Vrn-B1a* and i:Bez1*Vrn-B1c*, respectively (Efremova *et al.*, 2011). The donor of *Vrn-A1* gene was NIL Triple Dirk D (Pugsley, 1972), the donors of *Vrn-B1a* and *Vrn-B1c* alleles were substitution lines Diamant II(5A Mironovskaya 808) and Saratovskaya 29(5A Mironovskaya 808) (Maystrenko, 1992), respectively. In addition to three NILs we have also studied non-vernalized winter cultivar-recipient Bezostaya 1.

Plants were grown in the greenhouse in 14 h. photoperiod. We scored shoots, II, III, IV-th leaf, tillering, the 1st node, stem elongation and heading (Kuperman *et al.*, 1982). The tillering was scored in the day, when the second tiller had separated from the first tiller. The 1st node was scored , when it was above the surface. The stem elongation begun, when the first node had risen at the 5 cm, and the second node had separated from it. Heading begun, when the spike had completely appeared from the flag-leaf. The length of phases (days) was counted from the shoots. The significance of differences between average values of the traits was calculated by using the Student's *t*-test.

## Results

As is shown in figure 1, all three NILs and winter cultivar Bezostaya 1 didn't differ on appearance of the II, III, IV-th leafs and on tillering. These phases lasted for 2-4 days. NILs began to differ on the appearance of the 1st nodes. The earliest 1st nodes were in line i:Bez1*Vrn-A1* (24 days after shoots), after 8 days on average they appeared in i:Bez1*Vrn-B1c*, and after 13 days from that – in i:Bez1*Vrn-B1a* (Tab. 1). So, the period "tillering – the 1st nodes" accounted for 9, 17 and 29 days in lines with *Vrn-A1*, *Vrn-B1c* and *Vrn-B1a* alleles, respectively. The 1st nodes haven't appear in non-vernalized winter cultivar.

NILs didn't differ on the length of phases "stem elongation" and "heading". The heading time difference between lines with *Vrn-A1* and *Vrn-B1c* genes was 11 days, between *Vrn-B1c* and *Vrn-B1a* it accounted for 16 days, between *Vrn-A1* and *Vrn-B1a* – 28 days (for all three lines $P > 0.999$).

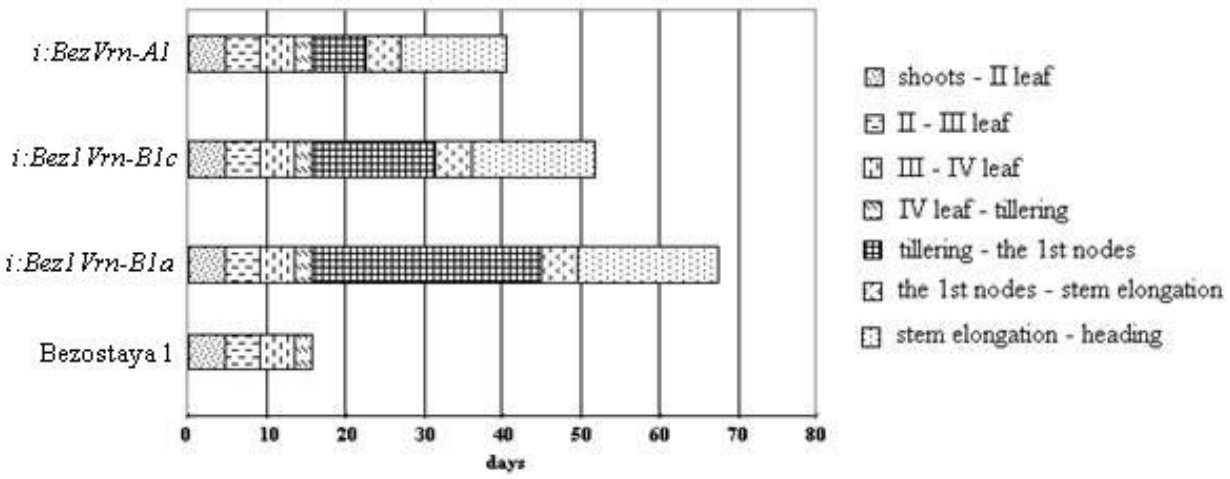


Fig. 1: Length of developmental stages (days) of isogenic lines with dominant *Vrn-A1*, *Vrn-B1a* and *Vrn-B1c* loci and of winter cultivar Bezostaya 1

As is shown in Fig. 2, the differences between NILs and winter cultivar can be observed also on development of their apexes. Apexes of winter cultivar until the harvest day (91 days after shoots) grew in length up to 1,5 mm.

Thus, lines with dominant alleles of *Vrn-B1* gene were later, than line with *Vrn-A1*, because their 1st nodes started to appeared later, or because they have later changed from vegetative to generative phase of development. Winter cultivar for the absence of vernalization had stayed at the vegetative stage.

Table 1: Mean amount of days from shoots to the developmental stages

| | II leaf | III leaf | IV leaf | tillering | The 1st nodes (1) | Stem elongation(1) | Heading (1) |
|---|---|---|---|---|---|---|---|
| Bez 1: *Vrn-A1* | 4,26±0,09 | 8,59±0,12 | 12,65±0,12 | 15,58±0,27 | 24,17±0,17 | 28,49±0,21 | 41,23±0,44 |
| Bez 1: *Vrn-B1c* | 4,25±0,08 | 8,56±0,09 | 13,05±0,09 | 15,38±0,18 | 32,3±0,17 | 35,92±0,18 | 52,29±0,3 |
| Bez 1: *Vrn-B1a* | 4,18±0,09 | 8,59±0,09 | 12,95±0,1 | 16,08±0,18 | 45,28±0,62 | 49,98±0,54 | 68,7±0,79 |
| Bez 1 | 4,1±0,08 | 8,58±0,09 | 12,86±0,12 | 15,5±0,22 | - | - | - |

(1) – differences between all lines are significant ($P > 0.999$).

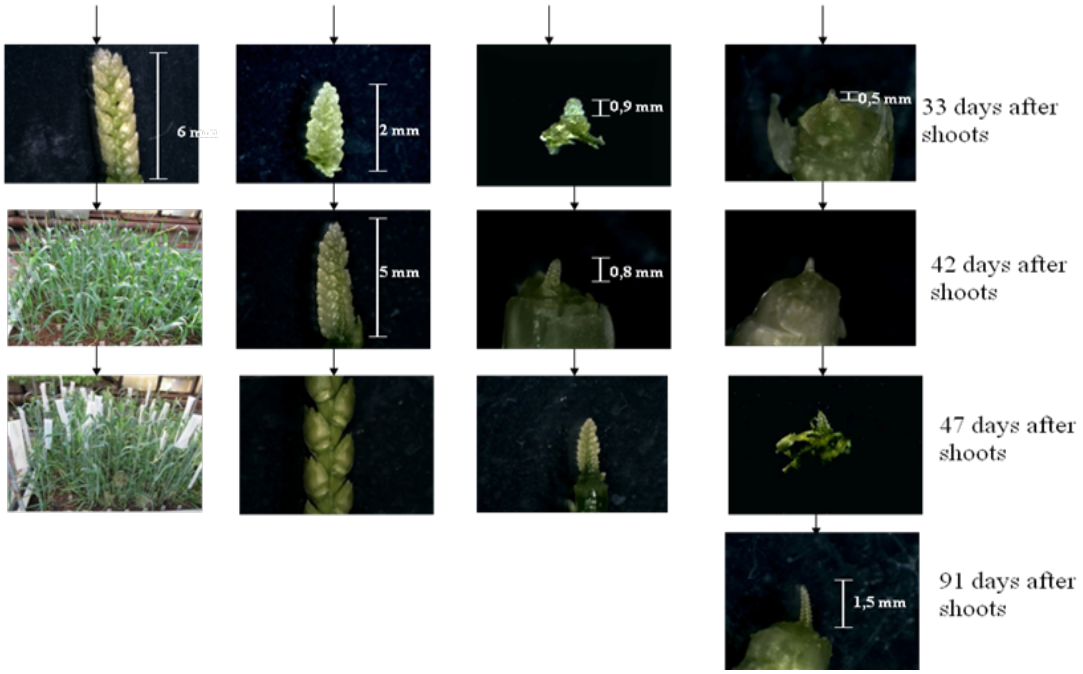


Fig.2: Apex development of NILs and winter cultivar

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# STS-PCR characteristics of genes coding HMW-GS in old cultivars of wheat *(Triticum aesitvum* spp. *vulgare* L.)


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Gluten is one of the storage protein fractions of wheat grain. It is characterized by flexibility, extensibility and viscosity, which determine the baking quality value of baking flour. Gluten is composed of subunits of gliadin and also subunits of HMW and LMW glutenin. HMW-GS glutenins have the greatest effect on the technological value of gluten and on flour quality. For this reason, their structure and genetic determination are the most intensively studied. The study presented here examines the genes coding HMW-GS in 28 old cultivars of wheat *(Triticum aestivum ssp vulgare* L.) and in two standard cultivars (Finezja and Kobiera). All the material was obtained from the collection of the Institute of Plant Breeding and Acclimatization in Radzików. Using the STS-PCR method, the composition of alleles at loci *Glu-A1, Glu-B1* and *Glu-D1* was identified. Differences were found in the frequency of allele occurrence at each locus in the different cultivars – the locus *Glu-A1* allelic differences were related to 3 alleles, as follows: *Glu-A1a* (coding *Ax1),* 1b *(Ax2 *)* and *1c (AxNull);* the locus *Glu-B1* to 5 alleles: *Glu-B1-1a (Bx7* ), *1b (Bx7 + y8), 1c (Bx7 + y9), 1d (Bx6 + y8) and 1e (By20);* and the *Glu-D1* locus to two alleles: *Glu-D1-1a (Dx2 + y12) and 1d (Dx5 + y10).* The presence of new allelic forms within the locus *Glu-A1-1 GluA1-1b-2* * in the range 145-330 bp were observed. These results indicate that the loci *Glu-A1* and *Glu-B1* are characterized by the greatest variability. Across all the cultivars a total of 42 loci that were 100% polymorphic were observed. The greatest diversity of genotypes occurred during the amplification of six pairs of primers for genes: *Dx2, Dx5, Dy10, Dy12, Ax1, By8, By9 and By20.* The results obtained showed that old cultivars of hexaploid wheat are a valuable source of new genetic variations that may be useful in breeding new cultivars.

## Material and methods

*Plant material:* The study presented here examines the genes coding HMW-GS in 28 old cultivars of wheat *(Triticum aestivum ssp vulgare* L.) and in two standard cultivars (Finezja and Kobiera). All the material was obtained from the collection of the Institute of Plant Breeding and Acclimatization in Radzików.

*DNA purification:* Genomic DNA was extracted from fresh 5-6 days old ethiolated coleoptyles from 28 cultivars of wheat and two standard cultivars (Finezja and Kobiera). The isolation was performed with Wizard® Genomic DNA Kit (Promega).

*PCR-STS analysis:* The oligonucleotides used as primers were designed based on the literature data (table 1) and synthesized in the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. PCR analyses were performed in a PTC-200 thermal cycler MJ MiniTM (BIO-RAD) with a heated lid in the final volume of 25µl. The single PCR reaction mixture contained: 1xbuffer (Novazym), 2mM MgCl2, 2 mM dNTP, 0,5 mM each primer, 50ng genomic DNA and 0,5 U Allegro Taq DNA Polymerase (Novazym).

Table 1: Set of alleles analysed in this study– specific markers for identification of HMW wheat glutenin genes

| Genes | Expected DNA fragment | Froward and reverse Primer sequences (5' – 3') | References |
|---|---|---|---|
| *Ax2** *Ax1* *AxNull* | 1400 bp 1500 bp | F:CCATCGAAATGGCTAAGCGG R:GTCCAGAAGTTGGGAAGTGC | Lafiandra et al. 1997 |
| *AxNull* | 920 bp | F:ACGTTCCCCTACAGGTACTA R:TATCACTGGCTAGCCGACAA | Lafiandra et al. 1997 |
| *Ax2** | 2650 bp | F:CCGATTTTGTTCTTCTCACAC R:CACCAAGCGAGCTGCAGAT | De Bustos et al. 2000 |
| *Bx6* *Bx7* *Bx20* | 3 groups: (1 band of 850-920 bp; 4-5 bands of 420-640 bp; 2-4 bands of 180-280 bp) | F:CAAGGGCAACCAGGGTAC R:AGAGTTCTATCACTGCCTGGT | Butow et al. 2004 |
| *By8* *By9* *By22* | 3 bands (290-400 bp) | F:GCAGTACCCAGCTTCTCAA R:CCTTGTCTTGTTTGTTGCC | Lei et al. 2006 |
| *By8* *By9* | 750 bp 710 bp 660 bp | F:TTCTCTGCATCAGTCAGGA R:AGAGAAGCTGTGTAATGCC | Lei et al. 2006 |
| *Bx7* | 2373 bp | F:ATGGCTAAGCGCCTGGTCCT R:TGCCTGGTCGACAATGCGTCGCTG | Ahmad 2000, Anderson, Greene 1989 |
| *By8* | 527 bp | F:TTAGCGCTAAGTGCCGTCT R:TTGTCCTATTTGCTGCCCTT | Lei et al. 2006 |
| *Dx2* *Dx5* | 413-430 bp 450 bp | F:GCCTAGCAACCTTCACAATC R:GAAACCTGCTGCGGACAAG | D'Ovidio, Anderson 1994 Anderson, Greene 1989 |
| *Dy10* *Dy12* | 516 bp 612 bp | F:GTTGGCCGGTCGGCTGCCATG R:TGGAGAAGTTGGATAGTACC | Ahmad 2000, Smith et al. 1994 |

Table 2. Identification of PCR –STS products confirming the presence of genes HMW-GS glutenin in studied cultivars of wheat

| Gens HMW-GS | Cultivars/ bands size bp | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | PIC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Dx5* | **453** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **0,50** |
| *Dx2* | **435** | **1** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **0** | **0,50** |
| *Dy12* | **552** | **1** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **0** | **0,50** |
| *Dy10* | **422** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **0,50** |
| *Ax1* | **1500** | **1** | **0** | **1** | **0** | **0** | **1** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **1** | **1** | **0,50** |
| | **943** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0,32** |
| | **472** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **0,28** |
| | **385** | **1** | **0** | **1** | **0** | **0** | **1** | **1** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **1** | **1** | **0,50** |
| | **245** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **1** | **0** | **0,36** |
| *Axnull* | **848** | **0** | **1** | **0** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0,50** |
| | **744** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0,48** |
| | **660** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0,48** |
| | **626** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **505** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0,48** |
| | **372** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |
| | **222** | **0** | **1** | **0** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **0,49** |
| *Ax2** | **30** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |
| | **241** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |
| | **145** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |
| *By8* | **751** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **1** | **1** | **0,28** |
| *By9* | **601** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **1** | **1** | **0** | **0** | **1** | **0,48** |
| *By20* | **506** | **0** | **1** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **1** | **0** | **1** | **0** | **1** | **1** | **1** | **1** | **1** | **0,48** |
| | **434** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **1** | **0** | **1** | **1** | **1** | **1** | **1** | **0,23** |
| | **288** | **1** | **0** | **1** | **1** | **1** | **0** | **1** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **1** | **1** | **0** | **1** | **1** | **0,50** |
| ***Bx6,*** | **2236** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |
| *Bx7* | **1195** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0,12** |
| | **980** | **0** | **0** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,32** |
| | **928** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **0,28** |
| | **720** | **1** | **0** | **1** | **1** | **1** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **1** | **1** | **1** | **0** | **0** | **0** | **0** | **0** | **0,48** |
| | **693** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **1** | **0** | **1** | **1** | **0,39** |
| | **554** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **1** | **1** | **0** | **0** | **1** | **0** | **0,44** |
| | **485** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **1** | **1** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **1** | **0** | **1** | **1** | **0,39** |
| | **289** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0,18** |
| | **221** | **0** | **0** | **1** | **1** | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **1** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0,78** |
| *Bx7* | **1585** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **1368** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **1011** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **767** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **545** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **354** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |
| | **321** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,12** |
| | **231** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **1** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0,06** |

*1-30 Studied cultivars: 1-Antonińska S.46, 2-Balta, 3-Biała Kaszubska, 4-Choryńska, 5-Dańkowska Biała, 6-Eka132, 7- Magnatka Rogalińska, 8-Mudczanka Czerwona, 9-Murzynka Lipińskieg, 10-Mydliczanka, 11-Niewylegająca, 12-Ostka Czerwona Łopustka, 13-Ostka Górczańska, 14-Ostka Kazimierska, 15-Ostka Mikulicka Selit, 16-Ostka Nadwiślańska, 17-Poznańska, 18-Sielecka Genetyczna, 19-Sobieszyńska 44, 20-Sobótka, 21-Squarhead Grodkowicka, 22-Srebrzysta, 23-Ślązaczka, 24-Tryumf Mikulic, 25-Udyczanka Czerwona, 26-Wysokolitewka Antonińska, 27-Wysokolitewka Sztywnosłoma, 28-Żelazna, 29-Finezja, 30-Kobiera.* PIC- *polymorphic information content (*Ghislain et al. 1999)

## Conclusions

Using the STS-PCR method, the composition of alleles at loci *Glu-A1, Glu-B1* and *Glu-D1* was identified. Differences were found in the frequency of allele occurrence at each locus in the different cultivars – the locus *Glu-A1* allelic differences were related to 3 alleles, as

follows: *Glu-A1a* (coding *Ax1),* 1b *(Ax2 *)* and *1c (AxNull);* the locus *Glu-B1* to 5 alleles: *Glu-B1-1a (Bx7* ), *1b (Bx7 + y8), 1c (Bx7 + y9), 1d (Bx6 + y8) and 1e (By20);* and the *Glu-D1* locus to two alleles: *Glu-D1-1a (Dx2 + y12) and 1d (Dx5 + y10)* (tab. 2). The presence of new allelic forms within the locus *Glu-A1-1 GluA1-1b-2* * in the range 145-330 bp were observed (tab. 2). These results indicate that the loci *Glu-A1* and *Glu-B1* are characterized by the greatest variability. Across all the cultivars a total of 42 loci that were 100% polymorphic were observed. The greatest diversity of genotypes occurred during the amplification of six pairs of primers for genes: *Dx2, Dx5, Dy10, Dy12, Ax1, By8, By9 and By20.*The results obtained showed that old cultivars of hexaploid wheat are a valuable source of new genetic variations that may be useful in breeding new cultivars.

This study distinguished two cultivars of wheat with favourable combination subunits for the: Sielecka Genetyczna, Sobieszyńska: 1/7+9/5+10, and as well as three cultivars with the unfavourable glutenin subunits: Żelazna, Balta: N/20/2+12 and Ostka Nadwiślańska: N/7/2+12.

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## Effects of wheat *Rht-B1b*, *Rht-B1c* and *Rht-D1b* genes on plant height and yield potential under the climatic conditions of Bulgaria


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Nearly 90% of current wheat cultivars throughout the world carry at least one of the major GA (gibberellic acid)-insensitive semi-dwarfing genes *Rht-B1b* (=*Rht1*, 'Norin 10' allele), *Rht-B1d* (=*Rht1*, 'Saitama 27' allele) or *Rht-D1b* (=*Rht2*) (Hedden 2003), or the GA-responsive one *Rht8* (Worland et al. 1998). The semi-dwarf cultivars generally have higher grain yield potential and improved lodging resistance and have been deployed in many breeding programmes since the Green Revolution. The distribution of *Rht-B1b* and *Rht-D1b* is restricted to cooler regions, whereas alleles *Rht-B1d* and *Rht8* prevail in regions with

warmer and drier climate, such as the countries in South-Eastern Europe, Australia, certain parts of China and USA. The gene *Rht-B1c* (=*Rht3*) is associated with extreme height reductions and is not commercially exploited though performs similarly as the tall isolines in certain stress conditions (Flintham and Gale 1983). The optimum semi-dwarf wheat cultivar is a function of local eco-climatic and genotypic variables (Flintham et al. 1997). The climate of Bulgaria is characterized with frequent periods of drought at various stages of crop vegetation, mainly in autumn around sowing time, and in spring around anthesis. *Rht8* is the dominating semi-dwarfing gene within Bulgarian bread wheat germplasm as revealed by GA-tests and analysis of allele polymorphism at the *Xgwm261* locus linked to *Rht8* (Ganeva et al. 2005). The recently observed trend of intensive introduction of GA-insensitive *Rht* alleles by Bulgarian wheat breeders (Ganeva et al. 2005, Landjeva et al. 2011) determined the objective of this study: to investigate the influence of *Rht-B1b*, *Rht-B1c* and *Rht-D1b* alleles on plant height and yield components under the drought-liable climate of Bulgaria.

## Experimental design

To study the *Rht* genic effects, we used a variety of wheat near-isogenic lines to achieve a wide range of heights governed by both the genotypic background (cultivars 'April Bearded', 'Bersée', 'Maris Huntsman' and 'Maris Widgeon'), and the *Rht* allele (*Rht-B1b*, *Rht-B1c* and *Rht-D1b*, or their combinations *Rht-B1b*+*-D1b* and *Rht-B1c*+*-D1b,* referred hereafter to as double dwarfs). The agronomic performance of the dwarf lines (plant height, number and length of internodes, tiller number, spike length, spikelet number, number and mass of grains per main spike, and number and mass of grains per plant) were compared with the corresponding tall cultivars (*rht*, *Rht-B1a*+*-D1a*) in a four-year trial in the experimental field of the Institute of Plant Physiology and Genetics, Sofia. The cultivars used and their isogenic lines are of winter habit with low vernalization requirement and low freezing tolerance. Since the temperature could reach -25°C in a typical winter in Bulgaria, sowing was performed in early spring (first half of March) after 1-month vernalization at 2-4$^{o}$C. Each line was sown as space-planted plots with 4 rows, each 2 rows representing a replication. Data were analyzed using the software package STATISTICA v.7 StatSoft.

## Results and Discussion

The variation for all traits was large (Table 1) and showed strong effects of the year (accounting for different climatic conditions), cultivar background and *Rht* allele, and their interactions ($P<0.001$, data not shown).

On average over the years of experiment and cultivar backgrounds, alleles *Rht-B1b* and *Rht-D1b* reduced plant height by 22% due to shorter last internode, but had no effect on the number of internodes. *Rht-B1b*+*-D1b*, *Rht-B1c* and *Rht-B1c*+*-D1b* reduced plant height by 53, 58 and 68%, respectively, on the account of both reduced number and length of internodes. Tillering was consistently greater in *Rht-B1c* isolines and the two double dwarfs. *Rht-B1b*, *Rht-D1b* and their combination increased the spike length; *Rht-B1c* had no effect on this trait, but in combination with *Rht-D1b* significantly reduced the spike length. All semi-dwarfing alleles increased the spikelet number up to 5% compared to the tall controls. *Rht-B1b* and *Rht-D1b* increased grain number per spike by 16% and 20%, respectively, did not affect the grain number per plant and grain mass per spike, and significantly reduced the grain mass per plant by 10% and 20%, respectively. *Rht-B1c*, *Rht-B1b*+*-D1b* and *Rht-B1c*+*-D1b* reduced considerably both the grain number and grain mass per spike and per plant. All semi-dwarfing alleles reduced the 50-grain mass by 12% (*Rht-B1b*) to 20% (*Rht-B1c*).

Table 1: Agronomic comparison of the semi-dwarfing alleles *Rht-B1b*, *Rht-B1c* and *Rht-D1b*, and their combinations, *Rht-B1b*+*-D1b* and *Rht-B1c*+*-D1b*: average data obtained in a four-year field trial with four series of *Rht* near-isogenic lines (in cultivars 'April Bearded', 'Bersée', 'Maris Huntsman' and 'Maris Widgeon'). Values within each column followed by a different letter are significantly different at $p<0.05$

| *Rht* allele | Height (cm) | Number of inter-nodes | Length of last internode (cm) | Tiller number | Spike length (cm) | Spikelet number | Grain number / main spike | Grain mass / main spike (g) | Grain number / plant | Grain mass / plant (g) | 50-grain mass (g) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *rht* | 80.7 a | 4.6 a | 34.0 a | 4.9 b | 11.0 d | 20.1 c | 42.3 b | 0.98 a | 127.3 a | 2.48 a | 1.23 a |
| *Rht-B1b* | 62.5 b | 4.5 a | 26.7 b | 4.8 b | 11.4 bc | 20.6 b | 48.4 a | 0.96 a | 134.4 a | 2.23 b | 1.08 b |
| *Rht-D1b* | 61.8 b | 4.4 a | 27.0 b | 4.9 b | 11.7 ab | 20.8 ab | 50.0 a | 0.98 a | 120.4 a | 1.99 c | 1.03 bc |
| *Rht-B1c* | 34.2 d | 3.9 b | 16.0 c | 5.9 a | 11.2 cd | 21.1 a | 35.3 c | 0.66 b | 70.3 c | 1.12 e | 0.98 c |
| *Rht-B1b*+*-D1b* | 37.5 c | 4.0 b | 16.6 c | 6.2 a | 11.9 a | 21.1 a | 38.4 c | 0.70 b | 100.8 b | 1.54 d | 1.01 bc |
| *Rht-B1c*+*-D1b* | 26.5 e | 3.8 c | 12.8 d | 6.0 a | 10.4 e | 20.8 ab | 20.7 d | 0.40 c | 66.5 c | 1.25 e | 1.03 bc |

The observed interaction of high temperature and limited water availability with the *Rht* genic effects (Blum et al. 1997, Botwright et al. 2001) on growth and dry matter partitioning leads to the expectation that in warmer and drier climates the effects of the semi-dwarfing genes could be more dramatic compared to those expressed in cooler regions with adequate precipitation.

The reductions in the plant height in presence of all *Rht* alleles and their combinations obtained in this study are considerably larger compared to those reported for the same series of near-isogenic lines under the conditions of UK and Germany (Flintham et al. 1997). The spring sowing of the lines may have contributed to the observed height reductions in addition to the effects of the semi-dwarfing genes. Under the warmer and drier climate of Bulgaria, the increases of grain number per spike was similar to (*Rht-D1b*) or less (*Rht-B1b*) than those obtained in the study by Flintham et al. (1997). Genes *Rht-B1c* and *Rht-B1b*+*-D1b* reduced the grain number per spike, whereas the same genes had increasing effect on this trait in the cooler regions. The present study showed approximately twice as lower values of the 50-grain mass in all the *rht*-lines and larger reductions in presence of all *Rht* alleles compared to the calculated data reported by Flintham et al. (1997).

