Detection of QTLs for Important Agronomical Traits in Hexaploid Wheat Using Association Analysis

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Abstract

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One of the main wheat breeder's goals is determining specific genomic regions which control important agronomical traits. Association analysis is a new strategy with high resolution in plant molecular breeding that could be used to improve the efficiency of marker assisted selection (MAS) for finding important QTLs (quantitative trait loci) or genes. A set of 96 diverse wheat genotypes was phenotypically measured during three growing seasons (2006/07, 2007/08, 2008/09). Microsatellite markers located near important QTLs were carefully chosen in accordance with existing literature data to validate marker trait associations (MTA). Genomic DNA was extracted using the CTAB method and PCR products were separated by capillary electrophoresis. The population structure was assigned based on molecular data in Structure v. 2.0 software, while association analysis was done by the Tassel program using the Q matrix. Nine significant associations were stable in all years investigated and eight MTA were detected to be significant in two growing seasons. Microsatellite markers which showed significant associations and stability in different seasons can be useful and suitable for marker assisted selection (MAS) in Serbian wheat breeding programs.

Keywords: agronomical traits; microsatellite markers; QTLs; Tassel; *Triticum aestivum*

One of the major challenges for breeders of economically important crop species, including bread wheat, is the improvement of yield and yield components. Yield is a complex trait controlled by a large number of major and minor QTLs and genes (Kumar *et al*. 2007). So far, a lot has been achieved in finding more efficient ways of selecting desired genotypes to develop high-yielding varieties by breeders worldwide. The implementation of new sophisticated molecular methods into existing conventional breeding has enabled considerable progress in reaching these goals. In the last decade, many studies have used specifically designed populations to analyse and find QTLs responsible for gene expression of yield components. A new approach for QTL detection, called association analysis, proved to have higher resolution than QTL mapping. The first association approach was done on the human genome to detect important alleles related with human diseases. Association analysis has expanded its uses for finding important agronomic QTLs/genes in many crop species in the last few years. Also, association studies have been successfully applied for detection of QTLs for milling quality, disease resistance, kernel size and high molecular weight glutenin, and seed longevity in hexaploid wheat (Breseghello & Sorrells 2006; Ravel *et al*. 2006; Rehman Arif *et al*. 2012). The novel studies

include genetic dissection of its whole genome, which significantly increases the fine mapping of the most important agronomic QTLs as shown in the study of Neumann *et al.* (2011).

The main goal of this study was to detect stable QTLs and marker trait associations in the investigated breeding material. Candidate markers located near important QTLs could facilitate and speed up the selection process of high-yielding varieties through marker assisted selection (MAS).

MATERIAL AND METHODS

Phenotyping. The population analysed consisted of 96 hexaploid wheat varieties originating from 10 worldwide breeding centres. Based on multi-year evaluations of yield *per se*, breeding materials were clustered into 5 groups: 20 high-yielding advanced lines, 20 low-yielding advanced lines, 20 high-yielding genotypes, 20 low-yielding genotypes, and 16 commercial cultivars developed in Serbia (Table 1). The genotypes were sown in a randomized block system in field trials at the Small Grains Department of the Institute of Field and Vegetable Crops in Novi Sad. Important agronomic traits such as heading, flowering, stem height and spike length were evaluated during three growing seasons (2006/07, 2007/08, 2008/09). Heading and flowering time were estimated from the first of January to the date when 50% of the plants were in certain phases. Stem height is given as stem length excluding the length of the spike, which was measured separately.

Genotyping. The genotypes were characterized using 16 microsatellite markers closely linked to target QTLs. The SSR loci (*gwm*95, *gwm*261, *wmc*89,