These observations are consistent with earlier reports on larger effects of drought and heat stress on plant height and yield in dwarfs or semi-dwarfs than in the tall controls (Richards 1992a, Blum et al. 1997). These effects have been attributed to the reduced water-use efficiency and higher susceptibility to heat stress in the shorter genotypes (Hoogendorn and Gale 1988, Richards 1992b), the outcome of which could be reduced number of competent florets, reduced pollen viability or impaired grain filling and could result in yield loss. These observations have important implications for wheat breeding aiming at optimizing the height-yield relation in environments susceptible to drought at different periods of crop vegetation. Earlier studies by Worland et al. (1998) suggested that the two major semi-dwarfing alleles were inappropriate for exploitation in hot and dry climates, such as the Southern European countries, where less potent dwarfing alleles or a combination with other height and yield related genes could be more efficient. In agreement with the above, results of a recent comparative analysis on Bulgarian wheat germplasm indicated that breeding for higher productivity had apparently led to preferential semi-dwarfing allele selection to combine the advantages of *Rht8* with the yield potential of *Rht-B1* alleles (Landjeva et al. 2011). This study showed that cultivars carrying the combination of *Rht8* and a *Rht-B1* allele produced shorter culms, but longer spikes with more spikelets, higher number and mass of grains per spike, and higher 50-grain mass in comparison with cultivars carrying *Rht8* alone. The pedigree analysis suggested prevalence of the 'Saitama 27' allele (*Rht-B1d*) among the *Rht-B1* carriers (Ganeva et al. 2005) which proved to be more suitable for this climate zone.

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# Cloning and mapping of the *Kao* genes in wheat

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The yield improvements achieved in cereals during the past decades have been accompanied by steady decreases in stem length. Gibberellins (GA) are essential regulators of many aspects of plant development, including seed germination, stem elongation, and flowering. *Ent*-kaurenoic acid oxidase (KAO) catalyses three steps in the gibberellin biosynthetic pathway from *ent*-kaurenoic acid to $GA_{12}$ (Helliwell et al. 2001). In bread wheat (*Triticum aestivum* L.), the *Kao* genes have been allocated to homoeologous group 7 chromosomes (Spielmeyer et al. 2004). In the current study, we performed partial gene cloning and comparative structural and mapping analysis among three homoeologous *Kao* genes in bread wheat.

## Materials and methods

The bread wheat cultivars 'Chinese Spring', 'Opata 85', 'Prinz', the synthetic hexaploid wheat 'W7984', and the diploids *T. urartu* and *Aegilops tauschii* were used for PCR-based cloning. A set of 'Chinese Spring' nullitetrasomic lines (Sears 1953) was exploited to establish chromosome locations of the cloned sequences and ESTs. ITMI mapping population (RILs obtained by cross 'W7984' / 'Opata 85'; Röder et al. 1998) and $F_2$ population 'CS' ('Hope' 7A) / 'TRI 15010' (Khlestkina et al. 2002) were used for genetic mapping of the *Kao* genes in hexaploid wheat with MAPMAKER 2.0 (Lander et al. 1987). Primer design and PCR analysis were performed according Khlestkina et al. (2010).

## Results and discussion

The use of the full-length genomic barley *Kao* sequence in BLAST search across the wheat EST Database revealed 13 homologous wheat ESTs. To cover unsequenced coding region of the wheat *Kao* gene three fragments of the *Kao* gene were isolated using PCR-based cloning approach. Multiple alignment was performed for 13 newly isolated genomic sequences and for the 13 ESTs, resulting in detection of 53 single nucleotide sequence variants within exons, from which 38 were silent mutations (Khlestkina et al. 2010). By these nucleotide sequence variants the sequences formed three groups, suggesting that three distinct copies of the *Kao* gene exist in bread wheat. A number of copy-specific primer pairs were designed and used in PCR analysis of 'Chinese Spring' nullitetrasomic lines. Thus, each of the three copies was assigned either to chromosome 7A (*Kao1*), 4A (*Kao2*) or 7D (*Kao3*).

Besides copy-specific single nucleotide sequence variants, 8 intervarietal SNPs were identified. Two SNPs detected within *Kao1* and *Kao3*, respectively, showed polymorphism between the parental lines ('Opata 85' and synthetic 'W7984') of the ITMI mapping population. The amplified fragments of *Kao1* and *Kao3* were sequenced to determine allelic status of each ITMI progeny at the SNPs. The $F_2$ population 'CS' ('Hope' 7A) / 'TRI 15010) was used for genotyping with primers to *Kao2*. Genetic maps were constructed on the basis of genotyping data at the *Kao,* microsatellite and RFLP loci (Fig. 1).

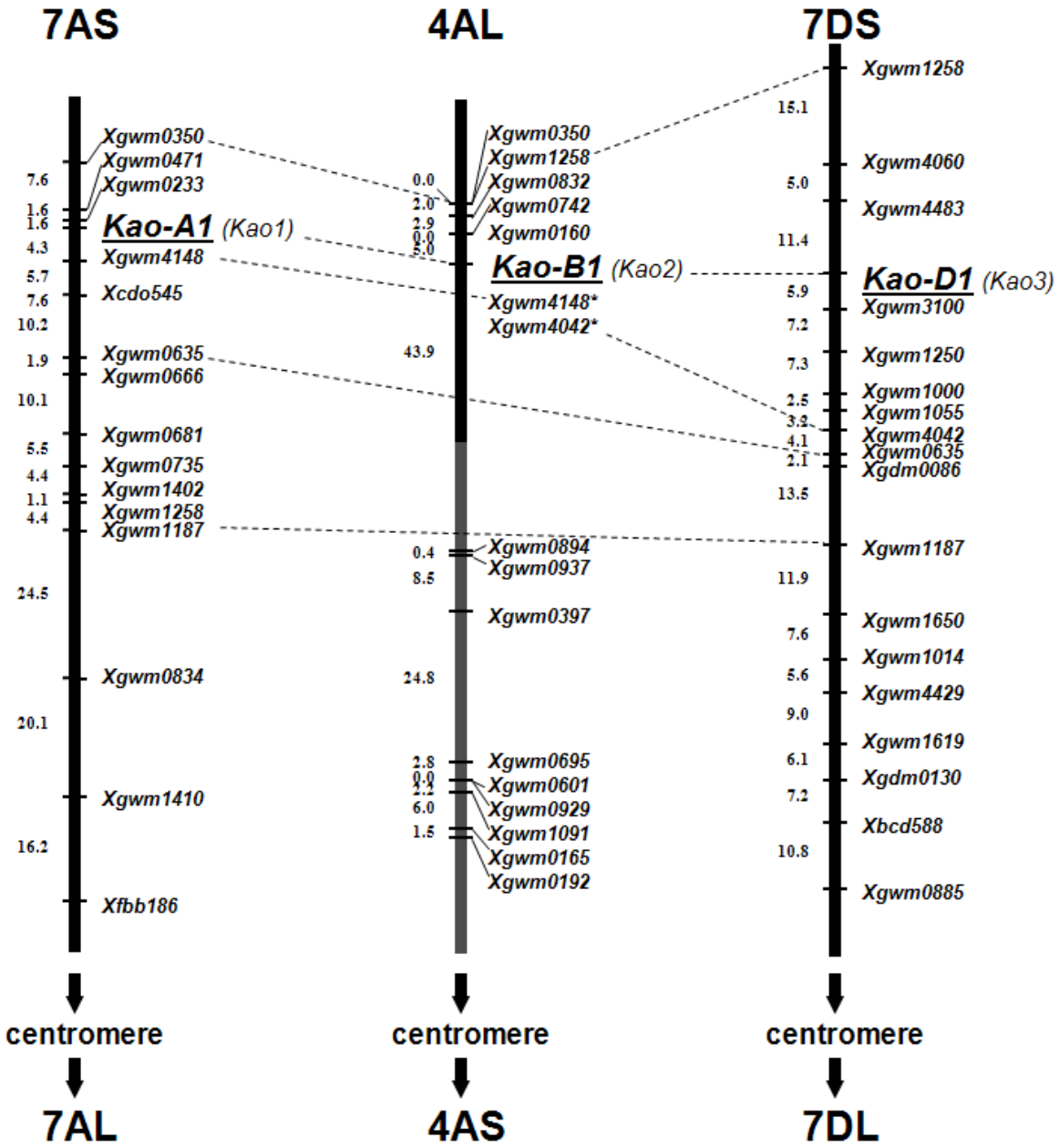


Fig. 1: Linkage maps of wheat chromosomes 7AS, 4AL and 7DL carrying *Kao* genes, constructed using the ITMI mapping population (7AS and 7DS) and $F_2$ population 'CS' ('Hope' 7A) / 'TRI 15010' (4AL). Genetic distances are given in centimorgans (cM). Homoeoloci are connected with dotted lines. * - Microsatellite loci indicated based on comparison with ITMI 4A map

The *Kao* loci were mapped to the distal ends of the chromosome arms 7AS, 4AL and 7DS, the regions known to be homoeologous in hexaploid wheat (Naranjo 1990). Mapping positions of the homoeologous microsatellite loci suggested co-linearity of the chromosomal regions carrying the *Kao* genes. Following the rules of wheat homoeologous gene designation (McIntosh et al. 2008) the wheat *Kao* genes mapped on chromosomes 7AS, 4AL and 7DS were designated *Kao-A1*, *Kao-B1*, and *Kao-D1*, respectively.

Thus, (1) molecular-marker based precise mapping demonstrated that the *Kao* loci map to the distal ends of the chromosome arms 7AS, 4AL and 7DS, corresponding to the 7BS/4AL translocation region; (2) co-linearity of the chromosome regions carrying the *Kao* genes is observed, and the three *Kao* genes we mapped represent a set of homoeologous loci; (3) exonic sequences of the three homoeologues differ from each other mainly by silent mutations, and each homoeologue is transcriptionally active.

**Acknowledgements**

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## Analysis of selected quantitative traits in *Triticum aestivum/Aegilops squarrosa* introgressive lines

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### Introduction

In common wheat breeding crosses with other species and types are often used in order to introduce genes responsible for resistance to diseases and pests, improved quality or

adaptability. Until now, many cases of introgression of different genes from wild species into wheat are described (Esser 1998; Cenci et al. 1999; Chevre et al. 1989, Simeone et al. 2001). Very often in breeding of common wheat crossing with *Aegilops squarrosa* L is used (Kerber, Dyck 1969; Chevre et al. 1989; Lutz 1994). The aim of the paper was the estimation of yield components of introgressive lines (*Triticum aestivum/Aegilops squarrosa)*.

## Materials and methods

82 lines carrying the segments of chromosomes from D genome were investigated, moreover Chinese Spring and Synthetic wheat were used as controls. Experiments were carried out over seasons 2006-2008 in Czeslawice near Lublin (Eastern Poland). Every year about 500 germinated kernels per $m^2$ were sown in 1 $m^2$ plots in three replicates. For each plot heading time was recorded as the number of days from 1$^{st}$ May until full ear emergence. Main shoot data were obtained from a random sample of 20 leading tillers from each sublines and used for the calculation of number of spikelets/spike, number of grains per ear, weight of grains per ear, 1000 grain weight and spikelet fertility. The results obtained were statistically analyzed individually for each year. The ANOVA programme was used, applying the F-Snedecor test.

## Results

During all three seasons the heading time of analyzed introgressive lines were significantly different than noticed for Chinese Spring. The earliest ones were the introgressive lines with 2D and 7D chromosome segments (Table 1). The latest were 5D introgressive lines. In 2006 the spring in Poland was rather hot (data not shown) and these lines needed more days from 1$^{st}$ May to ear emergence. Plant height of introgressive lines depended on the year. In 2008 lines were longest, and some lines in that year were longer than Chinese Spring (1D, 6D and 7D lines). 5D lines were the shortest in 2006 and 2007. Spike length and number of spikelets in introgressive lines depended on the year of investigation. Value of this traits in all lines were very similar (data not shown). More differences were found in number of kernels per spike. In 2006 chromosome 1D, 2D, 3D and 6D lines set more kernels in spike in comparison to control Chinese Spring. Value of this trait in 2008 for some lines was similar to control cultivar (Table 1). Weight of kernels per spike in all lines were smallest in 2006. In this year the smallest value of this trait was recorded for the 4D lines (0,85 g). Higher values of this trait were recorded in second and third year of investigations. All introgressive lines had similar spikelet fertility (data not shown). All studied lines were characterized by low value of 1000 grains weight. In 2006 and 2008 years value of these traits were the lowest (Table 1). Spike density in all studied lines was similar in all years of investigations (data not shown).

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Table 1: Average of some quantitative traits of common wheat introgressive lines with segments of D chromosome from *Aegilops squarrosa* (Czeslawice, Poland 2006-2008)

| Introgressive lines | Days from 1st May to ear emergence | | | Plant height (cm) | | | Number of kernels per spike | | | Weight of kernels per spike (g) | | | 1000 grains weight (g) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 | 2006 | 2007 | 2008 |
| 1D | 49,6 | 43,5 | 40,8 | 93,1 | 89,1 | 111,9 | 42,8 | 51,4 | 50,9 | 1,10 | 1,56 | 1,42 | 25,3 | 30,7 | 27,7 |
| 2D | 48,7 | 42,3 | 38,7 | 96,1 | 92,1 | 104,8 | 45,2 | 46,2 | 49,4 | 1,20 | 1,45 | 1,22 | 26,4 | 31,6 | 24,7 |
| 3D | 50,2 | 43,7 | 41,8 | 100,3 | 96,3 | 106,2 | 43,1 | 43,8 | 51,1 | 1,07 | 1,35 | 1,28 | 24,6 | 31,4 | 24,8 |
| 4D | 50,3 | 43,6 | 42,3 | 97,8 | 93,8 | 107,8 | 35,2 | 46,0 | 50,0 | 0,85 | 1,42 | 1,26 | 23,8 | 31,6 | 25,1 |
| 5D | 53,3 | 46,2 | 42,8 | 87,6 | 83,6 | 108,2 | 36,9 | 43,6 | 42,5 | 1,08 | 1,33 | 1,07 | 30,3 | 31,4 | 24,0 |
| 6D | 49,9 | 43,3 | 41,0 | 100,4 | 96,4 | 111,3 | 42,9 | 45,8 | 50,9 | 1,34 | 1,43 | 1,30 | 31,5 | 31,6 | 25,7 |
| 7D | 48,2 | 41,3 | 38,2 | 101,6 | 97,6 | 110,6 | 41,5 | 35,8 | 53,0 | 1,33 | 0,90 | 1,26 | 32,5 | 26,0 | 23,7 |
| Chinese Spring | 52,0 | 45,0 | 41,0 | 104,7 | 100,8 | 108,3 | 41,6 | 52,4 | 51,2 | 1,28 | 1,60 | 1,25 | 31,2 | 30,6 | 24,7 |

# Characterization of quantitative traits of Steptoe × Morex barley (*Hordeum vulgare* L.) population

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## Introduction

Barley is one of the major cereal crops. It serves as a animal fodder, a source of fermentable material for beer and certain distilled beverages, and as a component of various health foods.

From agronomic and breeding perspectives, yield and quality stability are normally seen in terms of genotype-by-environment interaction. Sometimes compensation among yield components is observed. It occurs when failure in formation of one yield component is at least partially compensated by enhancement of another yield component at later growth stages (Hardwick, Andrews 1980).

Physiological and genetic aspects of crops agronomic traits are subject of many different research. One of the important directions is analysis of QTLs related to different yield compounds and its influence on whole yield (von Korff et al. 2006, Xue et al. 2010, Comadran et al. 2011). Field examinations data are necessary for reliable identification and mapping of QTLs for quantitative traits of agronomic importance.

## Material and methods

The aim of examinations was estimation of the yield components of 91 barley lines derived from Steptoe × Morex cross combination and their comparison with both parental forms.

Experiments were carried out over 2008-2010 seasons at the Experimental Farm in Czeslawice, near Lublin (Eastern Poland). Every year about 400 germinated kernels per square meter were sown in 1 $m^2$ plots in three replicates. For each plot heading time was recorded as the number of days from 1$^{st}$ May until full ear emergence. Main shoot data were obtained from a random sample of ten leading spikes collected from individual plants in each plot and used for determination of spike lenght, number of spikelets per spike, number of grains per ear and weight of grains in ear. Moreover, plant height was measured and the 1000 kernel weight and spikelet fertility were calculated. Obtained results were statistically analyzed individually for each year using post-hoc HSD Tukey test at 0.05 significance level by means of SAS 9.2 software. Statistical analysis were performed for all traits except heading date.

## Results

During three years of examination main agronomic traits of parental forms were changing, what was connected with environmental condition. The largest differences were observed for plant height, number of spikelets per spike and 1000 kernel weight (Table 1).

Table 1: Mean values of the analyzed traits obtained for parental forms in field experiment

| | Steptoe | | | Morex | | |
|---|---|---|---|---|---|---|
| | *2008* | *2009* | *2010* | *2008* | *2009* | *2010* |
| Plant height *[cm]* | 90 | 71 | 72 | 104 | 86 | 84 |
| Spike lenght *[cm]* | 6.7 | 6.6 | 5.7 | 7.5 | 7.4 | 6.5 |
| Number of spikelets | 56 | 54 | 45 | 59 | 62 | 52 |
| Number of kernels | 50 | 32 | 34 | 54 | 51 | 39 |
| Spikelet fertility | 0.89 | 0.59 | 0.75 | 0.92 | 0.83 | 0.76 |
| 1000 kernel weight *[g]* | 53.64 | 44.55 | 52.02 | 43.16 | 34.96 | 37.25 |
| Heading time *[days from 1st May]* | 46 | 55 | 45 | 48 | 56 | 47 |

In most analyzed cases majority of the examined lines did not differ significantly from parental barley forms – Steptoe and Morex. However, for some traits differences between noticed patterns in different years were clearly visible. Many of the analyzed lines presented intermediate plant height and were significantly higher than Steptoe (68% of analyzed lines in 2008, 48% in 2009 and 40% in 2010), and lower than Morex (43%, 33% and 14%, respectively). The heading time was determined to the highest degree by the year of analysis. Each year the majority of analyzed lines showed earlier heading than their parental form Steptoe: 59% of lines in 2008, 60% in 2009 and 93% in 2010. The comparison of heading date of the tested lines with Morex showed that in the first year most of lines were later (56%), but in the next two years majority of them was earlier (53% in 2009 and 77% in 2010). The spike lengths of the analyzed lines usually did not differ significantly from parental forms during all three years of examinations, however considerable percentage presented significantly lower value of this trait in comparison to Morex (38% of lines in 2008, 32% in 2009 and 30% in 2010). Similar pattern was observed for number of spikelets (18% in 2008, 30% in 2009 and 16% in 2010) and number of kernels (29%, 45% and 8%, respectively). The largest differences between years of examinations were shown for spikelet fertility and 1000 kernel weight. The number of lines with spikelet fertility higher than noticed for Steptoe varied from 0% in 2008 to 48% of lines in 2009 and with lower value presented constant percentage during all three years (7%). Obtained results showed that 1000 kernel weight was determined by the conditions during year of analysis. The percentage of lines with lower value of this trait in comparison with Steptoe were 37% in 2008, 9% in 2009 and 63% in 2010. None of the analyzed lines showed lower 1000 kernel weight than Morex and the percentage of lines with higher value were 42% in 2008, 23% in 2009 and 63% in 2010.

## Conclusions

Obtained result showed that in many cases analyzed lines did not differ significantly in comparison to both parental forms in the course of three years of field experiments. In case of significant difference the values of analyzed traits were usually higher than noticed for

Steptoe and lower than for Morex. The largest diversity was noticed for heading time. Majority of analyzed lines were earlier than Steptoe and Morex parental cultivars. However this trait is prone to modifying environment influence, because in 2008 majority of lines were later than Morex.

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## Evaluation of wheat analogue-lines differing in alleles *Rht8*, *Rht-B1*, *Rht-D1*, *Ppd-D1* by quantitative traits

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### Introduction

The aim of the work was to determinate a characteristic, that would significantly reflect negative influence of variation of environmental conditions on wheat plant growth and development and to reveal the best genotype according to alleles *Rht* and *Ppd-D1* genes for growing in conditions of South Ukraine. The analogue-lines, parental and recurrent forms that differ in alleles of dwarfing (*Rht*) and photoperiod sensitivity gene (*Ppd-D1*) were studied.

### Material and methods

All analogues were obtained by the scheme Odesskaya semi-dwarf (or Karlyk 1) × R7, in which the recurrent form (R) lines were known varieties (Khangildin, 1993). Plants had grown on bare fallow in one breeding nursery with typical for southern Ukraine agrotechnology: fed with ammonium nitrate (N30 kg/ha) on frozen-thawed soil early spring. Alleles of *Rht* and *Ppd-D1* genes were identified by PCR-analysis and a test for sensitivity to exogenous gibberellic acid (Chebotar G.A. et al., 2010). Some traits were estimated for each plant (Chebotar G.A. et al., 2011): date of heading (DH), date of flowering (DF), productive

tillering (PT), length of culm (LC), length of the main ear (LE), number of fertile (NFs) and sterile spikelets (NSs) in the main ear, number of grains in the main ear (NGME), weight of the grains of the main ear (WGME), number of grains on secondary steams (NGSS), weight of the grains from secondary steams (WGSS). Were calculated: average number of grains in fertile spikelets (NGFs), number of grains from a plant (NGP), weight of the grains from a plant (WGP), ear density (D), weight of one thousand kernels (WTK).

**Results and discussion**

In many cases it is impossible to investigate and compare pure gene effects with full factor plan which contain all allele and gene combinations in the same genotype. In addition, the different growth conditions by years give rise to another well known permanent problem. In the good year (absence of stress conditions) as the best were estimated genotypes: *Rht8x Rht-B1a Rht-D1a Ppd-D1a* > *Rht8x Rht-B1a Rht-D1a Ppd-D1b*. In a bad year (water and temperature stress) the distinction between these two disappears, and as the most adapted genotype was estimated: *Rht8c Rht-B1b Rht-D1a Ppd-D1a*. The least adapted to the stress was genotype *Rht8c Rht-B1e Rht-D1a Ppd-D1a*.

To compare pure effects of genotype on the main crop traits we use sequential ANOVA to exclude the effects of year and parent variety (Fig. 1).

So, the best lines were: *Rht8a Rht-B1e Rht-D1a Ppd-D1b* and *Rht8x Rht-B1a Rht-D1a Ppd-D1a*. Combinations of *Rht8x Rht-B1a Rht-D1a Ppd-D1b* and *Rht8c Rht-B1b Rht-D1a Ppd-D1a* had shown a decrease of WGP. As we can see (Fig. 1), *Ppd-D1b* has a negative effect on WGP in compare with *Ppd-D1a*.

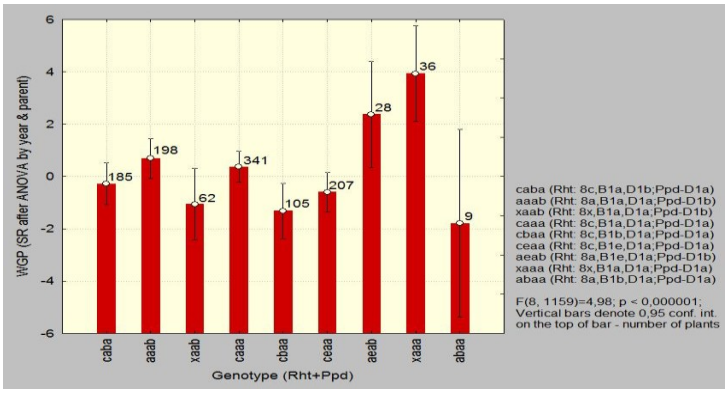


Fig. 1: Pure effects of the genotypes on WGP – weight of grains from a plant (SR – residuals after sequential ANOVA by year and parent variety)

It may be efficient to find the objective indicator which can at most reflect interaction between genotype and environment. Detailed data analysis had shown that the variation of the only one of biometric traits – NSs – was under impact of all sources of variation: year, gene background and their interaction. By another side, NSs is negatively correlated with all other biometric traits. For example, simple linear models of NSs shows that NSs is closely related to the main productive crop characteristics of wheat - WGP and PT:

WGP = (11,10±0,07) – (2,00±0,03) × NSs; R2 = 0,999; SEE = 0,097;

PT = (8,35±0,08) – (1,00±0,03) × NSs; R2 =0,996; SEE=0,10.

This circumstances allows to suppose NSs as well appropriate for quantitative estimation of the allele combinations success in environment and gene background. As one can see, *Rht-8x* has the negative and *Rht-8c* – positive effect in any allele combinations (Fig. 2).

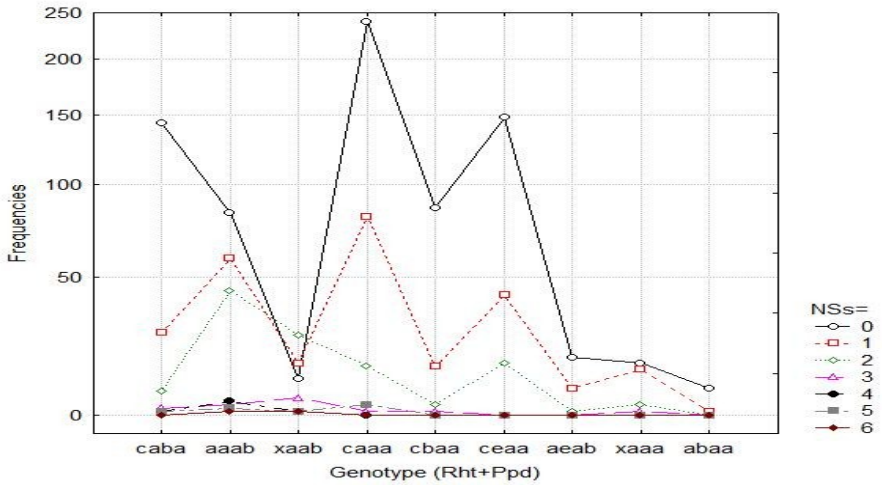


Fig. 2: Frequency distributions of sterile spikelets in main ear according to alleles combinations

## Conclusion

If the environment is close to optimal to realize given genotype productive potential, so the NSs should be less and tend to zero (complete fertility). The presence of sterile spikelets in the main ear indicates on not optimal conditions for the realization of the genetic potential at the early (II-IV) and later (V-VII) phases of ontogenesis (Kuperman, 1968) – the critical periods for formation of plant productivity. Excluding the variation of NSs and PT we are enable to estimate the allele combination effects on the whole plant productivity except of the most part of year or parent effects. So, NSs estimation can be used as an express (and prognostic) not destructive indicator of harvest.

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# Introgressive hybridization for production of spring bread wheat variety 'Pamyati Maystrenko' and new promising lines for breeding

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Western Siberia is a major region of wheat cultivation in Russia. Its environmental conditions require spring bread wheat that combines a high yield potential and the resistance to unfavorable biotic and natural constraints. A promising approach in bread wheat breeding is use of new useful genes incorporated into wheat from its wild relatives or cultivated species from other taxonomic groups. For instance, more than 70 genes conferring leaf rust resistance have been currently identified in wheat, and approximately half of them were incorporated from wild relatives (McIntosh, 2008). Two species, *T. timopheevii* and *Aegilops tauschii* are known to be important sources of resistance to fungal pathogens (Friebe et al., 1996). In this work we present the data on the development of commercial spring common wheat variety Pamyati Maystrenko using hybridization of wheat with an artificial amphiploid *T. timopheevii* x *Ae. tauschii*. In addition, we also demonstrate the perceptiveness of this variety in wheat breeding as the original material for the production of new promising lines.

## Materials and Methods

Earlier we reported about the development of a number of immune lines of Saratovskaya 29 resulted from cross between variety Saratovsksaya 29 and synthetic amphiploid *T.timopheevii* ($GGA^tA^t$) x *Ae. tauschii* (=*Triticum tauschii*) (DD), produced by Dr. P. Savov (Laikova *et al.,* 2008). These lines are characterized by resistance to powdery mildew and leaf rust (Laikova *et al.,* 2004). One of these immune lines, Im.L-10, was crossed as paternal parent with the variety Rang (Sweden). An advanced yield trials of the offspring (Rang/Im.L-10) were carried out during 2007-2009 on the fields of Siberian Agricultural Research Institute (SARI). Based on the results of these testing, in 2010 one breeding genotype was selected and submitted to the State yield trial as bread wheat mid-late variety Pamyati Maystrenko. Yield trials, evaluation of and testing of resistance to fungal pathogens were carried out on the fields of SARI. Drought resistance was assessed by *in vitro* method (Rosseev, 2007). In 2010 this variety was also tested for stem rust resistance using inoculation with TTKST (Ug99+Sr24) in Kenya Agricultural Research Institute (RARI). Varieties Saratovskaya 29 and Omskaya 28 were taken as controls in this experiment. Wheat variety Pamyati Maystrenko was also studied by C-banding technique following earlier published protocol (Badaeva et al., 1994).

## Results and Discussion

Chromosome analysis revealed two substitutions of 2B and 6B chromosomes by the homoeologous chromosomes of *T. timopheevii* (Fig. 1). Another substation involved wheat chromosome 1D, which was replaced by the orthologous chromosome of an amphiploid inherited from the parental line of *Ae. tauschii*. Comparison of karyotypes of the variety

Pamyati Maystrenko with karyotype of the parental forms showed that some chromosomes of this wheat are similar with those of the variety Range (3A, 5A, 3B, 4B, 7B, 2D); other chromosomes resemble those of cv. Saratovskaya 29 (2A, 1B, 5B), whereas the source of the remaining chromosome is not clear as they showed no polymorphism between two parental forms.

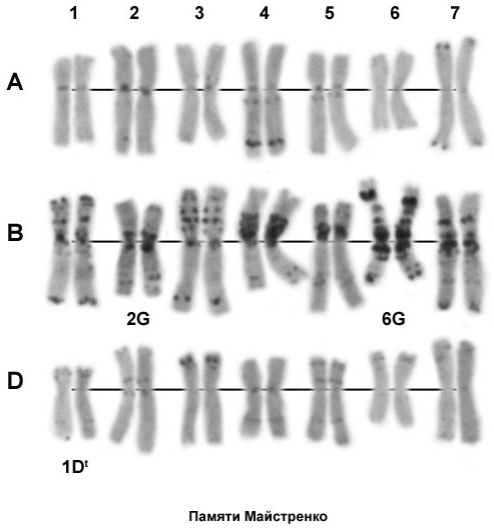


Fig. 1: Karyotype of wheat *T. aestivum* cv. 'Pamyati Maystrenko'

It was shown that the variety Pamyati Maystrenko combines high grain and bread-making qualities and the resistance to fungal pathogens (Table 1). In field conditions of SARI this variety was moderately resistant to powdery mildew, resistant to leaf rust, moderately resistant to stem rust (20MR). The variety Pamyati Maystrenko was found to be moderately resistant (10RMR) to the race of stem rust TTKST (Ug99+Sr24) under artificial inoculation trial in Kenya (KARI). The results of *in vitro* test have shown that this variety is highly resistant to drought.

Table 1. Test results of variety Pamyati Maystrenko

| Variety | Yield, t/ha | Protein content, % | Gluten content, % | Bread volume, $cm^3$ | Resistance to fungal pathogens (SARI) | | | Resistance to stem rust (RARI) |
|---|---|---|---|---|---|---|---|---|
| | | | | | Pow-dery mildew* | Leaf rust** | Stem rust | |
| Saratovskaya 29 | 3.32 | 15.79 | 31.3 | 990 | 3 | 4 | 60MS | 60MS |
| Omskaya 28 | 3.18 | 14.81 | 29.2 | 1025 | 6 | 4 | 70S | 60MS |
| Pamyati Maystrenko | 2.96 | 17.65 | 34.3 | 1075 | 6 | 1 | 20MR | 10RMR |

* Point rates for resistance to powdery mildew: 6 – moderate resistance; 3 – susceptible. ** Point rates for resistance to leaf rust: 1 – resistance; 4 – susceptible.

Immune lines and variety Pamyati Maystrenko were further included in other crosses with genotypes, which carry alien genes determining adaptive and economically important traits of

bread wheat. In particular, alloplasmic recombinant lines (*H.vulgare*)-*T.aestivum* (Pershina *et al.,* 2001), carrying wheat-rye translocation 1RS.1BL (Belan et al., 2010), wheat lines with genetic material of *Agropyron intermedium, T. dicoccoides, T. dicoccum* (Sibikeev et al. 2005) were used in these experiments.

On the basis of wheat variety Pamyati Maystrenko several new, cytologically stable introgression lines combining genetic material of *T. timopheevii-Ae. tauschii-T. dicoccum; T. timopheevii-Ae. tauschii-T. dicoccoides; T. timopheevii-Ae. tauschii-Agropyron intermedium; T. timopheevii-Ae. tauschii-S. cereale* have been produced. The identification of genes that determine resistance to fungal pathogens in new lines is being in progress. Some of the lines are already involved in breeding process.

**Acknowledgments**

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## Identification of *Lr19* gene in Polish common wheat (*Triticum aestivum* L.) breeding lines

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Leaf rust caused by *Puccinia triticina* Erikss. & Henn. is one of the most important foliar disease of crops. Leaf rust reduced wheat yields and deteriorates grain quality. This fungal disease appears every years with severity depending on weather conditions (McIntosh et

al.1995). Resistance to leaf rust in wheat is determined genetically by series of *Lr* genes. One of the most important gene determining resistance to *Puccinia triticina* in many part of the world is *Lr19*, introgressed into common wheat from *Thinopyrum* sp. (Tomar and Menon 1998; Pink 2002; Mesterhazy 2000). *Lr19* gene together with *Lr9*, *Lr23*, *Lr24* and *Lr25* are highly effective in Poland and no virulence to these genes was found up to now.

DNA based molecular markers have greatly increased the capacity for more precise prediction of a genotype in plant breeding. For high-throughput genotyping, simple PCR-based markers are highly desired by plant breeders. In the past few years STS as well as SCAR markers were developed for fast and certain identification of *Lr19* gene presence in wheat genetic material (Prins et al. 2001; Gupta et al. 2006).

The aim of presented study was identification of the leaf rust resistance gene *Lr19* in Polish common wheat lines obtained from plant breeding companies.

## Material and methods

The objective of the analyses consisted 250 breeding lines of common wheat. As a control line the Thatcher near isogenic lines with Lr19 gene was used.

Genomic DNA was isolated from 5-day-old seedlings by means of CTAB method. Extracted DNA was used as a template in PCR with pair of specific primers. Examinations were based on SCS253 SCAR marker specific for *Lr19* gene described by Gupta et al. (2006).

PCR products were separated in 1,5% agarose gel containing 0.1% EtBr. Gene Ruler$^{TM}$100bp DNA Ladder Plus was used to establish molecular weight of the amplification products. Fragments were visualized under a UV transiluminator and photographed using the PolyDoc system.

## Results

Analyses shown that the leaf rust resistance gene *Lr19* was present in only 16 tested breeding lines. Among analyzed 150 lines form Poznanska Breeding Station we identified 14 lines with *Lr19* resistance genes. We analyzed 100 lines from DANKO Breeding Station. Analyzes shown that *Lr19* gene was observed only in 4 among tested lines (Fot.1.).

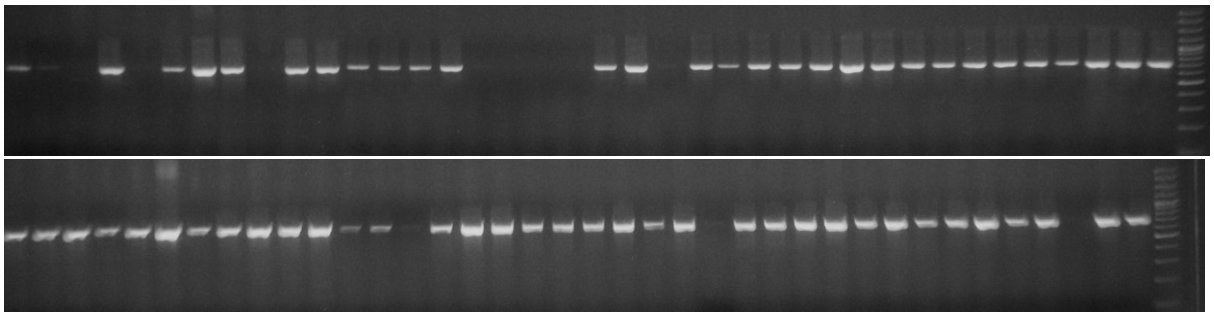

Fig. 1: Amplification of the SCAR marker $SCS253_{737}$ linked to *Lr19* on the some breeding lines of common wheat

## Conclusions

Obtained results suggest that *Lr19* gene does not occur widely in Polish wheat breeding materials. Analyzed gene is known to be very effective against leaf rust isolates occurring in Polish conditions. Because of this fact sixteen identified breeding lines containing *Lr19* gene can be considered as a valuable resistance source for wheat breeding in next few years.

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# Comparative mapping of loci determining pre-harvest sprouting and dormancy in wheat and barley


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## Introduction

Pre-harvest sprouting (PHS) is a phenomenon of cereal crops when germination of grains occurs in the spikes before harvest and therefore it is a big problem in wheat and barley production. It reduces crop yield to the point of a total damage of the harvest. Seed dormancy can prevent sprouting but can also for example delay the malting process in cultivated barley. Searching for dormancy and sprouting genes can help to avoid these effects. As complex traits with high genetic variation it can be assumed that both traits are controlled by multigenes or quantitative trait loci (QTLs).

## Material and methods

Three wheat and two barley populations were used to study PHS and dormancy. For wheat two biparental populations (ITMI and D-genome introgression lines) and one multiparental population based on 183 genebank accessions were investigated. For barley two biparental populations (OWB and "Steptoe" x "Morex") were studied. All material was cultivated in Gatersleben, Germany, on experimental fields, in greenhouses and/or in a foil tunnel in different years.

*Pre-Harvest Sprouting Test:* Five ears per line directly harvested at maturity are placed in a plate full of sand for 14 days. For the interpretation of the data a rating of seven score points is used. A replication followed after 14 days with afterripened ears. A mean value of both scoring results was calculated.

*Dormancy Test:* 60 seeds per line directly harvested at maturity are tested under two different temperature conditions: at 20°C for 7 d and at 10°C for 14 d under a light regime of 12 h light/12 h dark. The percentage of dormant seeds at 10 °C and at 20 °C was computed. In addition, the dormancy index (DI) was calculated in the following way:

DI = [2x (% dormant seed at 10 ◦C) + % dormant seed at 20 ◦C] /3.

## Results and discussion

A classical quantitative trait loci analysis was combined with an association mapping approach. Many quantitative trait loci and marker trait associations (MTAs) could be detected for the traits PHS, dormancy at 10 °C (D10), dormancy at 20 °C (D20) and the dormancy index (DI) on all seven chromosome groups of wheat and on the chromosomes 2H, 3H, 5H, 6H, and 7H of barley. Especially, the known regions on chromosomes 3A and 4A for wheat and 5H for barley were confirmed. Via a candidate homologues search and via expressed sequence tag annotation putative functions could be found. On chromosome 3A the *viviparous1* gene is located which is associated to pre-harvest sprouting and dormancy (fig. 1). On chromosome 4A a protein is detected which belongs to the aquaporin family (fig. 1). Aquaporins are responsible for water flow through the cell membrane. In barley an association with the *aleurain* gene on chromosome 5H was found. The expression of *aleurain* is regulated by abscisic acid and gibberellic acid. From both hormones an influence on dormancy and pre-harvest sprouting is known. It can be concluded that dormancy and pre-harvest sprouting are very complex traits regulated by multigenes and/or quantitative trait loci.

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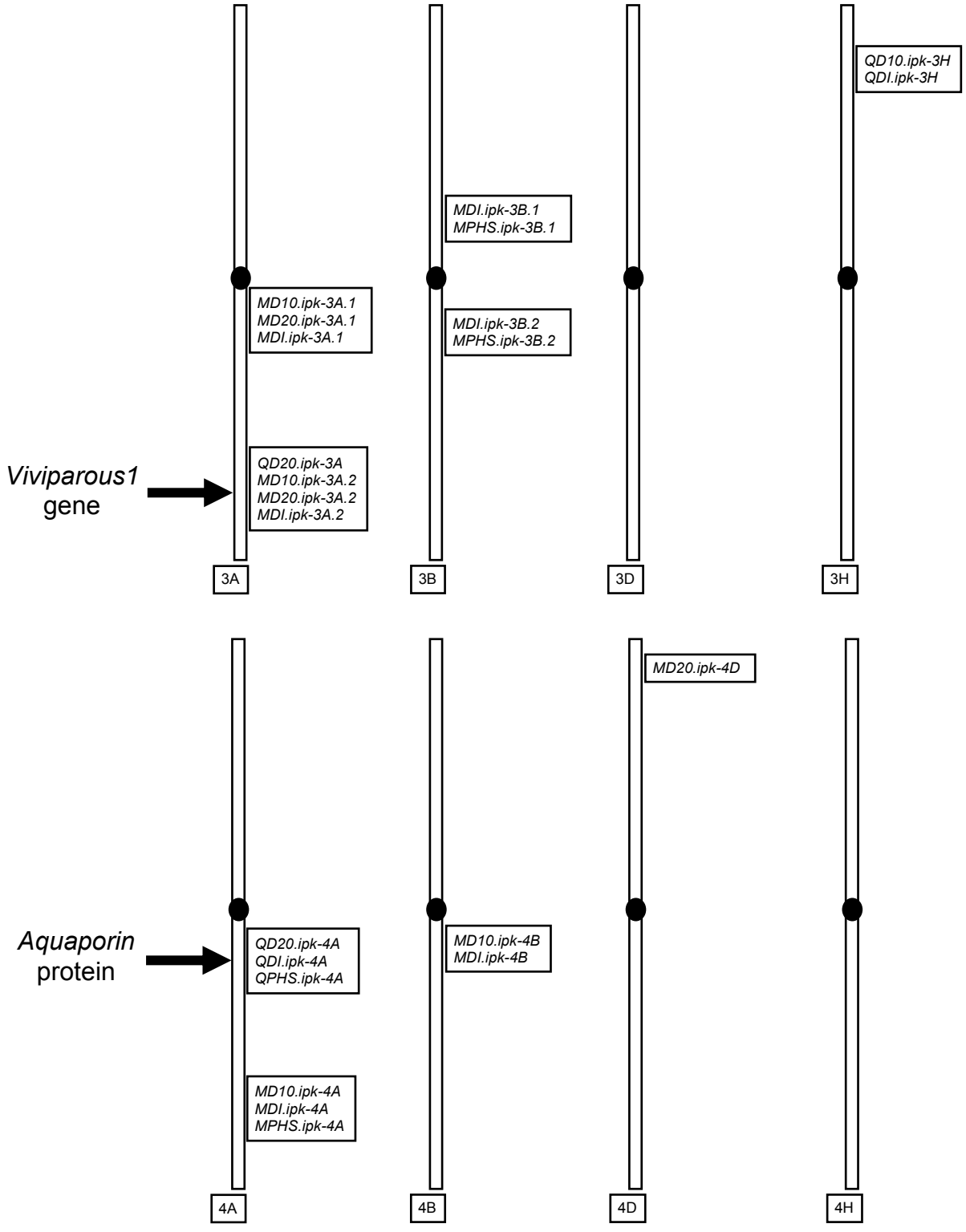


Fig. 1: Comparison of detected QTLs and MTAs in wheat and barley for chromosome groups 3 and 4 (● centromere region)

# Genetic control of grain morphology and characteristics in an Australian bread wheat mapping population

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## Introduction

Wheat production and quality are affected by a range of environmental conditions. Heat and moisture stress are common occurrences in Australian wheat production zones and are known to negatively affect wheat quality and often results in small and shrivelled grain. Grain morphology as deciphered by grain size and shape, and grain characteristics like protein content and hardness are important determinants of grain quality. Size constituents are thousand kernel weight, test weight, percentage screenings, seed area and grain shape dimensions are length, width, thickness, aspect ratio, roundness. Grain length and width tend to show no correlation with each other with an increase in length more often accompanied by decrease in width (Breseghello and Sorrells, 2007) indicating different genetic control mechanisms. The aim of this study was to investigate the genetic control of grain characteristics including grain shape and size in a recombinant inbred line population derived from a cross between the Australian cultivars Gladius and Drysdale grown under heat and drought stress conditions.

## Materials and methods

The grain samples analysed for grain traits came from field trials conducted in Leeton farm (New South Wales, Australia), Obregon (Mexico) and in the poly-house at the University of Adelaide (South Australia) under conditions involving heat and/or drought stress.

Percentage screenings, test weight and thousand kernel weight were measured. An NIR was used to estimate the total grain protein content, moisture content and particle size index. High particle size index corresponds to low hardness values, that is, softer grain. A seed imaging analysis scanner was used to measure and calculate the grain dimensions (width, length, thickness, area, aspect ratio and roundness). QTL analysis was done using composite interval mapping on GenStat (VSN International 2011).

## Results and discussion

Drought and heat stress lead to a reduction in grain size (Figure 1). All other traits were also negatively affected, with stress resulting in long and thin grain as opposed to round plump grain obtained in the control experiment.

QTLs were detected for each of the 11 traits measured in the Gladius/Drysdale population. Some of the QTLs exhibited QTL-by- environment interaction, and some showed “cross-over” interaction effects. QTLs were detected on 18 linkage groups, with some chromosome regions affecting several traits. Of the QTLs detected for grain characteristics, some corresponded with known photoperiod-sensitivity loci (chromosomes 2B and 2D) or with

QTLs that have been detected in other populations (chromosome 6A) but others were new and not associated with phenology genes.

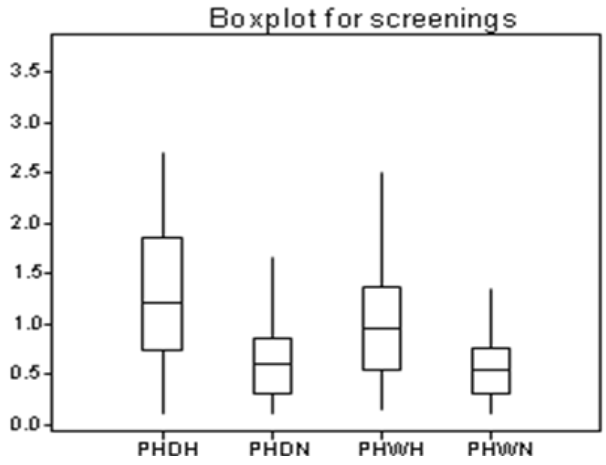


Fig. 1: The effect of heat and/or drought stress on grain size (percentage screenings) in an experiment conducted at the University of South Australia. PHDH = poly-house drought + heat; PHDN = poly-house drought + normal temperature; PHWH = poly-house well watered + heat; PHWN = poly-house well watered and normal temperature. Small grains results in higher percentage screenings.

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## Seed longevity in a barley collection - variation and gene identification

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About 7.4 million genebank accessions belonging to crop species and their wild relatives are stored beyond their natural environment (FAO 2010). Most of the material is kept as seeds in cold storage facilities. Unfortunately as soon as seeds have reached maturity, seed deterioration is initialised by a cascade of processes which are dependent on the storage conditions. Initial seed quality, seed moisture content and temperature are known as important parameters to influence seed life span but also intrinsic effects as species and genotype affect (Nagel et al. 2010, Nagel & Börner 2010).

In the current study we applied the seed viability equation developed by Ellis and Roberts (1980) to reduce initial seed quality effects and included storage conditions. Seeds of a spring barley association mapping panel were experimentally aged. Genome wide association studies (GWAS) using 918 mapped SNP markers revealed 54 significant marker trait associations. The results of different analyses are compared.

**Seed viability equation**

Ellis and Roberts (1980) were the first, who developed a model predicting seed storage periods and integrated initial viability ($K_i$), seed moisture content (smc) and temperature (T).

$$v = K_i - \frac{1}{10^{K_E - C_W \log(smc) - C_H T - C_Q T^2}} \cdot p$$

N – germination (probit) after storage for p days
$K_i$ – initial germination in probit
smc – seed moisture content in (%)
T – temperature in °C
P – storage period in days
$K_E$, $C_W$, $C_H$ & $C_Q$ – species specific constants

Additionally they transferred germination data for normalisation into the probit unit (Fig. 1). Using these parameters the so called viability equation is applicable to orthodox seeds in the range of −20 to 90°C and 5 to 25% seed moisture content. Due to different storage behaviour of species specific constants were integrated and estimated by experimental ageing procedures.

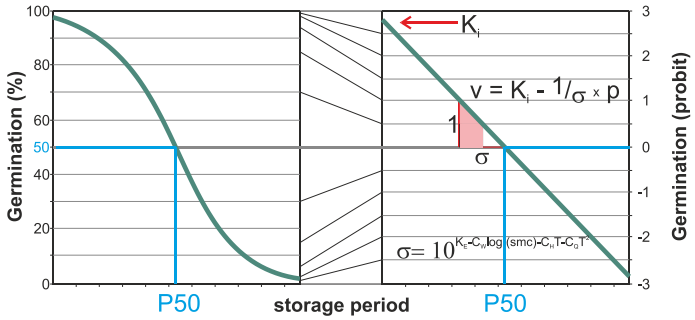


Fig. 1: Transformation of a seed survival curve from the percent scale to a line on the probit scale according to Ghiasi (2007). Abbreviation as above, s = days to lose one probit, P50 half-viability period

Up to now most quantitative studies investigating seed longevity traits used for their analysis either germination results per se or a quotient between germination results after ageing and initial germination.

Within this study we used the viability equation and tried to compare the different analysis.

## Quantitative analysis of seed longevity traits

Seeds belonging to 203 accessions of a diverse spring barley association panel of world-wide origin were experimentally aged using high seed moisture content (> 11%) and high temperature (> 43°C). Parameters were integrated in the viability equation and sigma (σ, days to lose one probit) and P50 (half-viability period) were calculated for every accession. The association analysis (TASSEL 2.1 (Bradbury et al. 2007)) using 918 SNPs (Pasam et al. 2012), PCA and two models (GLM, MLM) revealed 54 significant marker-trait associations having a LOD score > 2. Eight loci were linked with sigma. Three out of eight were further connected with P50 and germination after ageing. Interestingly, the GWAS confirmed one quantitative loci region (QTL) region on chromosome 2H which was previously identified by Nagel et al. (2009).

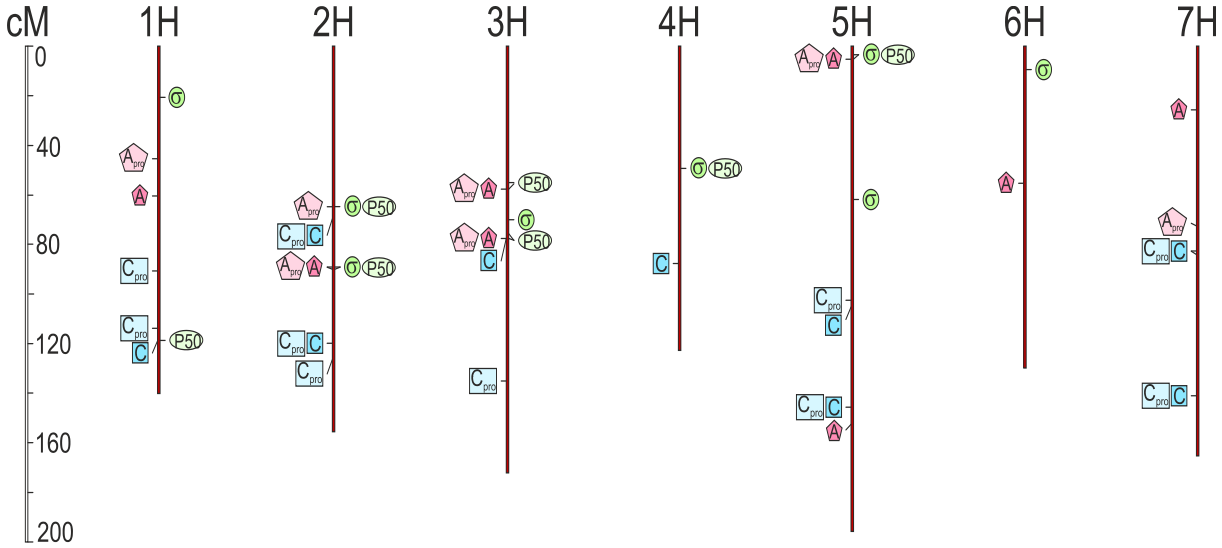


Fig. 2: Genome wide association analysis (GWAS) for germination related traits of 203 spring barley accessions. Germination related traits are: C – initial germination in %, $C_{pro}$ – initial germination in probit, A – germination after artificial ageing in %, $A_{pro}$ – germination after artificial ageing in probit, s – days to lose 1 probit, and P50 – half- viability period; cM – centi Morgan

## Summary

The usage of seed viability equation offers a new way of analysis to identify QTL. Several seed longevity loci were confirmed by the different parameters (P50, probit data). New loci detected by GWAS responsible for seed ageing could be discovered by sigma which might be interesting for future analysis.