Table 1. Diverse genotypes for molecular and phenotype analysis

I	Low yield genotypes from trials and origin	II	High yield genotypes from trials and origin	III	Low yield genotypes Core collection and origin	IV	High yield genotypes Core collection and origin	V	NS varieties and origin
1	NS71/02 (RS)	21	NS19/06 (RS)	41	L1 (HU)	61	NS66/92 (RS)	81	Evropa90 (RS)
2	NS3/05 (RS)	22	NS26/07 (RS)	42	Purdue/Loras (US)	62	NS79/90 (RS)	82	NSRana5 (RS)
3	NS2/06 (RS)	23	NS144/05 (RS)	43	Helios (US)	63	Mina (RS)	83	Rusija (RS)
4	NS7/06 (RS)	24	NS150/05 (RS)	44	Purdue39120 (US)	64	NS559 (RS)	84	Prima (RS)
5	NS91/05 (RS)	25	NS160/05 (RS)	45	Al-kan-tzao (CN)	65	Centurk (US)	85	Pesma (RS)
6	NS18/06 (RS)	26	NS42/07 (RS)	46	Saitama27 (JP)	66	Pobeda (RS)	86	Zlatka (RS)
7	NS31/068 (RS)	27	NS46/07 (RS)	47	AiBian (JP)	67	BCD1302/83 (MD)	87	Ljiljana (RS)
8	NS32/06 (RS)	28	NS47/07 (RS)	48	ZG987/3 (HR)	68	NS33/90 (RS)	88	Arija (RS)
9	NS39/068 (RS)	29	NS50/07 (RS)	49	Norin10 (JP)	69	Ana (HR)	89	Rapsodija (RS)
10	NS41/06 (RS)	30	NS52/07 (RS)	50	*T. compactum* (LV)	70	NS55-25 (RS)	90	Simonida (RS)
11	NS165/05 (RS)	31	NS55/07 (RS)	51	Timson (AU)	71	NS46/90 (RS)	91	Cipovka (RS)
12	NS166/05 (RS)	32	NS56/07 (RS)	52	INTRO615 (US)	72	Sofija (RS)	92	Dragana (RS)
13	NS171/05 (RS)	33	NS67/07 (RS)	53	Magnif41 (AR)	73	UC65680 (US)	93	NS40S (RS)
14	NS172/05 (RS)	34	NS75/07 (RS)	54	TibetDwarf (CN)	74	Slavija (RS)	94	Nevesinjka (RS)
15	NS174/05 (RS)	35	NS76/07 (RS)	55	ZGK238/82 (HR)	75	Sava (RS)	95	Venera (RS)
16	NS180/05 (RS)	36	NS77/07 (RS)	56	L1A/91 (RS)	76	Nizija (RS)	96	Nataša (RS)
17	NS181/05 (RS)	37	NS78/07 (RS)	57	L1/91 (RS)	77	Renesansa (RS)		
18	NS182/05 (RS)	38	NS80/07 (RS)	58	*T. spherococcum* (US)	78	TripleDirkB (AU)		
19	NS183/05 (RS)	39	NS81/07 (RS)	59	MinDwarf (AU)	79	NovaBanatka (RS)		
20	NS184/05 (RS)	40	NS84/07 (RS)	60	TomThumb (CN)	80	Rusalka (BG)		

AR – Argentina; AU – Australia; BG – Bulgaria; CN – China; JP – Japan; HR – Croatia; HU – Hungary; LV – Latvia; MD – Moldova; RS – Serbia; US – United States; NS varieties – varieties developed at Small Grains Department in Novi Sad

*wmc*420, *gwm*18, *gwm*11, *gwm*428, *psp*3200, *psp*3071, *gwm*337, *gwm*601, *gwm*539, *psp*3094, *wmc*48, *psp*3103, *wmc*31) were selected using existing data (available in GrainGenes 2.0 data base). Chosen markers were located on the ten following chromosomes: 1B, 1D, 2A, 2D, 4A, 4D, 6A, 6D, 7A,7D. Genomic DNA from young leaves (app. 10 leaves per genotype) was extracted using a modified CTAB method (Doyle & Doyle 1990). PCR reactions contained 25 ng genomic DNA, 1× buffer, 2mM $MgCl_2$, 0.2mM of each dNTPs, 2 units of Taq polymerase and 10 pmol of reverse and forward primers. PCR reactions were optimized according to protocols by Röder *et a*l. (1998). PCR fragments were separated by capillary electrophoresis using the ABI genetic analyser Prism3130 (Applied Biosystems, Foster City, CA, USA). Fragment analyses were genotyped by Gene Mapper Software v. 4.0. Polymorphism information content (PIC) was calculated according to Anderson *et al*. (1993). Population structure was estimated by the Structure v. 2.2 Program (Pritchard *et al*. 2000) using the allelic frequencies of polymorphic markers. The hypothetical number of expected populations (K) was set to range from 1 to 5. Data was processed by an admixture model using a burn-in in period of 100 000 and run length of 100 000. The presented clusters were corrected according to Evanno *et al*. (2005). Association analysis between SSR markers and agronomic traits was tested using the General Linear Model (GLM) in the Tassel v. 2.0.1 software program (Bradbury *et al*. 2007). Values of the Q matrix obtained in Structure were presented as covariates. The *P* value determines whether a QTL is associated with the marker and R^2 for a marker evaluates the magnitude of QTL effects.