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# Non-destructive phenotyping using the high-throughput LemnaTec-Scanalyzer 3D platform to investigate drought tolerance in barley

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The LemnaTec-Scanalyzer 3D system (Fig. 1) in the IPK Gatersleben was established in 2008 and was one of the first LemnaTec-platforms in the public domain. Plants are phenotyped fully automated with three different camera systems using I) visible light, II) fluorescence, and III) near infrared. A watering and weighing station offers controlled water supply. Plants grow in pots that are placed on a carrier having an RFID-chip. All data from imaging and watering are recorded by the chip and transferred to the database. The IPK system currently consists of 312 carriers located on six lanes of a conveyer belt system enabling an automated transport to the imaging chambers and watering station. Rotation of full or half lanes to overcome the effects of greenhouse inhomogeneities can be programmed in different ways.

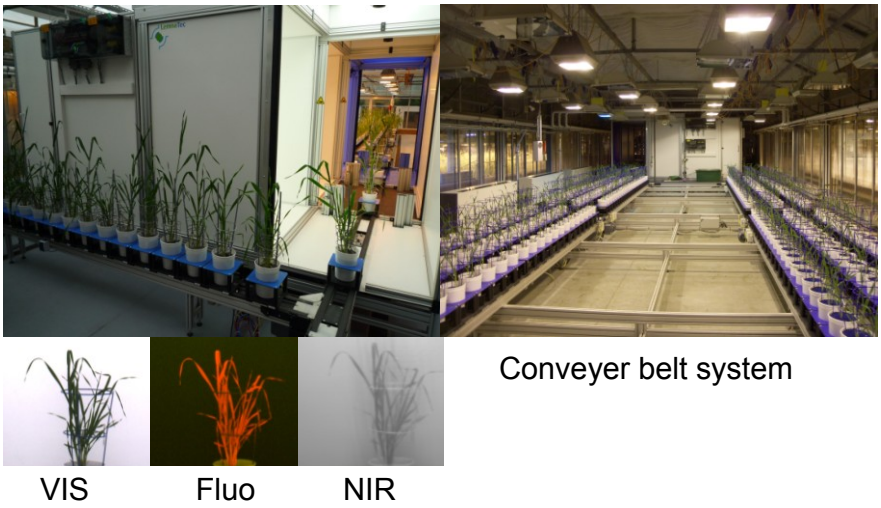


Fig. 1: The LemnaTec-Scanalyzer 3D system in IPK. Left side: imaging chambers for visible, fluorescence and near-infrared wavelengths; Right side: conveyer belt system

Since autumn 2010, the IPK is involved in the CROP.SENSe.net project. A barley core set of eight old and eight modern two-rowed German spring barley cultivars was screened in three repeated experiments for vegetative drought tolerance. Immediately after sowing, daily imaging and watering took place. Rotation jobs at night prevent a strong influence of greenhouse inhomogeneities. Watering was set to a target weight of 90% of field capacity (FC) in our standard organic soil. After four weeks of growth, drought stress was induced by withholding water to half of the plants of each genotype. After 18 days without water, plants were re-watered to the same level as before (90% FC) and underwent a recovery period of two more weeks. At the end, plant fresh and dry weight was measured and compared with the values for digital biomass. In house, an image analysis pipeline was developed to analyse the enormous amount of collected data (~500 Gb for one experiment). It is already giving good results for visible light and fluorescence images, though algorithm development is still in the process of fine-tuning (Fig. 2). The first experiment is already analysed while the remaining two are currently analysed. Correlation of digital biomass (in pixel) and measured fresh weight is high ($R^2$ ~ 0.95).

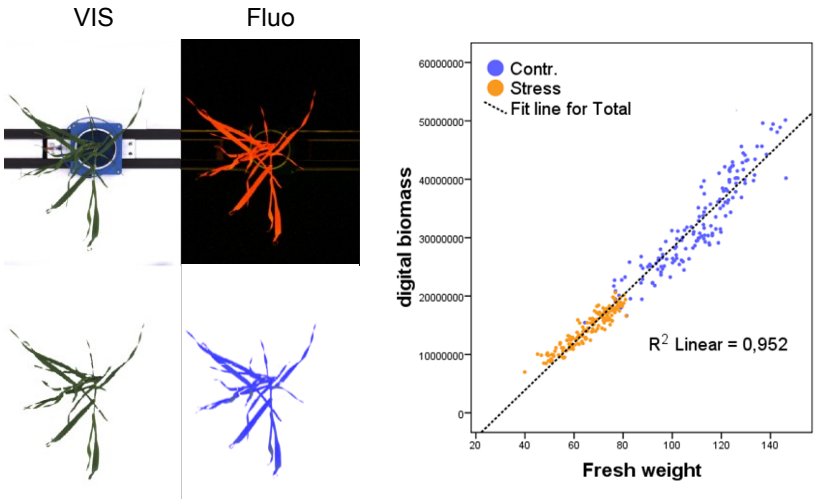


Fig. 2: left: original image of a plant in visible light (VIS) and with fluorescence (Fluo) camera , below extracted plant after image analysis; right: correlation of fresh weight and digital biomass of all 312 plants in one experiment.

Looking at two traits estimated from the digital images, a strong influence on plant biomass development due to drought stress is visible. Though after re-watering also the stressed plants start to grow again, biomass was reduced by 55% compared to control plants at the end of recovery period. Also plant height is reduced by water deficit, but here the reduction is only 16% (Fig. 3).

In the near future all three experiments will be analysed and we will try to develop a model to predict vegetative drought tolerance of genotypes. After increase of the system to 520 carriers in the middle of 2012, we will screen an association mapping panel to reveal loci relevant for drought tolerance in barley.

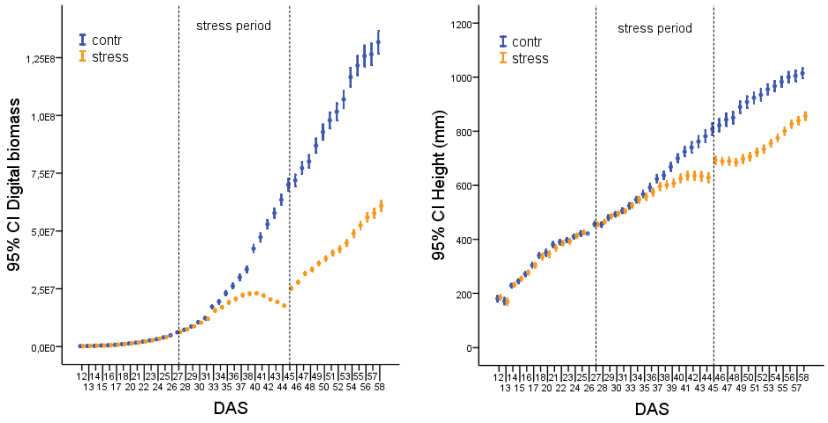


Fig. 3: Diagram of biomass and plant height development over time (in days after sowing – DAS) in control and stress treatment

**Acknowledgments**

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## Characterization of mitochondrial manganese superoxide dismutase (MnSOD) gene transcript level changes during activity of low temperature in wheat (*Triticum aestivum* L.)

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### Introduction

Reactive oxygen species (ROS) are often generated in live cells as a by-products of natural metabolic processes and under the influence of environmental stresses. ROS are serious danger for live cells, especially because of high oxidative potential (Bowler et al. 1992).

The first line of live cells defense against reactive oxygen species activity are superoxide dismutases (SOD) (EC 1.15.1.1) (Fink, Scandalios 2002). These enzymes dismutate two superoxide anions into $H_2O_2$ and $O_2$ (Baek, Skinner 2006).

The aim of presented studies was characterization of the MnSOD gene expression changes during low temperature activity in seedlings of Polish common wheat (*Triticum aestivum* L.) cultivars by means of real-time PCR method.

## Material and methods

For examination five Polish common wheat cultivars with winter growth habit (Nutka, Rywalka, Finezja, Fregata, Satyna) and 5 with spring growth habit (Bombona, Hewilla, Histra, Kosma, Żura) were used.

Five-day-old wheat seedlings derived from sterile kernels were placed in 4°C. Fragments of leaves were collected after 48 hours and 7 days of low temperature activity. At the same time points material from control plants placed in 24°C was harvested. For extraction of total RNA from plant material and its reverse transcription to cDNA commercial kits were utilized. RNA integrity was confirmed by means of 2% agarose gel electrophoresis, moreover nucleic acid quantity and purity was checked at each step spectrophotometrically.

Real-time PCR analyses were based on SYBR® Green I fluorescent dye. As a reference ubiquitin gene was used. The primers for MnSOD gene were used according to Baek and Skinner (2003) and for ubiquitin gene according to Kobayashi et al. (2005). Correctness of amplification for both genes were confirmed by means of melting curve analysis. Statistical analyses of expression differences were carried out using SAS 9.2 software and based on non-parametric Wilcoxon test according to procedure by Yuan et al. (2006).

## Results

Analysis of the MnSOD gene expression in spring wheat cultivars during low temperature treatment showed that reaction after two days of stress activity was not similar, however after seven days all tested forms presented the same pattern of response.

Expression of the MnSOD gene in Bombona and Histra cultivars after 2 days of experiment was reduced significantly (0.68 and 0.64 fold change, respectively). In the same time point expression of the analyzed gene in Hewilla and Żura cultivars did not differ significantly from their control forms. In wheat cv. Kosma significant increase of MnSOD transcript amount was noticed (Fig. 1).

After 7 days of low temperature activity for all examined spring cultivars statistically significant increase of the mitochondrial manganese superoxide dismutase gene expression was observed. Differences ranges from 0.56 fold change for cv. Kosma to 2.82 fold change for Żura (Fig. 1).

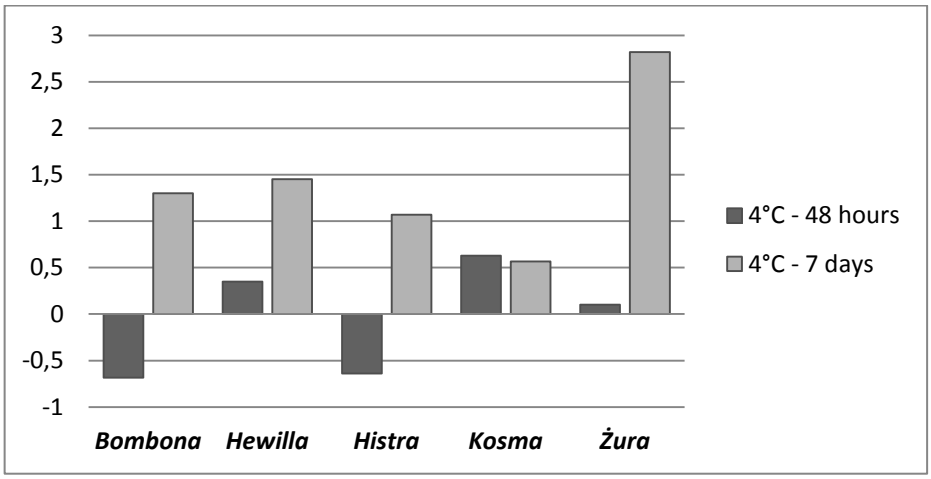


Fig. 1: Fold change of the MnSOD gene transcript level in analyzed spring wheat cultivars after low temperature treatment in comparison with respective control forms.

For majority of analyzed Polish winter wheat cultivars initial decrease of MnSOD gene expression during low temperature activity was noticed (from 0.24 fold change for Finezja to 3.17 for Fregata). Only for Satyna cultivar, a weak non-significant increase was observed (Fig. 2).

During next five days of experiment a progressive enhancement of analyzed SOD gene expression was observed. For almost all cultivars, with the exception of Rywalka, after seven days of experiment the level of expression was higher in comparison with control forms. The biggest difference was noticed for cv. Fregata (2.5 fold change in 7$^{th}$ day of experiment) and the slightest for cv. Nutka (0.29 fold change) (Fig. 2).

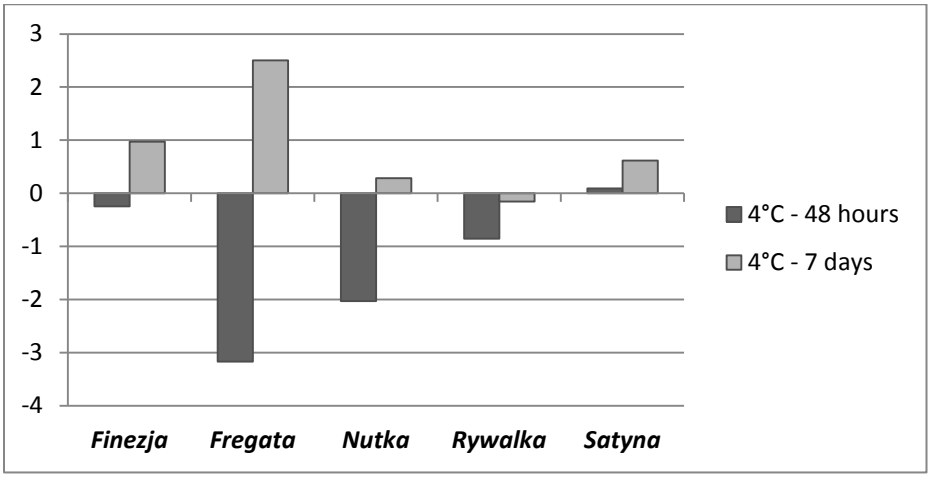


Fig. 2: Fold change of the MnSOD gene transcript level in analyzed winter wheat cultivars after low temperature treatment in comparison with respective control forms.

## Conclusions

Obtained results showed that level of the MnSOD gene expression in wheat is regulated by low temperature activity. It is possible that enzyme encoded by analyzed gene is one of the factors determined acclimation of wheat plants to low temperature in natural conditions. Moreover, our results suggest that exist differences in expression of the antioxidant enzymes encoding genes in response to low temperature between spring and winter wheat genotypes.

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# Biochemical marker-assisted development of new common wheat line with HMW-glutenin genes from *Triticum timopheevii* Zhuk.

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Complex study of introgressive lines of *T. aestivum* L. and *Triticum timopheevii* Zhuk with leaf-rust resistance was done. Active prolamine genes of homeological chromosomes 1 and 6 were established in 35 wheat lines. Protein, wet gluten, and prolamine fractions content, grain texture, and alveographic indices together with leaf-rust resistance scores were determined. Two lines were found with active HMW-glutenin genes from *Triticum timopheevii* Zhuk. Line 684 had HMW-GS encoded by both *1Ax* and *1Ay* (*1Ay* always silent in commercial cultivars) genes and could serve as a source of the *Glu-At1* locus. Line 186 carried the *Glu-B1/Glu-G1* substitution. The new stable form of common wheat was constructed under control of HMW- glutenins, carrying active *Glu-1* loci of *Tt* from the lines 684 and 186.

*Triticum timopheevii* Zhuk. (*Tt*) is one of the world immune wheat species (Dorofeev et al. 1987). In our institute, a collection of *T. aestivum,* introgression lines carrying resistance to brown rust from *Tt* has been generated (Budashkina and Kalinina 1998). Agronomically valuable traits of these new forms of common wheat, especially bread-making quality (BMQ) are of considerable interest for basic and applied research. It is known, that the gluten polymer is mostly formed by prolamins. These are HMW- and LMW- glutenin subunits (GS), linked by disulfide bounds, and gliadins Wrigley 1996). BMQ depends on allelic composition of glutenin subunits (Payne and Lawrence 1983, Payne et al. 1987, Gupta and Mac Ritchi 1994, Luo et al. 2001, Obukhova et al. 2001) and gliadins (Metakovsky 1991), and the volume of gluten fraction (Obukhova et al. 2008). It is known that in *T. aestivum* each *Glu-1* locus contains two genes for *x*- and *y* -type subunits. In the *Glu-A1* and *Glu- B1* loci, the *1Ax* and *1By* genes can be silent. At the same time, the *1Ay* genes are always silent in commercial cultivars. The higher is the number of HMW-GS the better is BMQ. It is known, that in *Tt* the *1Ay* gene is not silent, and this wheat species can potentially serve as a source of gene of interest (Obukhova et al. 2009a).

Earlier we investigated storage proteins in 11 introgression lines from the collection. In these studies, no active genes for HMW-glutenins from the genome of *Triticum timopheevii* were identified (Obukhova et al. 2007, Obukhova et al. 2009b). The present study is focused on screening of the *T. aestivum* collection for active *Tt*-HMW-GS gene accessions and construction of new lines of *T. aestivum* with active *Tt* genes.

## Materials and methods

Introgression lines of *T. aestivum* L. (2n=42, $F_{20-22}$ BC1), resistant to brown rust from *Tt,* were generated from the crosses beween commercial cultivars of common wheat, Saratovskaya 29 (S29), Novosibirskaya 67 (N67), Irtyshanka 10 (Ir10), Tselinnaya 20 (Ts20), Skala (Sk) and tetraploid endemic species *Triticum timopheevii* var. viticulosum (2n=28, $A^tA^tGG$) (Budashkina and Kalinina 1998).

Isolation, electrophoresis proteins, and identification of the *Glu-1* allelic states was performed according to (Payne and Lawrence 1983). The SDS-PAGE of gliadins and their identification

was carried out according to (Metakovsky 1991). Electrophoregrams were treated using the GelPro Analyzer 4.0 software program. The samples analyzed were represented by flour obtained from the kernel halves taken from 10 different accessions from each of the lines examined. In case of identification of polymorphism in the protein profile, analysis of individual seeds was performed. The *Glu-1* quality index was calculated, according to Lukow et al. (1989).

Technological properties of grain and flour, including contents of raw gluten, protein, and prolamine fractions, together with grain texture and flour physical properties from alveograph, have been investigated in common wheat seven introgressive lines and cultivars, and *Tt,* according Obukhova et al. (2008). Resistance of lines is evaluated to leaf rust (Mains and Jackson 1926) and to powdery mildew (Saari and Prescott 1975).

**Results and discussion**

Series of lines and their parental forms were carefully studied, including resistance to brown rust, allelic states of prolamin loci, *Glu1* score, bread-making quality, and the protein content (glutenin and gliadin) in flour (Tables 1 and 2) . Some of these results have been reported previously (Obukhova et al. 2007, Obukhova et al. 2009b). The resistance to the brown leaf rust was found for all lines; the line 821 is the best, while the line 676 has the best score in Omsk conditions.

As compared with parents, the weight of 1000 grains (GW) became larger for the lines 676, 175, 67, 140, 191. The diameter of flour particles (MD) became larger for lines 676, 175, 67 and dropped for lines 191 and 206. The protein content (PC) grew in lines 676, 175, 140, 206. The wet gluten content (WGC) grew in all lines. The glutenin fraction content grew in lines 821, 676, 191. The gliadin fraction content grew in lines 821,175. The alveograph index (W) grew for lines 821, 676 and 191. All changes are statistically significant.

According to the mentioned parameters, the lines meet the requirements of the State Committee Central Laboratory of the Russian Federation for bread wheat of grades no worse than valuable, except for lines 191 and 206, possessing soft grain textures. The line 821 is the best in all characteristics.

The information on grain quality obtained in the study is important for using the introgressive lines in breeding programs as donors of leaf rust resistance.

The Table 3 summarizes the new results on storage proteins. The study was focused on continuation of screening the collection for search HMW –glutenin subunits from *Tt*. As shown in the Table 3, some of the lines (10, 28, 38, 64, 134, 87, and 94) displayed polymorphism, which was associated with natural polymorphism of original cultivar Irtyshanka 10 (Morgunov et al. 1990). Lines 87 and 94 (Ir10) had better composition relative to HMW- GS with the *Glu-1 q*uality index equal to 7, versus 5 for the other lines.

For a number of lines (155, 157, 673, 838, and 842), the parental origin was not confirmed. These lines may have been re-pollinated. In the line-population 842, the genotype atypical to original cultivar, was removed under control of prolamins. The remaining lines (744, 783, 811, 742, 191,199, 212, 10, 28, 73, 134, 38, 87, 94, 64, 178, and 184) preserved the HMW-GS allele composition of the original cultivars. Line 64 has introgression of the *Tt* genome fragments in the form of two closely linked loci, *Gli-G1* and *Glu-G3,* which substituted the homeologous loci *Gli-B1* and *Glu-B3* of cultivar Irtyshanka 10. In line 178, a substitution of two pairs of linked loci was observed. Specifically, *Glu-A3* and *Gli-A1* were substituted with *Glu-*$A^t$*3* and *Gli-*$A^t$*1,* while *Glu-B3* and *Gli-B1* were substituted with *Glu-G3* and *Gli-G1*.

Lines 38 and 184 carry substitution of *Glu-B3/Glu-G3*. In addition, lines 38, 184 and 742 appeared to be polymorphic with respect to the *Gli-B1/Gl-G1* substitution. Specifically, individual kernels differ by the level of *Gli-B1*-locus-encoded γ-gliadin. For these reasons, lines 38, 184, and 742 need to be additionally partitioned with respect to this character.

Only two lines, carry the HMW- GS, which belong to the genome of *T. timopheevii.* Line 684, derived from cultivar Novosibirskaya 67 (N67), received the *Glu-A$^t$1* locus from *Tt.* Two genes, *1A$^t$x* and *1A$^t$y,* actively expressed all the proteins, produced by the genome of *Tt.* The *1A$^t$y* gene, which is usually silent in commercial cultivars, deserves special interest. In Line 186, derived from cultivar Tselinnaya 20 (Ts20), substitution of the *Glu-B1/Glu-G1* locus was observed. Both of the genes, *1Gx* and *1Gy,* were actively expressed. Similarly to original cultivars, in both of the lines (684 and 186) the *Glu-3* and *Gli-1* alleles remained unchanged.

The use of lines 684 and 186 enabled interpretation of the *T. timopheevii* genome products as expressed by the *Glu-A$^t$1* and *Glu-G1* loci. Figure 1 shows the densitogram of the HMW-GS from *Tt* with indication of chromosomal affiliation of the products. Both lines 684 and 186 in the field conditions have complex resistance to brown leaf rust, stem rust and powdery mildew, see Table 3.

The ability of line 684 to be the donor of the *1A$^t$y* gene was tested using a single cultivar. In the cross with the common wheat cultivar, Novosibirskaya 67, line 684 proved to be the donor of active *Glu-A$^t$1* locus.

The work on construction of the new form of *T. aestivum,* carrying the *Glu-1* loci of *Tt* from the lines 684 and 186 was done under control of HMW-glutenin genes from *T. timopheevii.* The combination of hybridization (186 x 684) is better than inverse one (684 x 186) due to the suppression of activity of the locus *Glu-A$^t$1*. The choice of the positive hybrid combination on the earliest stage permitted for the controlled selection of *Glu-1* homozygotes corresponding the genome of *Tt* in the second generation. The new form of common wheat has resistance to the brown leaf rust in the field conditions.

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Table 1: Characterization: *Glu-1* score, allelic states of prolamin loci, quality indices and brown leaf rust resistance of introgressive lines and their parental varieties.

| Cultivar, line | *Glu-1* score | *Glu-3* | *Gli-1* | *Gli-2* | GW | MD | PC | WGC | GliC | GluC | W | P/L | resistance,** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | 2004 | 2006 |
| S29 | 7 | | Ai + Af | | 31.5 | 19.8 | 15.3 | 34.5 | 132 | 49.6 | 374 | 0.9 | 4 | 4 |
| 821 | 7 | | Ai | | 29.7 | 18.5 c(-) | 15.2 | 43.0 a(+) | 147 c(+) | 58.4 b(+) | 568 a(+) | 1.1 c(+) | 0-1 | 0 |
| N67 | 8 | | | | 29.7 | 24.5 | 15.1 | 30.0 | 135 | 49.6 | 285 | 1.1 | 4 | 4 |
| 676 | 8 | | | | 32.5 | 27.7 b(+) | 16.1 a(+) | 37.0 a(+) | 143 | 62.8 a(+) | 553 a(+) | 1.7 a(+) | 0 | 2-3 |
| Sk | 7 | | | | 23.7 | 16.2 | 13.8 | 32.0 | 139 | 56.9 | 256 | 0.95 | 4 | 4 |
| 175 | 7 | $A^t$,G | $A^t$,G | (B+G)* | 31.9 | 19.6 a(+) | 16.1 a(+) | 39.5 a(+) | 150 c(+) | 55.5 | 270 | 1.3 a(+) | 2 | 2 |
| Ir10 | 7 | | | | 29.5 | 17.5 | 16.0 | 34.0 | 139 | 55.5 | 468 | 1.1 | 4 | 4 |
| 67 | 5 | G | G | (B+G)* | 33.5 | 19.6 b(+) | - | 40.0 a(+) | 147 | 58.4 | 384 b(-) | 1.7 a(+) | 1-2 | 2 |
| 140 | 5 | | | | 37.8 | 17.5 | 16.6 c(+) | 42.5 a(+) | 139 | 56.9 | 376 b(-) | 1.2 | 0-1 | 2 |
| Ts20 | 9 | | | | 28.4 | 18.9 | 15.0 | 33.0 | 150 | 56.9 | 440 | 1.5 | 4 | 4 |
| 191 | 9 | | | (B+G)* | 30.2 | 13.4 a(-) | 17.2 a(+) | 39.0 a(+) | 150 | 62.8 b(+) | 529 b(+) | 1.6 c(+) | 2 | 2 |
| 206 | 9 | | | (B+G)* | 24.7 | 13.4 a(-) | 16.1 a(+) | 44.0 a(+) | 150 | 58.4 | 451 | 0.9 a(-) | 2 | 2-3 |
| *Tt* | | *Tt* | *Tt* | *Tt* | 42.6 | 28.7 | 17.7 | 38.5 | - | - | 82 | 1.9 | 0 | 0 |

Notes to Table 1: *Glu-1* scores were determined as in Lukov et al. (1989). Only changed alleles are indicated. *- lines have possessing new *Gli-2* integration loci that involve individual genes of two loci: from the cultivars and from *Tt*, controlled by arms 6BS and 6GS. Technological parameters: GW =1000 grain weight, g; MD= flour particle mean diameter, μm; PC= protein content in grain, %; WGC= wet gluten content in grain, %; GliC=gliadin content per 1g of flour, mg; GluC=glutenin content per 1g of flour, mg; Alveograph: *W*=deformation energy, a.u.; *P*=stiffness, mm; *L* = extensibility, mm. a, b, c: significant differences observed in introgressive lines compared with their parent cultivars at $P<0.005$, $0.005<P<0.05$ and $0.05<P<0.15$ respectively. Signs show the directions of change. **- resistance, according to the scale Mains and Jackson (1926): 0-2 – resistant; 2004 year, Omsk, 2006 year, Novosibirsk.

Table 2: Allele variants of storage proteins (Obukhova et al. 2009a)

| Cultivar, line | *Glu-1* | | | *Glu-3* | | | *Gli-1* | | |
|---|---|---|---|---|---|---|---|---|---|
| | 1AL | 1BL | 1DL | 1AS | 1BS | 1DS | 1AS | 1BS | 1DS |
| S29 | *b* | *c+b#* | *a* | *f* | *b* | *a* | *i+f* | e | *a* |
| 842 | | *c* | *a+d#* | - | - | - | - | - | - |
| 744, 783, 811 | | *c* | | | | | *i* | | |
| 742 | | *c* | | | | | *i* | *Tt+e* | |
| 838 | *b+a#* | *c+b#* | *a+d#* | - | - | - | - | - | - |
| Ts20** | *b* | *c* | *d* | - | - | - | *f* | *e* | *a* |
| 191,199,212 | | | | Ts20 | Ts20 | Ts20 | | | |
| 186 | | ***Tt*** | | Ts20 | Ts20 | Ts20 | | | |
| N67** | *a+b* | *b* | *a* | *f* | *b'* | *a* | *f* | *e* | *a* |
| 673 | *a+Tt* | *b+c#* | | - | - | - | - | - | - |
| 684 | *Tt* | | | | | | | | |
| Ir10** | *b+c* | *c* | *a* | - | - | - | *k* | *b* | *a* |
| 10, 28, 73,134 | *c* | | | Ir10 | Ir10 | Ir10 | | | |
| 38 | *c* | | | Ir10 | *Tt* | Ir10 | | *b+Tt* | |
| 87, 94 | *b* | | | Ir10 | Ir10 | Ir10 | | | |
| 64 | *c* | | | Ir10 | *Tt* | Ir10 | | *Tt* | |
| Sk** | *c* | *c* | *d* | - | - | - | *k* | *b* | *a* |
| 157 | | | *a#* | - | - | - | - | - | - |
| 178 | | | | *Tt* | *Tt* | Sk | *Tt* | *Tt* | |
| 184 | | | | Sk | *Tt* | Sk | | *b+Tt* | |
| 155 | *b#* | *c+b#* | *a#* | - | - | - | - | - | - |

Notes to Table 2. Empty cells- alleles correspond to the parental cultivars; *Tt* - alleles corresponding *T.timopheevi* var. *viticulosum*; # - absent from cultivars (Morgunov et al. 1990). Sign «-» means that alleles were not interpreted; Sign ** marks cultivars containing additional fast ω–gliadins Obukhova et al. 2009b). *Glu-B3b*' corresponds to Gupta and Mac Richie (2001) and differs from *b* allele by absence of the slowest LMW-GS.

Table 3: Resistance of lines 186 and 684 to leaf rust (*Lr*), powdery mildew (*Pm*), and stem rust (*Sr*).

| lines | year | Lr | Pm | Sr |
|---|---|---|---|---|
| 186 | 2009 | 0 | 7 | - |
| | 2010 | 0 | 7 | 0 |
| 684 | 2009 | 0 | 3 | - |
| | 2010 | 0 | 8-7 | 0 |

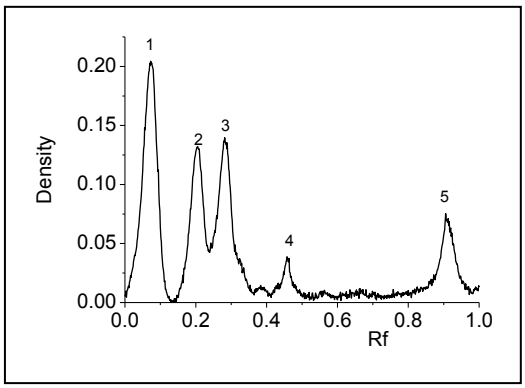


Fig. 1: Densitogram of Tt, based on electrophoregram of HMW-GS in SDS-PAGE. Proteins 2, 4, and 5 controlled by *Glu-A*$^{t}$1 locus are found in line 684. Proteins 1 and 3 found in line 186 are expressed by *Glu-G1* locus.

# Genetic variability of detoxification enzymes activity in leaves of inter-varietal substitution lines of bread wheat with different tolerance to water deficit

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Detoxification enzymes scavenging of excess reactive oxygen species play an important role in defense response of plants under water-deficit environment. Catalase (CAT) and ascorbate peroxidase (APX) prevent excessive accumulation of hydrogen peroxide; at the same time they maintain the constant low level of $H_2O_2$ necessary for redox signaling. Glutathione reductase (GR) and dehydroascorbate reductase (DHAR) are responsible for supporting the pool of redox pairs GSH/GSSG and ascorbate/dehydroascorbate in cells under water deficit. Ascorbate and glutathione are the key molecules participating not only in the hydrogen peroxide metabolism and signaling "but also in the wider contexts of plant development and environmental responses" (Foyer and Noctor, 2011).

In bread wheat, one of the most important crops, the genome organization of structural and regulatory genes controlling the activity of detoxification enzymes both in normal and stressful conditions is practically not studied. The aim of this work was to determine the chromosomes carrying the genetic factor(s) controlling the activity of CAT, APX, DHAR and GR under different regimes of water supply and to evaluate the association of enzyme activity with drought tolerance through yield components of wheat plants. We used two sets of inter-varietal substitution lines (ISCSLs) of different origin, Chinese Spring (Synthetic 6x) (Synthetic 6x formed from the wide cross *Triticum dicoccoides* (genomic constitution AB) x *Aegilops tauschii* (DD)), and Saratovskaya 29 (Janetzkis Probat).

ISCSLs and the donor and recipient cultivars were grown in a phytotron as described by Osipova et al. (2011) in two replicates, each of which consisted of two pots containing 10-15 plants. In one pair of pots, the plants were kept well-watered, while in the other pair, water was withheld from the three leaf stage until the soil moisture content had fallen to 30% of saturation. The moisture content of the soil for the well-watered control plants was maintained at 60% saturation. Soil moisture status was monitored three times per week by weighing the pots, and adjusted where necessary. Two uppermost expanding leaves were cut from three different plants per pot at the booting stage, frozen in liquid nitrogen and stored at -70ºC. The entire set was sampled on the same day. All enzyme activities were determined spectrophotometrically using a Hitachi U-1100 spectrophotometer: DHAR at 265nm ($E = 14\mu M^{-1}cm^{-1}$) (Baier et al., 2000), CAT at 240nm ($E = 42.5\mu M^{-1}cm^{-1}$) (Aebi, 1984), APX at 290nm ($E = 2.8\mu M^{-1}cm^{-1}$) (Nakano and Asada, 1981) and GR at 340nm ($E = 6.2\mu M^{-1}cm^{-1}$) (de Lamotte et al., 2000). Relation between the activity level of the CAT, DGAR, GR and APX and drought tolerance of wheat was evaluated through yield components and indexes of tolerance/susceptibility to drought. Indices are based on the parameters $\underline{Td}$ (experimental grand mean performance of all droughted plants), Td (mean performance of the droughted plants of a given line), $\underline{Tc}$ (experimental grand mean performance of all well-watered plants) and Tc (mean performance of the well-watered plants of a given line). IT was given by Td/Tc x 100 (Kuol 2004), SSI by $[1- (Td/Tc)]/[1- (\underline{Td}/\underline{Tc})]$ (Fischer and Maurer 1978).

In the first set the lines carrying chromosomes 1B, 1D, 2D, 3D, 4D and also Synthetic 6x were able to increase the low constitutive level of DHAR activity in response to stress (Fig. 1). Having done so, they appeared better able to retain the moisture status of their flag leaf, and their grain yield components were less compromised by drought. The lower sensitivity of the grain yield components shown by CS/Syn1B, /Syn1D, /Syn2D, /Syn3D and /Syn4D may reflect their superior adaptation to stress occurring early in development (Osipova et al., 2011). The best performing CS/Syn lines were derived from D genome substitutions. This suggests at least that *Ae. tauschii*, the natural range of which is characterized by arid soils, may as a species be well adapted to water deficit conditions.

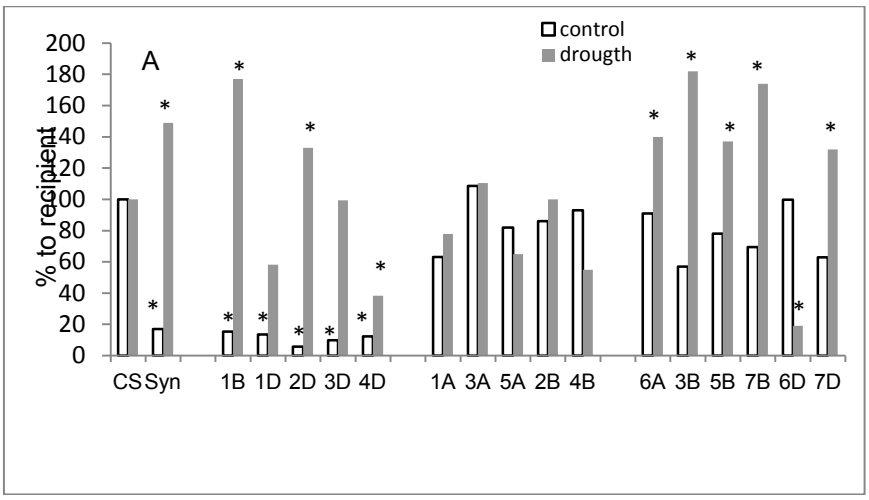


Fig: 1. Relative leaf DHAR activity in the CS (Syn 6x) substitution lines and their parents at tillering stage (in percentage to the recipient CS). *- $P < 0.01$; comparing to the recipient CS

In the other set of ISCSLs the recipient cultivar Saratovskaya 29 (S29) is highly tolerant to drought in contrast to the donor cultivar Janetzkis Probat (JP). The most critical chromosome substitutions with pronounced biological effects were revealed by non-metric multidimensional scaling. Replacement of chromosomes 2A, 2B, 2D and 4D resulted in reduction of yield component tolerance under drought stress (Table 1).

For the same lines reduction in activity of the investigated enzymes was observed. Genetic variability for antioxidant enzyme activity among the S29/JP ISCSLs allowed the identification of the key chromosomes underlying the control of this trait. There was also clear evidence of the involvement of genes present on the homoeologous group 2 chromosomes in the control of GR activity (Fig. 2), which suggests the presence of either structural or regulatory genes for GR activity on these chromosomes. The average GR activity of lines S29/JP2A, S29/JP2B and S29/JP2D was reduced by almost 4 times compared to the average GR activity across all the lines studied under both conditions (0.5 U / mg versus 1.9 U/mg, $P < 0.01$). GR catalyses the reduction of oxidized glutathione (GSSG) which is necessary to support a high glutathione (GSH) to GSSG ratio in the cell. Reduced GSH is a co-factor of DHAR. DHAR and GR activity positively correlated with one another under well-watered conditions ($r = 0.43$, $p < 0.05$), and the strength of this correlation was higher under drought stress conditions ($r = 0.70$, $p < 0.001$). Average DGAR activity in the lines S29/JP2A, S29/JP2B and S29/JP2D was reduced by 3 times comparing to the entire population of lines (4.6 U / mg versus 13.8 U / mg, $P < 0.001$).

Table 1: Indexes of tolerance / sensitivity in the ISCSLs S29 (JP) grouped according to drought tolerance

| Parameters | I group, ISCSLs for chromosomes of the homoeologous groups 1, 3, 5, 6, 7; 4A and 4B substitutions | | II group, ISCSLs for chromosomes 2A, 2B, 2D and 4D | |
|---|---|---|---|---|
| | IT,% | SSI | IT,% | SSI |
| Stem length | 97.0 | 0.3 | 68.5** | 3.7** |
| Spikelet number | 88.0 | 0.5 | 56.5* | 1.1 |
| Grain number | 99.0 | 0.2 | 77.4* | 3.9* |
| Grain weight | 82.8 | 0.7 | 38.0* | 1.7* |
| Reproductive fertility | 82.3 | 0.4 | 65.1** | 1.5* |

*- P < 0.05; ** - P < 0.01; compared to I group of lines
IT,% - index of tolerance (Kuol 2004); SSI – index of sensitivity (Fischer and Maurer 1978)

The recipient S29 differed from JP by a higher CAT activity. ISCSL S29/JP4D under drought stress conditions had the lowest CAT activity (15.2 U / mg) among all the lines (60.2 U/mg).

Thus, our work revealed the association between the genetic variability for tolerance to water deficit of yield and variability for the activity of antioxidant enzymes. We believe that the fine mapping using the recombinant lines based on S29/JP2A, S29/JP2B, S29/JP2D and S29/JP4D substitutions will discover genes and QTL, associated with these traits.

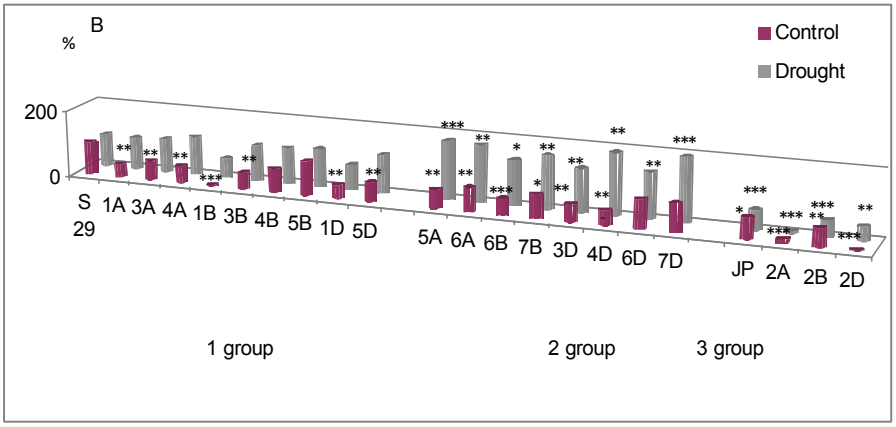


Fig. 2: Relative leaf GR activity in S29 (JP) ISCSLs and their parents under well-watered and drought conditions. * P < 0.05; ** P < 0.01; *** P < 0.001, comparing to the recipient S29

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# New flowering time genes and alleles in wheat; the study of their effects

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Different approaches and methods of identification and characterization of new genes and alleles influencing yield of wheat are presented.

Two mapping populations Sandra (Sandra 3B / CP 3B) and Zlatka (Zlatka 3B / CP 3B) for a flowering time gene *QFt.cri-3B.1* that was recently located near the centromere of the chromosome 3B (Pánková et al. 2008) have been subject of further work aiming at a more detailed characterization of the gene, its location (fine mapping) and effects.

Molecular checks of flowering time genes present in the set of 40 spring wheat varieties have resulted in identification of a new allele of the gene *Vrn-B1*. Changes of the DNA sequence were analysed in the new allele and multiplex PCR was designed for distinguishing among all of the known *VRN-B1* in one PCR reaction.

## Materials and methods

*Mapping of the c 3B and analysis of its effects on flowering:* The $F_1$ generation for near isogenic line (NILs) development had been produced by backcrossing selected recombinant substitution lines (RSLs) of the existing mapping populations of Sandra (Sandra 3B / CP 3B) and Zlatka (Zlatka 3B / CP 3B) to the parental varieties, Sandra and Zlatka. . Fine mapping populations from the $F_1$ were then produced by a series of backcrosses between marker-selected recombinant lines of the mapping populations and the parents.

Flowering time and development of the apexes of plants of substitution lines Zlatka (CP 3B), Sandra (CP 3B) including parental lines for the NILs under defined conditions (air-conditioned room - photoperiod 10 hours, temperature 24°C/16°C day / night) was carried out with the aim to define phenotyping conditions for the gene *QFt.cri-3B.1*. Changes of morphology of plant apexes were checked in weekly intervals and heading time of the lines was recorded as the date when a half of first spike headed.

*Identification of a new allele Vrn-B1c:* A set of 40 spring wheat varieties (*T. aestivum* L.) was sown on 15 April (no photoperiodic response expected in the latitude of Prague) into the field plots in three repetitions, in a randomized scheme. Heading time was recorded in parallel with molecular checks of DNA of the varieties for present alleles of genes influencing considerably growth habit and earliness (*VRN1, Rht-B1, Rht-D1, Rht8, Ppd-D1)*. Extraction of DNA from young leaves of the wheat plants was done using commercially available DNeasy Plant Mini Kit (Qiagen).Within the set 7 varieties were found (Granny, Kaerntner Frueher, Linda, Paragon, Quarna, Septima and Sirael) with neither dominant nor recessive allele of the gene *VRN-B1* detected. For those varieties sequential analysis was done using data from the database NCBI (http://www.ncbi.nlm.nih.gov/genbank/index.html) on the lines Triple Dirk B (TDB) for the allele *vrn-B1* (No. AY747604.1), and Triple Dirk C (TDC) for the allele *Vrn-B1a* (No.AY747603.1), and new primers were suggested (Milec et al. 2011).

**Results and discussion**

*Mapping of the QFt.cri-3B.1 and analysis of its effects on flowering:* We have developed 139 NILs for the Sandra (Sandra 3B / CP 3B) cross, and 49 NILs for the Zlatka (Zlatka 3B / CP 3B) cross. Marker selection has been continued for maintenance of the non-recombined substituted chromosome segment containing *QFt.cri-3B.1* until a higher level of homozygosity of the recipient background is obtained. Nearly 2000 $F_2$ plants per parental population were derived by selfing the heterozygous backcross plants. Segmental substitution lines were identified and developed from segregating progeny to fine map the gene. These lines were sent for DArT analysis (Diversity Arrays Technology Pty Ltd, Canberra, Australia) to delineate the substituted segment.

To distinguish the effect of the *QFt.cri-3B.1* it is necessary to define cultivation conditions that clearly differentiate between the early and late plants. Once we succeed to calibrate the cultivation condition for phenotyping, it will be possible to combine phenotyping results with the marker information of the mapping populations to fine map. For this purpose we have used the original substitution lines and parental varieties where both difference in genotype and flowering time was clear, and added available parental lines, used at backcrosses for Sandra (Sandra 3B / CP 3B) NILs production. The limiting phenotyping factor is mainly duration of the photoperiod combined with the temperature. The difference in flowering time of contrasting genotypes was only found under a short photoperiod combined with a higher day temperature (24°C). Vernalization did not have effect on expression of *QFt.cri-3B.1* (Table 1).

Table 1: Days to heading of wheat plants under controlled regime: 10 h photoperiod, temperature 24°C day /16°C night

| Line | Sandra | Sandra (CP 3B) | Zlatka | Zlatka (CP 3B) | Sandra (CP 3B / San3B) 151 17 | Sandra (CP 3B / San3B) 169 12 |
|---|---|---|---|---|---|---|
| Average | 155,22 | 148,53 | 162,54 | 149,13 | 145,31 | 139,50 |
| St. Dev. | 9,51 | 6,72 | 19,84 | 16,82 | 8,71 | 7,74 |
| Signif. | | * | | * | ** | ** |

*Identification of a new allele Vrn-B1c:* Three exon-based primer pairs (Ex1/B/F3 and Ex2/B/R3; Ex2/B/F1 and Ex3/B/R1; Ex3/B/F2 and Ex8/B/R2; numbers next to 'Ex' refer to the numerical order of exons) covering nearly the complete *Vrn-B1* sequence were designed. Amplicon sizes corresponded to expected sizes for two primer pairs only. The first pair showed differences between real and calculated product sizes. Therefore, further work was focused on the region between the first and second exon. The new forward primer Ex1/B/F3 (5´GAAGCGGATCGAGAACAAGAT 3´) was combined with a reverse primer Intr1/B/R3 and tested on those seven varieties. Two winter wheat varieties carrying the recessive allele *vrn-B1* and four spring wheat varieties carrying the dominant allele *Vrn-B1a* were added as a control. The amplicon size for spring varieties with the *Vrn-B1a* allele was slightly over 1200bp (as expected) and winter varieties did not amplify since the expected product size was larger than 8000bp which would need different PCR conditions for successful amplification.

The remaining seven varieties gave products of size of 850bp approximately. This indicated a possible deletion of 350 to 370 bp. However, sequencing of cloned PCR products showed that the sequence of these products was more complicated. Alignment with the sequence of the TDB line showed that the new sequence can be divided into three "regions": region 1 starting

from 528 to 797bp, region 2 from 1615 to 1762bp, and region 3 covering sequence from 2019 to 2452bp.

There was apparently an 818bp deletion (from 797 to 1615bp upstream) which was in contradiction with the predicted deletion size. The answer to this was found : a region of 433bp (from 2019 to 2452bp upstream - region 3) had been moved downstream in front of the region 2 .This gives, together with region 1, band size of 849bp which is in agreement with gel electrophoresis results. We have designated this new allele *Vrn-B1c* and its sequence has been annotated on the NCBI database as HQ593668.

The designed primers were then combined to get bands distinguishing clearly among the alleles *Vrn-B1a, Vrn-B1b, Vrn-B1c* and *vrn-B1* after amplification in one multiplex PCR (Milec et al., 2011). The characteristics of *Vrn-B1c* corresponds with another very recently reported discovery of *Vrn-B1c* (Shcherban et al. 2011)

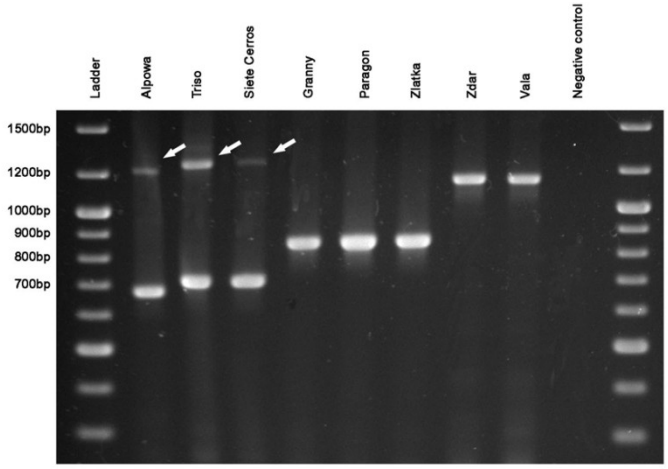


Fig. 1: Gel electrophoresis of multiplex PCR to distinguish among all *VRN-B1* alleles with two ´forward´ primers (Ex1/B/F3 and Intr1/B/F) and two ´reverse´ primery (Intr1/B/R3 and Intr1/B/R4: spring varieties: Alpowa – *Vrn-B1b* (673bp and 1199bp); Triso and Siete Cerros – *Vrn-B1a* (709bp, 1235bp); Granny, Paragon, Zlatka – *Vrn-B1c* (849bp); winter varieties: Zdar, Vala – *vrn-B1* (1149bp); negative control – water. White arrows show the bands larger than 1kbp, that can also amplify (Milec et al., 2011).

**Acknowledgement**

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# Lipoxygenase isozymes activity in bread wheat: inheritance and relationship to drought tolerance

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## Introduction

Lipoxygenase (linoleate: oxygen oxydoreductase, LOX, EC 1.13.11.12) catalyzes the oxidation of polyunsaturated fatty acids. Existence of a lot of isozymes initiating various pathways of lipid degradation is explained by lipoxygenase multifunctionality and its involvement in many physiological processes of plant ontogenesis and adaptation to stresses (Feussner, Wasternack 2002).

Droughts are the most strong and unpredictable abiotic stressors, which considerably limit wheat productivity. Search for the factors influencing crop stability under conditions of moisture deficit will be helpful in wheat breeding considering drought tolerance. The aims of the work were identification of chromosomes participating in the genetic control of activity levels of certain lipoxygenase isozymes under water deficiency and study of the relationship between LOX activity and wheat yield stability under drought.

## Material and methods

The set of substitution lines (SL) Chinese Spring (Synthetic 6x) (CS / Syn6x) in which a pair of chromosomes from the recipient CS is substituted for a homologous pair from the donor (the artificially synthesized allohexaploid Syn6x) was the object of the study. The seed material of SL and their parents was kindly provided by A. Börner (Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany).

Wheat was grown under controlled conditions in the phytotron growth chamber at the Siberian Institute of Plant Physiology and Biochemistry as it has been described earlier (Osipova et al., 2011). In the control group, the plants received optimal watering while in the experimental group - only 50% of the optimal amount of water. The relative water content in leaves (RWC,%) was measured at the flowering stage by a standard method. At the phase of full ripeness, the productivity indices including grain number per spike (GN), grain weight per spike (GW, g) and weight of one grain (GS, mg), as ratio GW/GN, were studied.

Preparation of enzymatic extracts and subcellular protein fractions, polyacrylamide gel electrophoresis, LOX activity and protein concentration measurement were in detail described in preliminary works (Permyakova et al. 2010; Permyakova et al. 2012). The specific activity was represented by a ratio of the units of enzymatic activity (E) to the extract protein concentration (mg per ml). In order to detect the effects of chromosome substitution on the genetic background of the recipient cultivar, all comparisons were made with CS.

**Results and discussion**

The enzyme molecular form mLOX with relative electrophoretic mobility (Rf) 0.04, was detected in the protein microsomal fractions of wheat leaves (Fig.1). It was found only in the samples of plants exposed to drought conditions. The absence of mLOX and its enzymatic activity in the control samples indicated that the induction of this membrane bound LOX isozyme was caused by soil drought.

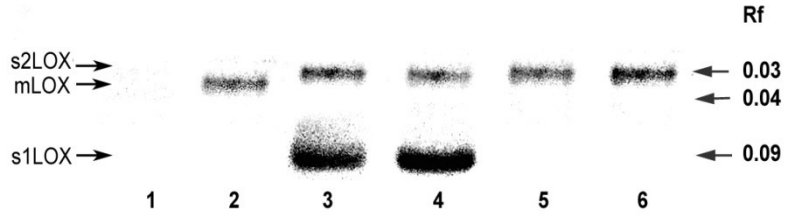


Fig. 1: Electrophoretic profile of LOX isozymes extracted from leaves of Chinese Spring (Synthetic 6x) lines grown under drought. 1, 2 – membranes fraction; 3, 4 - fraction of soluble proteins with exception of chloroplasts soluble proteins; 5, 6 - fraction of chloroplasts soluble proteins. 1, 3 and 5 – under control conditions; 2, 4 and 6 – under drought conditions

The activity of mLOX in the recipient cultivar CS was very low while in the donor Syn6x it was considerably higher (Fig. 2a). All studied SLs considerably exceeded the recipient with the exception for the lines with substitution of 5D and 6D chromosomes. An interesting fact is that these SL were most resistant to the drought and one of the most sensitive line (SL for chromosome 3A) had the highest mLOX activity (data on the genotype drought resistance are provided in Osipova et al. 2011). This may prove the involvement of LOX isozymes in a protective mechanism which probably connected with a jasmonate dependent protective signaling (Kasan, Manners, 2008).

Two molecular forms s1LOX and s2LOX differing in Rf were detected in the fraction of soluble proteins with the exception for the chloroplasts soluble proteins. The s2LOX enzyme was also found in the fraction which contained soluble proteins of chloroplasts (Fig. 1).

The levels of the enzymatic activity in this protein fractions in SLs with respect to the recipient cultivar are presented in Fig. 2 a, b and c. The use of wheat lines with substituted pairs of chromosomes and detection of polymorphism for a specific LOX activity allowed us to demonstrate that the enzymatic activity of all three molecular forms in leaves had a polygenic control involving many genes located on the chromosomes of different homoeologous groups. However, precise substitution effects have been found only in the fraction of soluble proteins with the exception of chloroplasts soluble proteins. The obvious effects of chromosome substitution on the total s1LOG and s2LOG enzymatic activity facilitated the identification of the critical chromosomes associated with its genetic control. These were chromosomes 1A, 1D, 3A, 5A, 5B, and 5D under a sufficient water supply and chromosomes 1B and 1D under the drought conditions (Fig.2b).

Three LOX isozymes were found in grain of all investigated SL as well as in the previous researches (Trufanov et al. 2006). Only 4D chromosome participated in the genetic control of lipoxygenase isoforms activity in grain grown at the optimum water delivery. Chromosomes 4B, 1D, 2B, 3A and 3B took part in the genetic control of the trait in addition to a chromosome 4D under the conditions of simulated drought (Fig. 3).

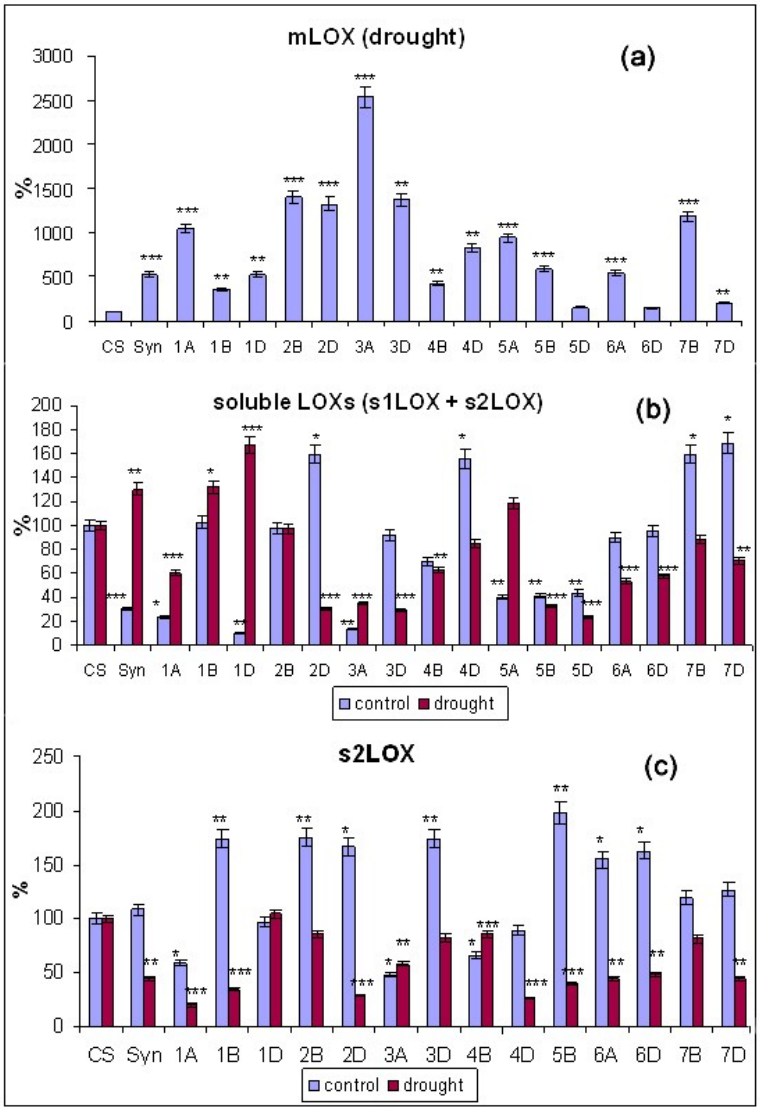


Fig. 2: Specific LOX activity in leaves of single chromosome substitution lines of wheat Chinese Spring (Synthetic 6x) comparing to the recipient

Using nullisomic-tetrasomic lines of the CS cultivar it has been demonstrated that the structural genes of LOX isozymes are located on the chromosomes of homoeologous groups 4 and 5 (Li et al. 1999). Earlier we have shown that the genetic control of LOX activity in mature wheat grain is connected with the chromosomes of 4 homoeological group (Permyakova et al. 2006). Probably, the chromosomes of homoeologous group 5 carry the structural genes responsible for LOX activity level in leaves. Chromosomes belonging to homoeologous groups 1, 2 and 3 possibly associated with the genetic regulation of their activity level. More complex genetic regulation of enzymes activity under drought is explained by a wide participation of lipoxygenase in defense signaling, plant development and grain formation in stressful conditions.

The correlation analysis has shown a positive differential influence of the lipoxygenase isozymes activity on yield components under drought (Table). LOX isoenzymes activity level may be one of the factor associated with the maintenance of productivity under a moisture deficiency used as a marker trait in genetic programs of wheat improvement.

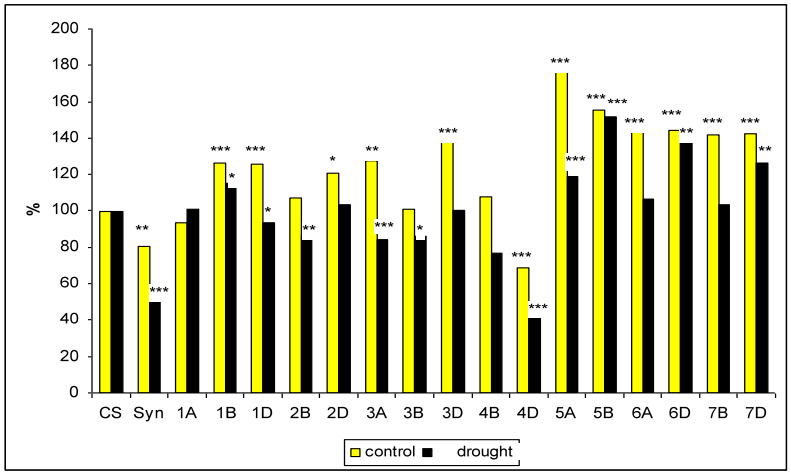


Fig. 3: Specific LOX activity in grain of single chromosome substitution lines of wheat Chinese Spring (Synthetic 6x) comparing to the recipient

Table 1: Correlation relationships between isozymes activity of LOX, relative water content, stem length and yield components

| | mLOX | s2LOX | | | grain LOXs | | |
|---|---|---|---|---|---|---|---|
| Parameters | drought | control | drought | D/C | control | drought | D/C |
| RWC, C | 0,187 | 0,06 | 0,292 | 0,152 | -0,154 | -0,178 | -0,162 |
| RWC, D | 0,577** | -0,117 | 0,466** | 0,443* | 0,148 | 0,264 | 0,346 |
| RWC, D/C | 0,409* | -0,108 | -0,137 | 0,006 | 0,209 | 0,281 | 0,308 |
| Stl, C | -0,165 | -0,434 | -0,206 | -0,07 | -0,027 | 0,089 | 0,218 |
| Stl, D | 0,323 | -0,12 | 0,514** | 0,44* | 0,361 | 0,566** | 0,528** |
| Stl, D/C | 0,434* | 0,078 | 0,496** | 0,418* | 0,367 | 0,454** | 0,332 |
| GW, C | 0,261 | --0,669** | -0,021 | 0,4 | 0,247 | 0,378 | 0,553** |
| GW, D | 0,222 | 0,013 | 0,275 | 0,124 | 0,324 | 0,437* | 0,471** |
| GW, D/C | -0,238 | 0,598** | 0,254 | -0,208 | -0,013 | 0,024 | 0,012 |
| GN, C | 0,359 | -0,572** | 0,243 | 0,53 | 0,261 | 0,294 | 0,393 |
| GN, D | 0,48** | -0,169 | 0,36 | 0,336 | 0,292 | 0,201 | 0,178 |
| GN, D/C | -0,237 | 0,51** | 0,025 | -0,311 | -0,129 | -0,232 | -0,319 |
| GS, C | -0,12 | -0,25 | -0,422* | -0,156 | 0,215 | 0,456** | 0,515** |
| GS, D | -0,12 | 0,286 | 0,162 | -0,078 | 0,22 | 0,457** | 0,48** |
| GS, D/C | -0,065 | 0,444* | 0,434* | -0,082 | 0,047 | 0,128 | 0,121 |

RWC – relative water content; Stl - stem length; GN – grain number per spike; GW – grain weight per spike; GS – weight of one grain. C - control, D - drought.

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## Genetic diversity of winter wheat germplasm


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One of the main prerequisite for successful future winter wheat breeding programs is genetic diversity and great efforts are aimed toward its maintenance and development. Genetic variability is based on very complex statistical relationship assigning to the variance among allele at individual gene loci, among several loci, between individuals within populations and between populations (Smale, 1997). Danger of genetic erosion is a constant threat in plant breeding it can easily appear in smaller breeding programs and selection for limited production area with similar growing conditions (Fu et al. 2009; Ganeva et al. 2010). Selection of diverse and divergent parents is essential for creation of superior new varieties. Assessment of genetic diversity is conducted by deployment of different criteria markers: morphological, agronomical, coefficients of parentage, biochemical and molecular. Today different combination of molecular markers is intensively used because it diminishes all subjective assessments (Manifesto et al. 2001; Ahmad, 2002; Khlestkina et al. 2002). Aim of this study was to evaluate genetic diversity of wheat varieties using SSR and AFLP markers as a powerful tool for assessing genetic diversity.

### Materials and methods

Forty winter wheat varieties, originating from Croatian, Austrian, French, Russian and Italian breeding centres were included in the study. Selection of varieties was done according to date of release (from 1936 to 2008) and by production area. DNA was isolated using CTAB method (Doyle and Doyle, 1987). Amplification of 26 SSR markers was conducted according

to Röder et al. (1998). For AFLP analysis we used the restriction enzymes *Sse8371* and *MseI* and selective amplification was conducted using two selective nucleotides (Buerstmayr et al. 2002) which is all together four AFLP primer combinations. Both molecular marker analyses were conducted in Wheat Research Department, Agricultural Research Institute of the Hungarian Academy of Science (Martonvásár, Hungary).Using Powermarker (Liu, 2002) we calculated expected heterozigosity ($H_e$) and polymorphic information content (PIC) (Botstein et al. 1980) for each locus (SSR), and for each selective primer pair (AFLP. Rogers distance was used to estimate genetic diversity between varieties according to SSR markers, and Dice coefficient was used to estimate genetic similarity using AFLP data. Data from both molecular methods was input data for cluster analysis based on UPGMA (NTSYS ver. 2.2.). Mantel test was used to evaluate correlation between distance, and similarity matrices.

## Results and discussion

SSR markers produced total of 108 alleles with the average gene diversity ($H_e$) of 0.58 and genetic distance ($d_{ij}$) of 0.66. Mostly similar results were estimated by other authors (Ahmad et al. 2002; Landjeva et al., 2006; Christiansen et al. 2002). A total of 110 polymorphic fragments were detected using AFLP markers, with average number of polymorphic fragments ($np_i$) of 28 per primer combination used and genetic similarity coefficient ($s_{ij}$) was 0.54. Very similar results were estimated by Manifesto et al. (2001) with average $s_{ij} = 0.55$. Grouping of varieties were in accordance with their origin (breeding centre) and pedigree.

Mantel test (Table 1) showed significant ($p<0.01$) but weak correlation ($r=0.191$) between two methods used justifying the deployment of these two methods used in this study. High coefficient of correlation estimated by Maccaferri et al. (2007) was explained with good molecular marker coverage of wheat genome.

Table 1: Mantel's test (below diagonal) and significance (above diagonal)

| Mantel's test | SSR | AFLP |
|---|---|---|
| SSR | - | 0.01 |
| AFLP | 0.191 | - |

Results showed large genetic diversity among tested varieties and therefore can be used as parents in future wheat breeding programs.

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## The use of monosomic lines of bread wheat for verification of quantitative trait loci (QTL)

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Earlier, a number of quantitative trait loci (QTLs) associated with technological properties of grain were identified in ITMI mapping population of bread wheat (Pshenichnikova et al. 2008). One of them named *QGlc.ipk-5B* responsible for gluten content in grain was localized on 5B chromosome in the region of molecular markers *Xwg899* and *Xtam72c*. Synthetic R-93, one of the parental forms of ITMI population was the donor of the high meaning of the trait.

The QTL associated with flour strength and dough tenacity named *QDse.ipk-7D* was localized near the marker *Xfba204a* on 7D chromosome with improving effect from another parental form of ITMI population cv. Opata-85.

Monosomic lines for 5B and 7D chromosomes of the cultivar Saratovskaya 29 (S29) were used for verification of QTLs performance in another genotypic environment. This cultivar has very high physical properties of dough but contains relatively low gluten content in grain. The donor of *QGlc.ipk-5B* allele improving the trait performance was the line ITMI-43. The same line was the donor of 7D chromosome carrying *QDse.ipk-7D* allele adversely affecting physical properties of dough.

Single chromosome substitution lines have been developing on the genetic background of S29 through introducing of the corresponding chromosomes from ITMI-43 line which carries the marker loci. Monosomic and disomic $F_3$ families were selected from $BC_4$ and $BC_5$ single chromosome substitution lines for further technological investigations. The families of the line S29 (ITMI-43 5B) were grown under green house and field conditions in two replicates. The families of the line S29 (ITMI-43 7D) were grown under the field conditions in two replicates. Milling parameters and wet gluten content in grain were studied according the methods accepted in Russian State variety testing, rheological parameters were studied using Chopin alveograph.

It was found that both Synthetic R-93 and ITMI-43 line have significantly higher gluten content in grain (Table 1). Introducing of 5B chromosome from the line ITMI-43 into the background of S29 significantly increased this parameter (Table 1). Both monosomic and

disomic lines contained more wet gluten content by 2-3 percent comparing to the recipient S29. Therefore, *QGlc.ipk-5B* may be effective in another genetic background.

Table 1: Milling parameters and gluten content in grain in $F_3$ families of single chromosome substitution line S29(ITMI-43 5B), $BC_4$

| Lines and cultivars | Technological properties of grain | | | |
|---|---|---|---|---|
| | TGW, g | Vitreousness, % | Gluten content in grain % | Mean diameter of flour particles, μm |
| S29, recipient | 26,3 | 89,3 | 31,1 | 24,2 |
| $BC_4$ $F_3$ S29/ITMI 43 5B, disomic | 25,4 | 89,8 | 33,1 | 24,1 |
| $BC_5$ $F_3$ S29/ITMI 43 5B, monosomic | 29,8 | 93,4 | 34,5 | 23,0 |
| ITMI-43, donor | 24,4 | 97,1 | 46,8 | 27,2 |
| Synthetic R-93 | 44,5 | 78,6 | 42,6 | 13,6 |
| Opata-85 | 32,2 | 67,4 | 36,0 | 17,8 |
| $F_{crit}$ | 3,11 | | | |
| $F_G$ | 43,5*** | 305,2*** | 83,6*** | 52,2*** |
| $LSD_{05}$ | 2,3 | 1,3 | 1,2 | 1,4 |

The line ITMI-43 has very low physical properties of dough: low flour strength (16.5 u.a.) and low tenacity (25.5 mm). Introducing of 7D chromosome from the line ITMI-43 carrying the allele of *QDse.ipk-7D* negatively associated with dough properties did not influence these traits in disomic family (Table 2). However, both monosomic families had significantly lower meanings of flour strength, tenacity and their ratio comparing to the recipient S29 and disomic family. It may mean that 7D chromosome of ITMI-43 carries the unknown genetic factor of quantitative nature determining physical properties of dough in bread wheat. A significant decrease of dough strength was found earlier in 7DS ditelosomic line of cv. Chinese Spring (Maystrenko et al. 1973).

Table 2: Physical properties of dough (alveograph) in substitution line S29(ITMI-43 7D), $BC_5$

| Genotypes | Alveograph parameters | | |
|---|---|---|---|
| | Dough strength | Tenacity | P/L |
| S29, recipient | 418.8 | 147.7 | 1.7 |
| $BC_5F_4$ S29(ITMI-43 7D), | | | |
| disomic | 378.4 | 133.3 | 1.6 |
| monsomic 1 | 234.3 | 99.7 | 1.2 |
| monosomic 2 | 169.6 | 84.6 | 1.2 |
| $F_{crit}$ | 4.07 | | |
| $F_G$ | 54.7*** | 61.0*** | 26.8*** |
| $LSD_{05}$ | 32 | 7.5 | 0.1 |

To this moment, eight backcrosses have been made for both lines and the field experiments will be carried out in the future.

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## Genome-wide association mapping of seed longevity, dormancy and pre-harvest sprouting in bread wheat (*Triticum aestivum* L.)

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### Introduction

Seed longevity is defined as the time during which a seed remains viable when stored under optimal conditions. Genebanks are interested in increasing the shelf lives of seeds stored in plant germplasm repositories. Identification of loci responsible for long life of seeds is the first step towards this mammoth task. Seed dormancy which is defined as the temporary failure of seed germination inspite of suitable conditions is a trait that seems related to seed longevity. Pre-harvest sprouting (PHS) results from the lack of dormancy and is characterised by seed germination on intact spikes. This research was conducted with an aim to identify loci responsible for seed longevity, dormancy and pre-harvest sprouting to get a better understanding of these phenomena in the wheat genome.

### Materials and methods

We used two sets of association mapping panels in this study. The first panel consisted of an elite gemplasm collection of 96 winter wheat accessions assembled at the Institute of Field and Vegetable Crops, Novi Sad, Serbia. The members of the panel were selected on the basis of their phenotypic diversity with respect to a group of key agronomic traits, and their provenance is spread over 21 countries (Neumann et al. 2011). The second panel consisted of 183 (129 spring and 54 winter wheats) accessions originating from 32 countries selected from the Gatersleben genebank repository based on the differential behaviour of longevity over long term storage. The first panel was genotyped with 525 mapped DArT (Diversity Arrays Technology) markers (Neumann et al. 2011) whereas second panel was genotyped with 416 mapped DArtT markers (Rehman Arif MA, PhD thesis; in prep).

To assess longevity, germination of genebank collection after 34 years of storage (natural longevity) and two experimental ageing (accelerated ageing (AA) and controlled deterioration (CD)) methods were used. Software TASSEL was used to find out marker trait associations for all traits.

## Results and discussion

Analysis of elite germplasm collection resulted in detection of numerous marker trait associations (MTAs) for longevity after experimental ageing, for dormancy and pre-harvest sprouting distributed on all the three genomes of wheat (Rehman-Arif et al. 2011). Genebank collection revealed many MTAs after long term cold storage, experimental ageing, dormancy and PHS, respectively. The main gene contents of the deletion bins harboring these MTAs are shown in Figure 1.

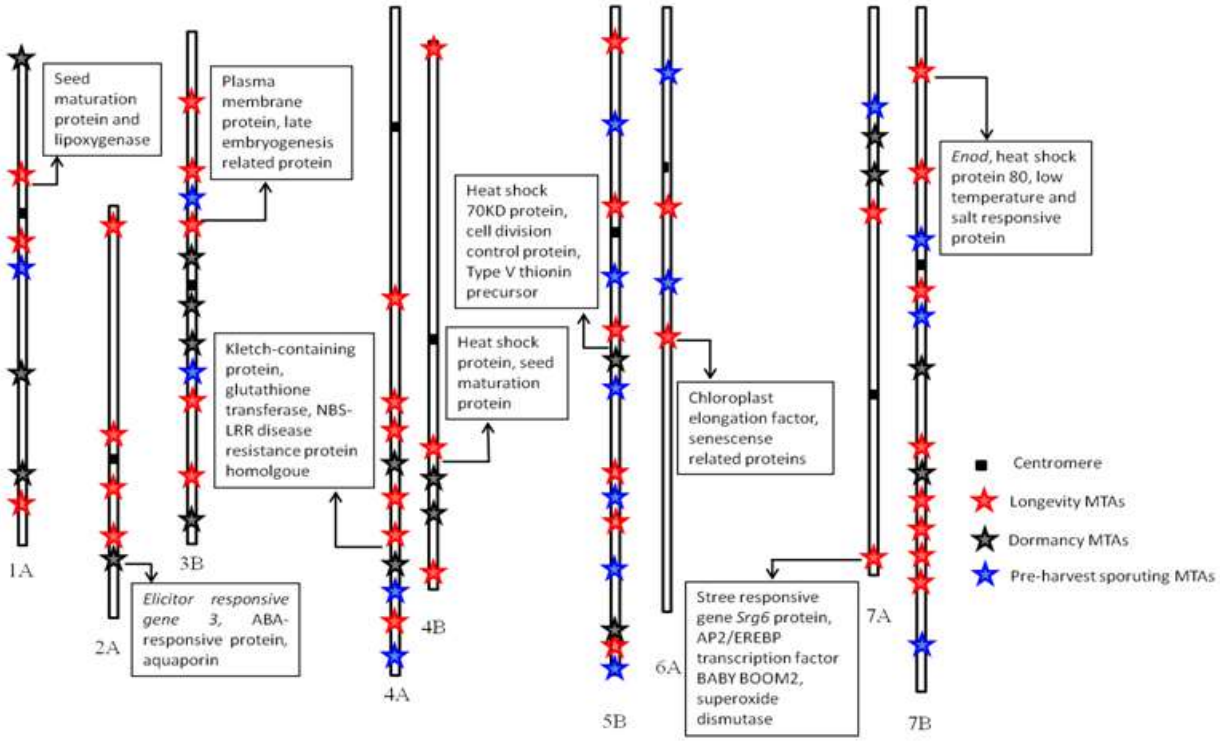


Fig. 1: Association analysis of seed longevity, dormancy and pre-harvest sprouting of 'elite germplasm' and 'genebank' collections

Some of these genes have known effects in regulating dormancy and pre-harvest sprouting such as aquaporin. The findings of this study match with similar studies in barley (Nagel et al. 2009) and rice (Miura et al. 2002, Sasaki et al. 2005, Zeng et al. 2006 and Xue et al. 2008) for longevity (Figure 2).

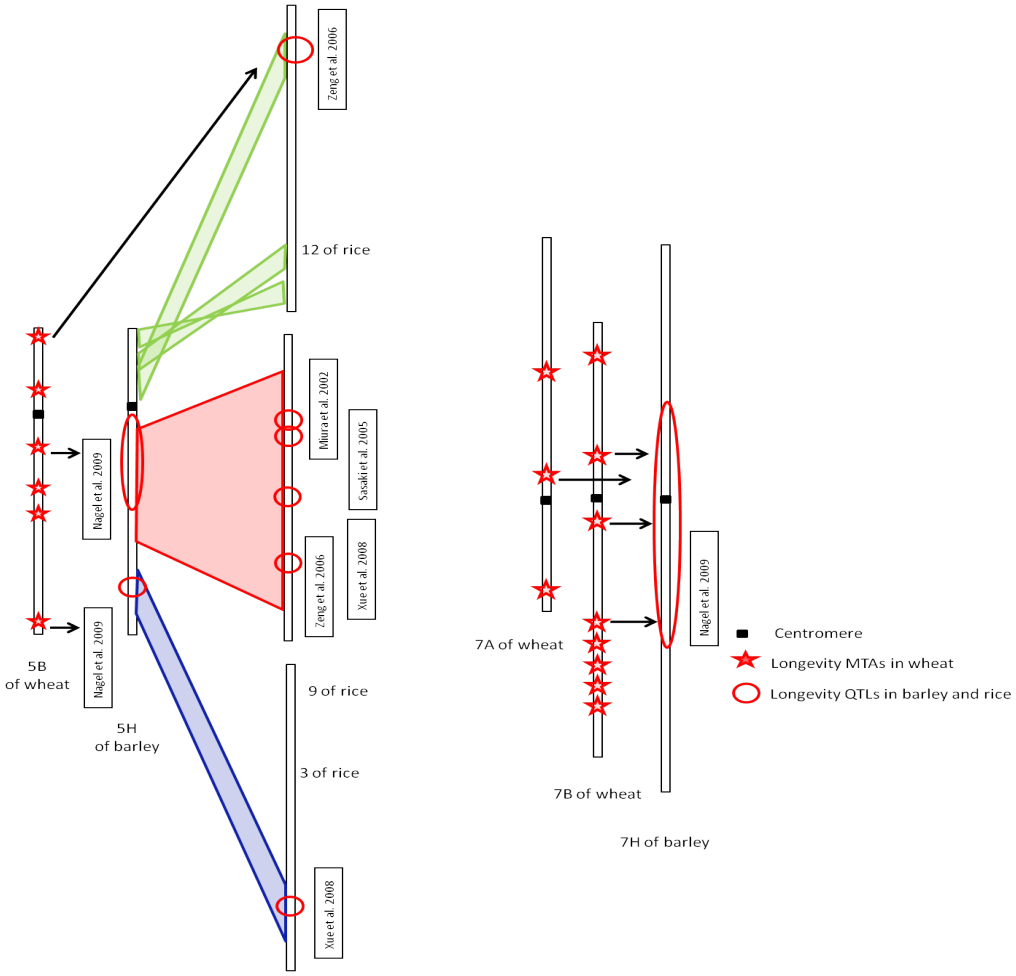


Fig. 2: Comparison of seed longevity in wheat with barley and rice

## Conclusion

Seed longevity is a quanitative trait and may be controlled by several genes/loci**.** Colocated MTAs for dormancy and pre-harvest-sprouting indicate that these phenomena might be controlled by similar genes in the upstream such as abscisic acid regulation. It also indicates that there are loci for longevity, dormancy and pre-harvest sprouting that control these traits indepedently. Synteny of investigated traits in wheat, barley and rice open new questions for future research of these traits.

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# Evaluation of genetic diversity among Egyptian bread wheat (*Triticum aestivum* L.) varieties during the period 1947-2004 using microsatellite markers

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## Introduction

Wheat (*Triticum aestivum* L.), a self-pllinating crop, is the world's most important cereal crop based on cultivation area and the second most important after maize in total production (FAO, 1999). Information on the extend and patterns of distribution of genetic variation of a crop species is essential for effective utilization of germplasm in plant breeding programmes. Intensive plant breeding is generally considered to be a practice that leads to reduced genetic diversity. In Egypt, there are four wheat breeding programs performed at Sakha, Gemmiza, Giza and Seds that have developed new cultivars. Collectively, these programs have released the cultivars that are included in this study.

The use of molecular markers for the evaluation of genetic diversity of wheat has recently received a great deal of attention from molecular geneticists and wheat breeders. With the help of molecular markers, geneticists can monitor the changes in DNA sequences in genotypes released at different times (Ortiz, 2001). This approach would help breeders to assess the allelic combinations selected generation after generation, and thus provide a genetic ideotype for future marker-assisted selection (Christiansen *et al*., 2002). This information would help breeders to incorporate useful genetic variation into adopted gene pools by selecting for marker alleles linked to loci controlling important agronomic traits (Tanksley and McCouch, 1997).

Genetic diversity is the foundation for crop genetic improvement and thus the most important consideration in any plant breeding program. A major concern for many modern plant breeding programs is the narrow genetic base of their germplasm (Velle, 1993). Several authors have argued that the narrowness in genetic diversity could lead to an increased vulnerability to diseases and pests, as well as the ability of plants to respond to changing environmental conditions (Tripp, 1996). Thus, quantifying genetic diversity among existing germplasm helps to address this concern.

Genetic diversity in wheat was characterized using morphological traits (Salem *et al*., 2008), isozymes (Guadagnuolo *et al*., 2001) and DNA based markers such as random amplified polymorphic DNA (RAPD) (Cao *et al*. 2000), amplified fragment length polymorphism (AFLP) (Bohn *et al*., 1999), restriction fragment length polymorphism (RFLP) (Ward *et al.,* 1998; Bohn *et al*., 1999) or microsatellites (Plaschke *et al.* 1995; Prasad *et al.,* 2000; Ben Amer *et al*., 2001; Christiansen *et al*. 2002; Huang *et al.* 2002; Dreisigacker *et al.*, 2004; Dreisigacker et al., 2005; Fufa *et al.*, 2005).

Microsatellites or simple sequence repeats (SSR) combine many desirable marker properties including high levels of polymorphism and information content (PIC), high reproducibility, co-dominance, rapid and simple genotyping assays, uniform genome coverage, and specific polymerase chain reaction (PCR) based assays (Röder *et al.*, 1998 a, b). Furthermore, the analysis of microsatellites based on PCR is simple to perform. In wheat, microsatellites have

been successfully used in a wide range of applications such as genotype identification (Prasad *et al.*, 2000), diversity studies (Prasad *et al.*, 2000; Ben Amer *et al.*, 2001; Huang *et al.*, 2002, Alamerew *et al.,* 2004; Salem *et al.*, 2008) and quantitative trait locus analysis (Salem, 2004, Salem *et al*., 2007). The objective of this study is to (i) characterize the allelic diversity of thirty-three Egyptian wheat genotypes released from 1958 to 2004 using eighteen microsatellite markers, (ii) provide a deep insight into the genetic diversity of the Egyptian bread wheat collection and (iii) assess the potential application of our results for future studies on the evaluation and conservation of wheat genetic resources.

## Materials and methods

*Plant material:* In total thirty-three diverse wheat (*Triticum aestivum* L.) genotypes released from 1947 to 2004 and from different wheat breeding programs in Egypt were used in this study. Wheat varieties represent four wheat breeding programs in Sakha, Khafr El-Sheikh Governorate; Gemmiza, Gharbia Governorate; Giza, Giza Governorate and Sids, Bani Suef Governorate. In addition, the two wheat varieties Aztec and Chinese Spring were included as standards. Grains of all Egyptian varieties were obtained from the Agricultural Research Center (ARC), Giza, Egypt. A List of the wheat genotypes, their breeding program and pedigree is presented in Table 1.

*DNA extraction*: Total genomic DNA was performed according to Plaschke *et al.* (1995).

*PCR amplification:* PCR reaction were carried out as described by Röder *et al.* (1998a). The amplification products were resolved on 10% polyacrylamide denaturing gels (PAGE) (Röder *et al.*1998a).

*Microsatellite loci analysis:* Seventeen Gatersleben Wheat Microsatellite (GWM) markers were selected from Röder *et al*. (1998b). The microsatellite primers used were described by Röder *et al.* (1998a) (Table 2). Fragment detection for SSR markers was carried out as given in Röder et al. (1998b).

*Statistical analysis:* Gels were scored as binary data matrix. The presence (1) and absence (0) of alleles for each microsatellites marker were recorded for each variety. The data were analyzed using the Numerical Taxonomy and Multivariate Analysis System (NTSYS), version 2.1 (Rohlf 2002). Gene diversity was calculated according to formula of Nei (1973) using the equation $\mathrm{PIC} = 1 - \sum_{i=1}^{k} P_i^2$ , where k is the total number of alleles detected for a locus of a marker and *Pi* is the frequency of the *i*th allele in the set of 12 wheat varieties investigated.

## Results

*Characteristics of SSR markers:* Seventeen SSR markers dispersed across the genome were used to test the genetic diversity of 33 genotypes. The 17 Gatersleben wheat microsatellite markers used revealed a total of 17 loci on 15 chromosomes (Röder *et al.,* 1998 a,b and Devos

*et al.,* 1995) (Table 2). All the 17 SSR markers generated polymorphic patterns, yielding a polymorphism rate of 100% (Table 2).

Table 1: List of bread wheat cultivars released by the Wheat Research Department, ARC, Giza, Egypt during the last 50 years

| No | Genotype Name | Origin | Pedigree |
|---|---|---|---|
| 1 | Giza 139 | 1947 | Hindi 90/ Kenya B256 |
| 2 | Giza 144 | 1958 | Rgent/2* Giza 139 |
| 3 | Giza 150 | 1960 | Mida-Cadet/2* Giza 139 |
| 4 | Giza 155 | 1968 | Regent/2* Giza 139//Mida-Cadit /2* Hindi 62 |
| 5 | Giza 157 | 1977 | Giza 155//Pit 62 /LR 64/3/Tzpp/Knott |
| 6 | Sakha 8 | 1977 | Indus/Norteno “s” |
| 7 | Sakha 61 | 1980 | Inia/RL 4220//7C/Yr “s” |
| 8 | Sakha 69 | 1980 | Inia/RL 4220//7C/Yr “s” |
| 9 | Giza 160 | 1982 | Chenab70/Giza 155 |
| 10 | Sakha 92 | 1987 | Napo 63/Inia 66//Wern “s” |
| 11 | Giza 162 | 1987 | Vcm//Cno67/7C/3/Kal/BbCM8399-D-4M-3Y-1M-1Y-1M-0Y |
| 12 | Giza 163 | 1987 | *T.aestivum*/Bon//Cno/7C CM33009-F-15M-4Y-2M-1M-1M-1Y-0M |
| 13 | Giza 164 | 1987 | Kvz/Buha “s”//Kal/Bb CM33027-F-15M-500y-0M |
| 14 | Gemmieza 1 | 1991 | Maya 74/On//1160.147/3/Bb/Gall/4/Chat”s” CM58924-1GM-OGM |
| 15 | Sahel 1 | 1994 | N.S.732/Pim//Vee”s” |
| 16 | Giza 167 | 1995 | Au/Up301//Gll/Sx/Pew”s”/4/Mai”s”/May”s”//Pew”s”CM67245-C-1M-2Y-1M-7Y-1M-0M |
| 17 | Sids 1 | 1996 | HD 2172/Pavon”s”//1158.57/Maya 74 “s” SD46-4SD46-4Sd-2SD-1SD-0SD |
| 18 | Sids 2 | 1996 | HD 2206/Hork”s”/3/Napo63/Inia66//Wern “s” SD635-4SD-1SD-1SD-0SD |
| 19 | Sids 3 | 1996 | Sakha 69/Giza155 SD723-7SD-1SD-0SD |
| 20 | Sids 4 | 1994 | Maya "s"/Mon "S"/CM H74.A592/3/Giza 157*2 |
| 21 | Sids 5 | 1994 | Maya "s"/Mon "S"/CM H74.A592/3/Giza 157*2 SD10001-7sd-4SD-2SD-0SD |
| 22 | Sids 6 | 1994 | Maya "s"/Mon "S"/CM H74.A592/3/Sakha 8*2 SD10002-4SD-3SD-1SD-0SD |
| 23 | Sids 7 | 1994 | Maya "s"/Mon "S"/CM H74.A592/3/Sakha 8*2 SD10002-8SD-1SD-1SD-0SD |
| 24 | Sids 8 | 1994 | Maya "s"/Mon "S"/CM H74.A592/3/Sakha 8*2 SD10002-14SD-3SD-1SD-0SD |
| 25 | Sids 9 | 1994 | Maya "s"/Mon "S"/4//CM H72.428/MRC//jip/3/CMH74A582/5/Giza157*2SD10003 |
| 26 | Gemmieza 3 | 1997 | Bb/7C*2//Y50/Kal*3//Sakha8/4/Prv/WW/5/3/Bg”s”//OnCGM.4024-1GM13 GM2GM-0GM |
| 27 | Gemmieza 5 | 1998 | Vee”s”/SWM 6525 CGM.4017-1GM-6 GM-3 GM-0GM |
| 28 | Gemmiza 7 | 2000 | CMH74 A. 630/5x//Seri 82/3/Agent CGM.4611-2GM-3GM-1GM-0GM |
| 29 | Gemmiza 9 | 2000 | Ald”s”/Huac”\s”//CMH74A.630/5x CGM.4583-5GM-1GM-0GM |
| 30 | Giza 168 | 1999 | Mil/Buc//Seri |
| 31 | Sakha 93 | 1999 | Sakha 92/TR 810328 |
| 32 | Sakha 94 | 2004 | Opata/Rayon//Kauz |
| 33 | Gemmieza 10 | 2004 | Maya 74 “s”/On//1160-147/3/Bb/4/Chat”s”/5/Ctow |

Table 2: Description of seventeen wheat microsatellites, their position, size range of alleles, number of alleles and gene diversity

| Locus | Position | Size range of alleles (bp) | | Number of alleles | Gene diversity |
|---|---|---|---|---|---|
| | | Min Allele | Max Allele | | |
| *Xgwm3* | 3D | 77 | 84 | 3 | 0.561 |
| *Xgwm18* | 1B | 186 | 192 | 4 | 0.578 |
| *Xgwm 46* | 7B | 147 | 187 | 7 | 0.765 |
| *Xgwm 95* | 2A | 109 | 131 | 6 | 0.521 |
| *Xgwm155* | 3A | 129 | 147 | 4 | 0.501 |
| *Xgwm160* | 4A | 177 | 189 | 6 | 0.756 |
| *Xgwm165* | 4A | 187 | 202 | 7 | 0.786 |
| *Xgwm186* | 5A | 122 | 134 | 6 | 0.629 |
| *Xgwm190* | 5D | 204 | 212 | 5 | 0.659 |
| *Xgwm261* | 2D | 165 | 192 | 3 | 0.538 |
| *Xgwm389* | 3B | 119 | 136 | 5 | 0.685 |
| *Xgwm408* | 5B | 178 | 194 | 5 | 0.657 |
| *Xgwm437* | 7D | 91 | 130 | 11 | 0.841 |
| *Xgwm458* | 1D | 109 | 122 | 5 | 0.659 |
| *Xgwm513* | 4B | 141 | 150 | 6 | 0.780 |
| *Xgwm631* | 7A | 190 | 200 | 3 | 0.339 |
| *Xtaglgap* | 1B | 212 | 280 | 9 | 0.845 |
| Total | | | | 95 | ------ |
| Mean | | | | 5.58 | 0.668 |

In total, 95 microsatellite alleles of 17 microsatellite markers for 33 genotypes in Egyptian bread wheat were detected, and the number of alleles per marker was *Xgwm*437-7DL (11) > *Taglgap*-1BS (9) > *Xgwm*165-4A (7) = *Xgwm*46-7B(c) (7) > *Xgwm*513-4BL (6) = *Xgwm*186-5AL (6) = *Xgwm*160-4AL (6) = *Xgwm*95-2AS (6) > *Xgwm*458-1D(c) (5) = *Xgwm*408-5BL (5) = *Xgwm*389-3BS (5) = *Xgwm*190-5DS (5) > *Xgwm*155-3AL (4) = *Xgwm*18-1BS (4) > *Xgwm*631-7AS (3) = *Xgwm*3-3DL (3) (Table 2). The number of alleles per locus ranged from 3 to 11 with an average number of 5.58 alleles per locus (Table 3). The largest number of alleles per locus occurred in the B genome with 36 compared to 32 and 27 for genomes A and D, respectively (Table 3). The lowest and the largest number of alleles per locus among the homeologous groups was observed in homeologous group 2 and 7 with 9 and 21, respectively (Table 3).

*Genetic diversity:* Gene diversity for 17 microsatellite loci varied from 0.339 for *Xgwm631-7AS* to 0.845 for *Taglgap-1BS* with an average of 0.668. Gene diversity for the three genomes A, B and D was 0.589, 0.654 and 0.606, respectively (Table 3).

Table 3: Genetic diversity in different genomes and chromosomes across the 17 microsatellite loci in the 33 wheat genotypes

| | Number of loci checked | Number of alleles | | Gene diversity |
|---|---|---|---|---|
| | | Total | Average | Mean |
| Genomes | | | | |
| A | 6 | 32 | 5.33 | 0.589 |
| B | 6 | 36 | 6.40 | 0.654 |
| D | 5 | 27 | 5.50 | 0.606 |
| Chromosomes | | | | |
| 1 | 3 | 18 | 6.00 | 0.694 |
| 2 | **2** | 9 | 4.50 | 0.529 |
| 3 | 3 | 12 | 4.00 | 0.653 |
| **4** | 3 | 19 | 6.33 | 0.792 |
| **5** | **3** | 16 | 5.33 | 0.648 |
| **7** | 3 | 21 | 7.00 | 0.648 |

The correlation coefficient between gene diversity and the number of alleles was high, $r = 0.578$ ($P < 0.01$).

*Analysis of relationship:* UPGMA cluster analysis of SSR genetic similarity (gs) matrix resulted in the dendrogram presented in figure 1. Five groups can be distinguished by truncating the dendrogram at gs value of 0.48. The major group (A) consists of 12 genotypes and includes the Egyptian varieties Sakha 8, Sakha 69, Gemmiza 3, Sakha 61, Gemmiza 5, Gemmiza 7, Giza 168, Gemmiza 9, Sids 1, Sids 2, Sids 3 and Sids 4. Another group (Group B) contains ten Egyptian wheat genotypes and includes Giza 163, Sahel 1, Sahel 6, Giza 164, Sids 5, Sids 6, Sids 7, Suds 8, Sids 9 and Sakha 94. Group C consists of seven genotypes Giza 139, Giza 144, Giza 167, Gemmiza 10, Giza 157, Giza 160 and Giza 162. Group D includes the three Egyptian wheat genotypes Sakha 92, Sakha 93 and Giza 155. Interestingly, the wheat genotypes Gemmiza 1 is not clustered to any other varieties/cultivar and form a separate group (group E).

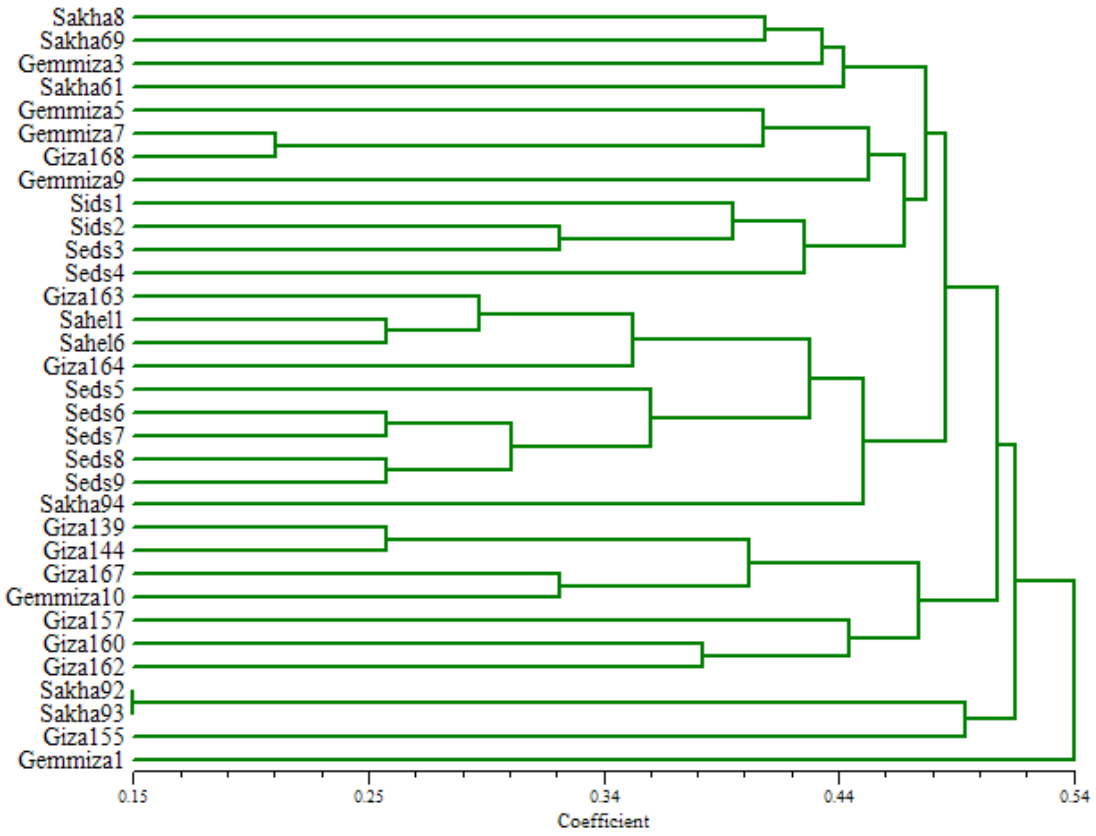


Fig. 1: UPGMA cluster analysis-based dendrogram depicting genetic relationships among 33 Egyptian bread wheat genotypes and based on data of 17 SSRs primer pairs

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# Characterization and exploitation of barley-genetic resources for resistance to frost

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Breeding of high yielding elite barley cultivars has narrowed the genetic diversity in cultivated barley. This gene erosion hampers the selection of new cultivars with features adapted to changing climate conditions. Improving frost resistance in barley as a deciding component of winter hardiness is an important breeding aim as winter barley is very susceptible to frost injury in comparison to winter rye or winter wheat. Improved frost resistance could contribute to better overwintering in strong winters as well as to expansion of cultivation areas to more continental climates. Progress of improving cold hardiness by conventional breeding in winter cereals has been slow during the last decades, possibly due to limited genetic variation within the pool of barley cultivars. The gene pool of the genus *Hordeum* could possibly provide a valuable resource for resistance to biotic and abiotic stresses like frost.

## Material and Methods

Seeds from the wild barleys of the secondary and tertiary gene pools (Table 1) were sown and plants were vegetatively propagated. Leaves were freshly cut and plants were cultivated for about 4 weeks at 18°C/14°C 10 h day/14 h night till new leaves were grown. Fully developed leaves were used for all measurements. Membrane stability was determined by electrolyte leakage (Chandrasekar 2000) before hardening and after a hardening period of 5 weeks at 4°C/2°C 12 h day/12 h night. Samples for the determination of proline and soluble sugars were taken weekly during the hardening period. Proline accumulation was determined according to Bates et al. (1973), total content of soluble sugars was estimated by the anthrone reagent method basing on Yemm and Willis (1954). Experiments were conducted in 2008 and repeated in 2010.

Crosses between the *H. vulgare* cultivars 'Igri' and 'Nikel' and the wild barley accessions (Table 1) were performed to transfer freezing resistance into the cultivated barley. About 12 days after pollination embryo rescue was used to regenerate plants. Genomic in situ hybridisation (GISH) was performed to analyse the hybrid character of the progenies.

Table 1: Wild barley accessions provided by IPK-Genebank

| Scientific name | IPK Accession number | Scientific name | IPK Accession number |
|---|---|---|---|
| *H. bulbosum* L. subsp. *bulbosum* | GRA 945 | *H. bogdanii* Wilensky | GRA 969 |
| *H. bulbosum* L. subsp. *bulbosum* | GRA1029 | *H. chilense* Roem. et Schult. | GRA 972 |
| *H. bulbosum* L. subsp. *bulbosum* | GRA 949 | *H. violaceum* Boiss. et Huet. | GRA 615 |
| *H. bulbosum* L. subsp. *bulbosum* | GRA655 | *H. brevisubulatum* (Trin.) Link | GRA 894 |
| | | *H. murinum* L. | GRA 1097 |

**Results**

<u>*Freezing resistance of wild barley accessions:*</u> The wild barleys *H. violaceum, H. chilense* and *H. bogdanii* showed the highest resistance to frost according to their membrane stability after hardening in repeated experiments (Fig. 1). High membrane stability is an indirect measure for frost resistance of the leaf tissue. *H. murinum* was characterized by an exceptionally high accumulation of proline and soluble sugars during hardening and a good frost resistance. All species mentioned above are members of the tertiary barley gene pool, which therefore turns out to be a valuable source of resistance characters. Concerning *H. bulbosum,* the sole member of the secondary gene pool, the accessions evaluated showed lower membrane stability when exposed to temperatures between -5°C and -15°C compared to the barley species of the tertiary gene pool investigated. Among the *H. bulbosum* accessions, GRA 655 that originated from the Tien Shan mountains (Himalaya) was characterised by a relatively high accumulation of proline and soluble sugars (Fig. 1) and exhibited good resistance to frost.

<u>*Interspecific hybridisation to transfer frost tolerance from H. bulbosum (GRA 655) from Tien Shan to H. vulgare:*</u> Interspecific crosses of *H. vulgare* and these wild accessions have been performed to transfer resistance to frost into cultivated barley. No seeds or embryos were obtained when *H. bogdanii* and *H. chilense* were used as pollinators. Embryos were regenerated in *H. vulgare* × *H. jubatum* and *H. vulgare* × *H. murinum* crosses, only.

However, the embryos did not survive *in vitro* after applying embryo rescue. Results confirm that the tertiary gene pool is not useable till now. But 54 plants resulted from *H. vulgare* × *H bulbosum* crosses. Out of these, 18 plants were derived from GRA 655 (Table 2). The hybrid character of the F1 has been verified by genomic in situ hybridisation (GISH Fig. 2) and the respective offspring will be investigated regarding its response to frost stress. Respective lines may be a useful source to improve frost tolerance in cultivated barley.

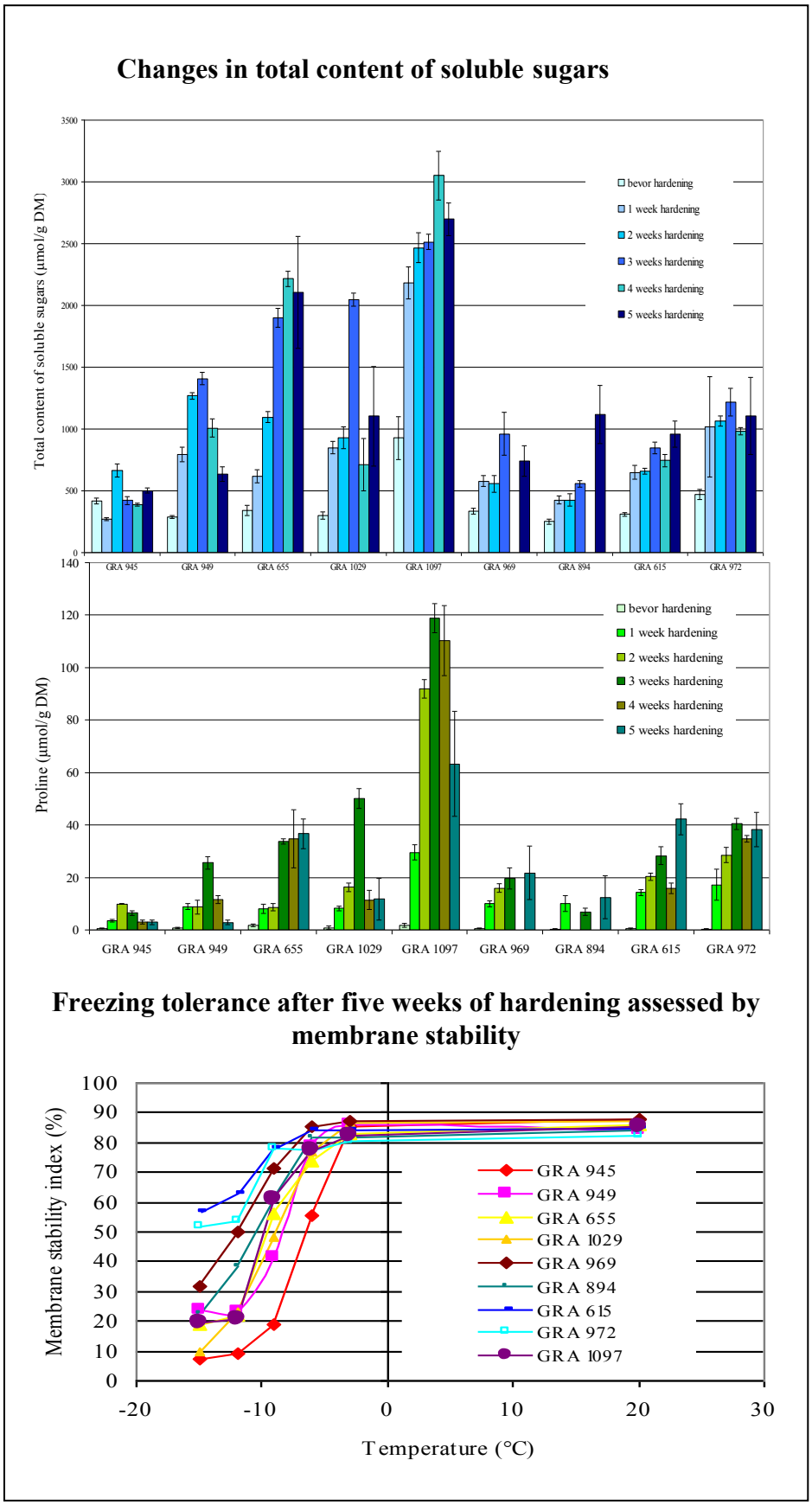


Fig. 1: Total contents of soluble sugars, contents of free proline over the hardening period and freezing tolerance after hardening of nine wild barley accessions

Table 2. Yields of hybrid plants obtained from *H. vulgare* × *H. bulbosum* crosses

| Cross | | Florets | Embryos cultures | | Hybrid Plants | |
|---|---|---|---|---|---|---|
| ♀ | ♂ | pollinated | Number | (%) | Number | (%) |
| *H. v.* cv. 'Igri' | GRA 655 | 331 | 29 | 8.8 | 17 | 5.1 |
| *H. v.* cv. 'Nikel' | GRA 655 | 54 | 1 | 1.9 | 1 | 1.9 |

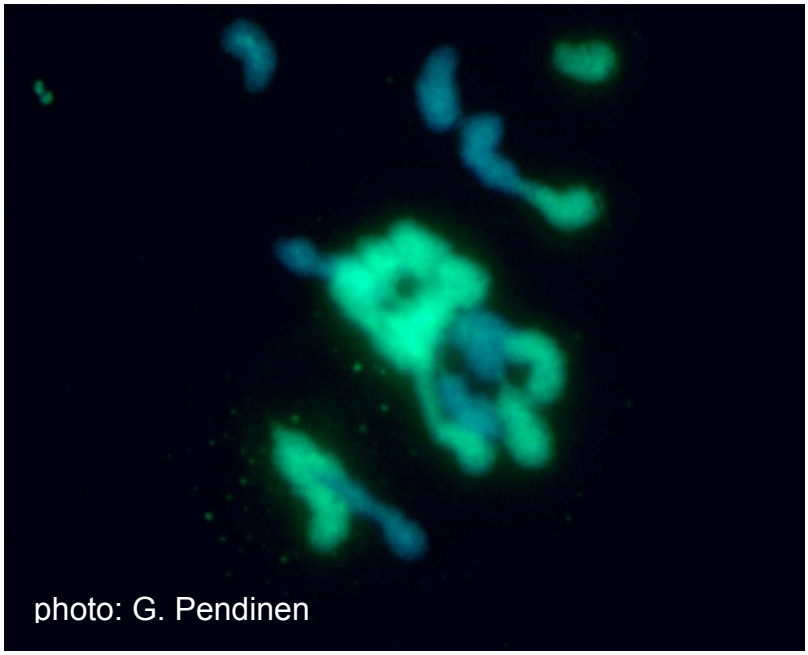


Fig. 2: Interspecific *H. vugare* × *H. bulbosum* hybrid. A meiotic metaphase cell showing 14 Chromosomes of *H. bulbosum* (green) and 7 chromosomes of *H. vulgare* (blue) (GISH)

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# Effect of the grain softness locus *Ha-Sp* introgressed from *Aegilops speltoides* Tausch. on the phenotype of endosperm of soft-grain and hard-grain bread wheat cultivars

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The 'Arsenal' collection of bread wheat introgression lines with a genetic material from *Ae. speltoides* Tausch. was developed by I.F. Lapochkina with co-workers on the genetic background of cv. 'Rodina' (Lapochkina et al. 2003). The purpose of our work was search for the new genetic factors influencing milling parameters of grain in the line of this collection and study of its interaction with the gene *Ha* of bread wheat determining the same trait. The winter introgressive line 84/98$^{w}$, hard-grain cultivars Saratovskaya 29 (S29) and Diamant 2 (Dm2), soft-grain cultivar Chinese Spring (CS) and monosomic lines of S29 and Dm2 were used as the genetic material. The milling parameters - flour particles size and vitreousness - were determined according the methods and techniques accepted in Russia (Anonymous, 1988).

The initial cultivar Rodina is characterized with a high vitreousness of grain and large flour particles. The locus *Ha-Sp* was discovered in the line 84/98$^{w}$ which had a low vitreousness and small particle size comparing to cv. Rodina (Pshenichnikova et al., 2010). Using monosomic analysis *Ha-Sp* locus was localized in 5A chromosome of cv. Rodina. The recombinant forms with a vitreousness ranging from 50 to 90% were obtained among the hybrids of the line 84/98$^{w}$ with the 5A monosomic lines of hard-grain cvs. S29 and Dm2. Differences in milling parameters were found between $F_3$ plants grouped according their vitreousness (Table 1). The selected $F_3$ progenies from homozygous $F_1$ genotypes (*Ha-Sp / Ha-Sp*) had all semi-vitreous grains like the line 84/98$^{w}$ and had significant differences from corresponding recipients (columns A). The $F_3$ progenies from heterozygous $F_1$ genotypes (*Ha-Sp / ha-sp*) are presented in columns B and C. Plants from the columns B could be heterozygous for *Ha-Sp* gene, because they have intermediate flour particle size and vitreousness. Plants from the columns C have a high vitreousness and large flour particles as they did not inherit *Ha-Sp* gene. Probably, they carry minor genes modifying flour particle size and vitreousness because they showed the differences from the corresponding recipients.

Interaction of introgressed locus *Ha-Sp* with *Ha* locus on 5D chromosome manifested in the genotypes with a very soft endosperm texture in hybrids $F_2$ CS x 84/98$^{w}$. The recombinants with vitreousness ranging from 25% to 95% were found among them. A continuous variability for vitreousness and flour particles size was observed in the population (Figures 1 and 2). Plants with very low vitreousness and small flour particles size inherited both the gene *Ha-Sp* from line 84/98$^{w}$ and the gene *Ha* from CS. Plants with high vitreousness and flour particles size inherited the recessive alleles of these genes (Figure 3). Positive correlation of vitreousness and flour particles size was detected. At the same time, variability for vitreousness inside the groups with similar flour particles size was found. This again may be due to the minor genes influencing grain texture. Thus, the new gene *Ha-Sp* can be use for expansion of genetic variability of wheat for milling parameters.

Table 1: Grain texture characteristics in $F_3$ progenies of 5A monosomic populations

| Milling properties | S29 | $F_3$ mono 5AS29 × 84/98$^W$ | | | Dm2 | $F_3$ mono 5ADm2 × 84/98$^W$ | | | 84/98$^W$ |
|---|---|---|---|---|---|---|---|---|---|
| | | A | B | C | | A | B | C | |
| Total vitreousness, % | 99 | 51*** | 77* | 84*** | 84 | 52*** | 77* | 96** | 50 |
| Mean diameter of flour particles, µk | 21,5 | 11,4** | 16,1 | 18,1* | 16,5 | 10,8* | 15,7 | 19,8 | 12,0 |
| Specific particles surface, sm$^2$/g | 1874 | 3524*** | 2496 | 2225* | 2433 | 3742** | 2570 | 2036 | 3367 |

* P<0,05; ** P<0,01; *** P<0,001, comparing to the respective recipient
A – progeny of *Ha-Sp/Ha-Sp* (or *Ha-Sp/0*) genotypes; B, C – progeny of *Ha-Sp/ha-sp* genotypes

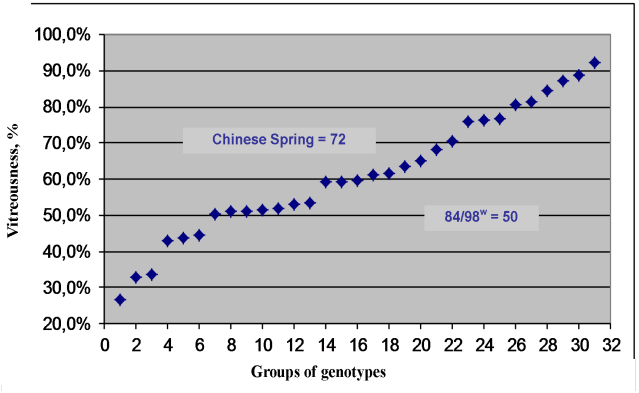


Fig. 1: Variability of vitreousness among the hybrids $F_2$ Chinese Spring x 84/98$^W$

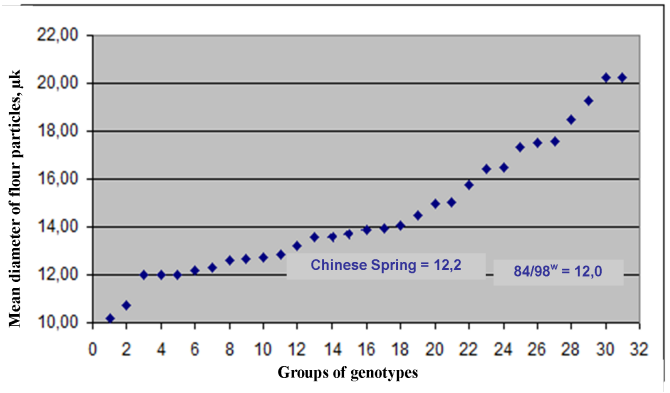


Fig. 2: Variability of flour particle size among the hybrids $F_2$ Chinese Spring x 84/98$^W$

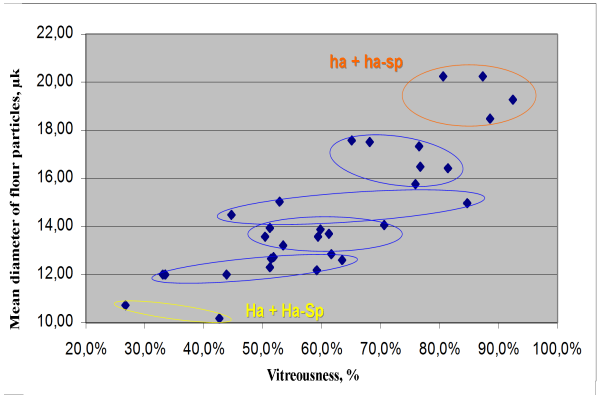


Fig. 3: Range for vitreousness inside the groups of hybrid plants with similar flour particle size in $F_2$ Chinese Spring x 84/98$^W$

**Acknowledgments**

We are grateful to Dr. Andreas Börner for growing $F_2$ CS x 84/98$^w$ population in greenhouse of IPK. Participation in the Conference was support by Russian Foundation for Basic Research.

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## The change of the structure of *atp1* mitochondrial gene are connected with presence of additional heterochromatin in 2R chromosomes in *Secale vavilovii* Grossh. lines

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Investigated material were lines of *Secale vavilovii* Grossh. (52, 109, 121, 225), characterized by mosaic colored grains and anthers as well as reduced pentozan content and additional heterochromatin (JNK1) on 2R chromosomes. Plant with heterochromatin bands on both 2R chromosomes grew very faintly and died quickly. In aim of examining the reason of this phenomenon, from lines and from standard (population *S. vavilovii* Grossh.) DNA was isolated and five mitochondrial genes (*atp1, atp6, atp9, pol - r, orf -25*) were chosen for digestion with four restriction enzymes: *Eco*RI, *Bam*HI, *Hind*III and *Pst*I (MBI Fermentas).

Restriction patterns were identical for all genes except *atp1,* which in lines with additional heterochromatin showed presence of restriction site, reporting changes in this gene. Sequence analysis of this gene confirmed these changes. *Atp1, atp6* and *atp9* genes are connected with mitochondrial ATPase. It was allege, that the change in *atp1* gene structure could influence ATPase activity in these lines.

## Methods

DNA isolation from etiolated rye coleoptiles was carried out using the Genomic DNA Purification Kit (Fermentas) by means of methods recommended by the manufacturter. The quantity and quality of isolated DNA were determined spectrophotometrically.

The amplification was carried on in a Personal Cycler thermocycler v. 3.15 (Biometra). The volume of the reactive solution was 50 μl. PCR products were separated electrophoretically in a 0,8% agarose gel in 1xTAE buffer, and then purified with Amicon Microcon-PCR firmy Millipore (USA) and digested with four restriction enzymes *Eco*RI, *Bam*HI, *Hind*III and *Pst*I (MBI Fermentas). The reactive solution was composed of: 1 x digestion buffer, 20U endonuclease, 8 μl PCR product and water. The digestion was carried out at $37^0$C for 4 hours. The inactivation of enzymes was performer through the heating of the samples to $65^0$C (*Hind*III and *EcoR*I) or $80^0$C (*Pst*I and *Bam*HI) for 20 minutes. The PCR-RFLP reaction products were separated on a 3% agarose gel and sized using the MassRuler™ DNA Ladder Mix (Fermentas).

Sequencing reactions were performed with BigDye® Terminator v3.1 (Applied Biosystems) in capillary 3730xl DNA analyzer (Applied Biosystems) (Laboratory of DNA Sequencing and Oligonucleotides Synthesis Laboratory, Institute of Biochemistry and Biophysic Polish Academy of Sciences, Warsaw, Poland). The computer analysis of sequences was executed in MEGA version 4 (Tamura, Dudley, Nei, and Kumar 2007).

## Results and discussion

Plant mitochondrial genome has many specific features (diversity in size, complex structure, various inter-molecular and intra-molecular recombinations). Genetic information contained in mtDNA makes it possible to synthetise only a small part of proteins found in mitochondria. One of such genes is *atp1*, connected with mitochondrial ATPase (ATP synthetase), identified in the internal mitochondrial membrane.

The *atp1* gene is responsible for the synthesis of the protein subunit $\alpha$ $F_1$ (catalytic segment) of ATP-ase. The $F_1$ is a structure of spherical shape protruding out of the internal membrane to the mitochondrial matrix. The role of subunit $\alpha$ is to bind adenylic nucleotides and, thanks to that property, to control the rate of ATP synthesis. The gene coding the subunit $\alpha$ $F_1$ of ATP-ase is plant mtDNA- and cpDNA specific, it is lacking in the mtDNA of animals and fungi.

The restriction analysis of the *atp9, pol-r* and *orf25* genes did not reveal differences among studied lines and *S. vavilovii* Grossh. as a standard (Tab. 1).

The analysis of *atp1* indicated polymorphism between studied lines (Tab. 1). Polymorphism was observed in case of digestion *atp1* with *Bam*HI in two of analyzed *Secale vavilovii* lines - 52 and 225. In this situation the enzyme recognized one restriction site giving the fragments of length 860bp and 740bp (Tab. 1). This shows differences within *atp1* gene in lines with additional heterochromatin and reduced pentozan content. Sequence analysis of this gene confirmed these changes (Fig. 1).

Differences in presence of restriction site within *atp1* among analyzed *S. vavilovii* lines and the standard were detected (after *Eco*RI digestion) and also within *atp6* (after *Bam*HI digestion). Nevertheless in these cases all studied rye lines differed from standard with lack (*atp1*/*Eco*RI) or occurrence (*atp6*/*Bam*HI) restriction site (Tab. 1).

Table 1: Restriction fragment length polymorphism of analyzed genes in examined rye lines

| Gene | Enzyme | Length [bp] | *Secale vavilovii* Grossh. lines | | | | *Secale vavilovii* Grossh. |
|---|---|---|---|---|---|---|---|
| | | | 52 | 109 | 121 | 225 | |
| *atp1* | *Eco*RI | 1600 | + | + | + | + | - |
| | | 1550 | - | - | - | - | + |
| | | 50 | - | - | - | - | + |
| | ***Bam*HI** | **1600** | - | + | + | - | + |
| | | **860** | + | - | - | + | - |
| | | **740** | + | - | - | + | - |
| | *Hind*III | 1600 | + | + | + | + | + |
| | *Pst*I | 1600 | + | + | + | + | + |
| *atp6* | *Eco*RI | 1220 | + | + | + | + | + |
| | *Bam*HI | 1220 | - | - | - | - | + |
| | | 750 | + | + | + | + | - |
| | | 470 | + | + | + | + | - |
| | *Hind*III | 770 | + | + | + | + | + |
| | | 450 | + | + | + | + | + |
| | *Pst*I | 1220 | + | + | + | + | + |
| *atp9* | *Eco*RI | 400 | + | + | + | + | + |
| | *Bam*HI | 400 | + | + | + | + | + |
| | *Hind*III | 400 | + | + | + | + | + |
| | *Pst*I | 400 | + | + | + | + | + |
| *pol-r* | *Eco*RI | 950 | + | + | + | + | + |
| | *Bam*HI | 950 | + | + | + | + | + |
| | *Hind*III | 950 | + | + | + | + | + |
| | *Pst*I | 950 | + | + | + | + | + |
| *orf25* | *Eco*RI | 680 | + | + | + | + | + |
| | *Bam*HI | 680 | + | + | + | + | + |
| | *Hind*III | 440 | + | + | + | + | + |
| | | 240 | + | + | + | + | + |
| | *Pst*I | 680 | + | + | + | + | + |

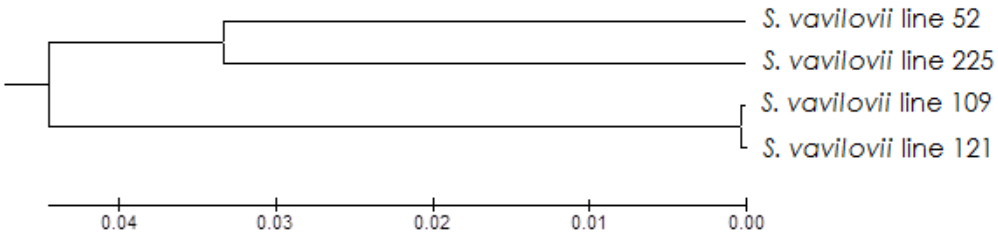


Fig. 1: Relationships of 4 lines

The relationships of 4 lines was inferred using the UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length = 0.12272132 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the distances used to infer the tree. The distances were computed using the Maximum Composite Likelihood method (Tamura, Nei & Kumar, 2004) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1248 positions in the final dataset. Analyses were conducted in MEGA4 (Tamura, Dudley, Nei & Kumar, 2007).

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## Mitochondrial and chloroplast DNA variability during the development of euploid and aneuploid lines produced using barley-wheat hybrids *H. marinum* subsp. *gussoneanum* Hudson x *T. aestivum* L.


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Crossing of different species are carried out to produce experimental materials and new sources for cultivar plants improvement. Alloplasmic lines (or nuclear-cytoplasmic hybrids) in which alien plasmons are combined with given nuclear genomes through repeated substitution backcrosses, are valuable experimental tools for studying interaction of nuclear genomes and plasmons during the development of new genotypes derived from wide hybrids. Despite the maternal mode of cytoplasmic DNA inheritance in cereals, there are some facts that biparental transmission may occur in wide crosses (Soliman et al. 1987, Tsukamoto et al. 2000, Aksyonova 2005). As a result, plasmon heteroplasmy, which is defined by the presence of different DNA molecules within individuals (Woloszynska, 2010), has been widely recognized in the progenies of wide hybrids (Hattori et al. 2002; Aksyonova 2005). Although

the nuclear genome has a predominant role on the plasmon inheritance and function, nuclear-cytoplasmic interactions are also important, especially their influence on plant fertility.

The aim of this work was to study the variability of some regions of mitochondrial (mt) and chloroplast (cp) DNA in these lines depending on their nuclear genome constitution and degree of fertility.

## Materials and Methods

Euploid (2n=42) and aneuploid (2n=42+2t) alloplasmic wheat lines obtained by backcrossing and self-pollination of barley-wheat hybrids *H. marinum* subsp. *gussoneanum* x *T. aestivum* were studied. Euplasmic line contained wheat plasmon and the same type of wheat-barley chromosome substitution $7H^mL(7D)$ as one of the alloplasmic lines. Euplasmic line was produced according early developed strategy (Trubacheeva et al. 2009). Chromosome number and wheat-barley substitution was identified by GISH and C-banding. Characteristics of lines are shown in Table 1.

The mitochondrial (mt) 18S/5S repeat was amplified by PCR using specific primer sets published in Trubacheeva et al. (Trubacheeva et al. 2005). Chloroplast regions *ndhH, rpoB, psaA, infA* was studied by PCR-RFLP using primer sets developed from http://gobase.bcm.umontreal.ca/

## Results and Discussion

Table 2 shows genome composition of lines studied. Lines L-32 and L-36 were euploid (2n=42), L-32 contained wheat-barley chromosome substitution $7H^mL(7D)$ and L-36 wheat chromosomes only. Aneuploid line (2n=44) L-37 had two additional barley $7H^mL$ chromosomes. It seems that seven group chromosomes of barley *H. marinum* subsp. *gussoneanum* can compensate the absence of 7D chromosomes of common wheat.

Table 1. Description of the lines used in this study

| Lines (generation) | Origin | Cytoplasm donor | Fertility |
|---|---|---|---|
| L-37 ($BC_1F_{8,}F_9$) | (*H.mar* xPyr)Amph x Pyr | *H.marinum* (alloplasmic) | fertile |
| L-32 ($BC_3F_8$, $F_{10}$) | (*H.mar* x Pyr)x Pyr x Nov$^{(2)}$ | *H.marinum* (alloplasmic) | fertile |
| | | | partly fertile |
| | | | sterile |
| L-36($BC_4F_7$,$F_9$) | (*H.mar* x Pyr)x Pyr x Nov$^{(3)}$ | *H.marinum* (alloplasmic) | fertile |
| JI-411 | (monosomic 7D x L-37) | *T. aestivum* (euplasmic) | fertile<br>partly fertile |

*H.mar* - *H. marinum* subsp. *gussoneanum*; Pyr – wheat cultivar Pyrotrix 28; Nov – wheat cultivar Novosibirskaya 67.

Barley and wheat copies, i.e. heteroplasmy of 18S/5S mitochondrial repeat were revealed in line L-37, fertile and partly fertile plants of line L-32 (Table 2). Sterile plants of line L-32 contained barley mtDNA copies only (homoplasmy of barley type). Fertile line L-36 contained wheat copies of 18S/5S repeat.

Table 2. Nuclear and cytoplasmic genome composition in lines studied.

| Lines | Genome composition | Plant fertility | 18S/5S mtDNA | cpDNA (*rpoB, infA, psaA*) | cpDNA (*ndhH)* |
|---|---|---|---|---|---|
| L-37 | 2n=44=42w+2(7H$^{m}$L) | fertile | barley+wheat | barley | barley+wheat |
| L-32 | 2n=42=40w+7H$^{m}$L(7D) | fertile | barley+wheat | barley | barley+wheat |
| | | partly fertile | barley+wheat | barley | barley+wheat |
| | | sterile | barley | barley | barley |
| L-36 | 2n=42w | fertile | wheat | wheat | wheat |
| L-411 | 2n=42=40w+2(7H$^{m}$L) | fertile<br>partly fertile | wheat | wheat | wheat |

w – wheat chromosome ; 7H$^{m}$ – barley *H. marinum* chromosome; L – long arm; t – telocentric chromosome

Using *infA, rpoB, psaA* polymorphic marker chloroplast loci, barley copies were detected in alloplasmic lines L-37 and L-32 (Table 2). But at the *ndhH* region heteroplasmy (barley+wheat copies) was detected in these lines.

It appears that cytoplasmic DNA transmission from both parents occurred in barley-wheat hybrids $F_1$ and then mt- and cp- DNA transmitted through backcrossed and self-pollinated generations depending the nuclear genome structure. Heteroplasmic and barley homoplasmic copies were associated with the presence of barley chromosomes in nuclear genomes (L-32 and L-37); homoplasmic wheat copies with the presence of wheat chromosomes only (L-36). The reason of wheat homoplasmy in L-36 may be a complete elimination of barley cytoplasmic DNA or its presence in a small substoichiometric amount which were not detected by methods used. Different types of nuclear-cytoplasmic interactions also influenced on the degree of plant fertility. Sterile plants in line L-32 possessed barley homoplasmic mt-copies, which were not detected in partly fertile and fertile plants in lines L-32 and lines L-37. It seems that heteroplasmic or wheat homoplasmic copies are necessary for fertility restoration in alloplasmic lines. As for line L-32, different degrees of fertility also may be caused by different nuclear genome recombination between parental wheat cultivars Pyrotrix 28 and Novosibirskaya 67 used for backcrossing. The physiological significance of heteroplasmic state remains elusive but its maintenance during self-pollinated generations is an evidence that heteroplasmy detection wasn't accidental in alloplasmic lines.

The study of mt- and cp- loci in euplasmic line L-411, carrying the same type of wheat-barley chromosome substitution as in alloplasmic line L-32, showed that it contained wheat ctDNA copies only. These data confirmed that DNA copies amplified using our primer sets in alloplasmic lines derived from cytoplasmic genomes, not from barley chromosomes in nuclear genome.

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