RESULTS

Genetic diversity

In this study, allelic diversity was assigned on the set of 96 varieties using 16 SSR markers dispersed through all three wheat genomes (Table 2). The total number of 136 alleles was detected in all loci analysed with an average number of 8.5 per locus. The largest number of alleles (19) was found at *psp*3094 locus, whereas only three allelic forms were detected at

Table 2. Description of microsatellite markers

SSR markers	Chromosome	Allele size (bp)	Allelic No./locus	PIC	Heterozygote	Relative frequency of the most frequent allele
GWM95	2A	114–120	4	0.513	–	0.649 (116 bp)
GWM261	2D	165–196	6	0.646	1	0.484 (192 bp)
WMC89	4A	120–172	10	0.616	26	0.549 (126 bp)
WMC420	4A	118–132	5	0.532	10	0.575 (118 bp)
GWM18	1B	184–196, null	8	0.786	2	0.306 (196 bp)
GWM11	1B	186–212, null	11	0.726	2	0.479 (192 bp)
GWM428	7D	121–135	4	0.342	9	0.8 (121 bp)
PSP3200	6D	159–177	6	0.723	–	0.417(162 bp)
PSP3071	6A	148–167	10	0.811	6	0.323 (153 bp)
GWM337	1D	173–209	14	0.828	–	0.292 (187 bp)
GWM601	4A	120–124	3	0.318	9	0.816 (120 bp)
GWM539	2D	129–165	7	0.554	27	0.566 (137 bp)
PSP3094	7A	105–199	19	0.886	5	0.22 (199 bp)
WMC48	6D	121–143	8	0.616	–	0.558 (139 bp)
PSP3103	4D	157–181	11	0.700	17	0.416 (157 bp)
WMC31	7D	110–155	10	0.586	9	0.617 (128 bp)
Total number			136			

PIC – polymorphism information content

Table 3. Descriptive statistics for heading time (HT), flowering time (FT), stem height (SH) and spike length (SL)

Trait	Mean	Confidence (%)		Min	Max	Var.	SD	CV	SE
		–95.00	+95.00						
HT	126.3	125.5	127.2	102	144	51.1	7.2	5.7	0.42
FT	131.6	130.7	132.3	111	150	46.1	6.8	5.2	0.42
SH (cm)	84.6	82.7	86.5	19	132.3	262.1	16.2	19.1	0.95
SL (cm)	10.0	9.8	10.2	4.3	19.7	3.0	1.7	17.4	0.1

SD – standard deviation; CV – coefficient of variation; SE – standard error

*gwm*601 locus in the genotypes investigated. The PIC values ranged from 0.318 (*gwm*601) to 0.886 (*psp*3094), with an average of 0.602. Heterozygous genotypes were detected in 12 out of 16 SSR loci analysed. Null alleles were detected at loci *gwm*11 and *gwm*18.

Trait analysis

Mean values of the evaluated traits in three years analysed showed considerable levels of phenotypic diversity (Table 3). Early heading and flowering date was recorded first in the Chinese variety Alkan-tzao, while the Serbian spring variety Nevesinjka has the latest date of heading and flowering time. Stem height is the most variable trait with a coefficient of variation of 19.1. The shortest spike was in *Triticum aestivum* spp. *compactum* with an annual average length of 4.3 cm, whereas variety ZGK238/82 has a spike length 19.7cm on average.

Population structure

The determination of population structure on the obtained molecular data is the main prerequisite for association analysis. Population structure of

Table 4. Significant associations between markers and traits detected during two/three years (R^2)

Trait	Marker	*P*			*R* (%)*		
		2007	2008	2009	2007	2008	2009
HT	WMC89-4A	0.0829	0.011*	0.0057**	15.60	22.29	24.08
	GWM261-2D	9.60E-06**	2.10E-06**	7.65E-06**	28.28	32.92	29.09
	GWM18-1B	0.0271*	0.0018**	0.1358	16.77	23.61	11.96
	GWM11-1B	5.38E-05**	5.03E-04**	2.84E-05**	37.02	32.17	38.56
	GWM337-1D	1.95E-04**	0.0284*	7.64E-04**	35.4	23.41	32.85
	WMC48-6D	0.0107*	0.0042**	0.0012**	17.32	21.02	22.42
FT	WMC89-4A	0.1017	0.0281*	0.0106*	15.05	19.66	22.52
	GWM261-2D	1.92E-05**	1.19E-06**	1.15E-05**	27.48	34.24	28.61
	GWM18-1B	0.033*	0.0016**	0.0798	16.41	24.19	13.79
	GWM11-1B	8.40E-05**	1.55E-04**	2.58E-05**	36.37	35.01	38.83
	GWM337-1D	2.99E-04**	0.0299*	7.17E-04**	34.91	23.42	33.14
	WMC48-6D	0.0104*	0.0095**	0.0023**	17.56	19.26	21.11
SH	GWM18-1B	0.0011**	0.0046**	4.32E-04**	24.45	21.24	26.08
	PSP3200-6D	0.0316*	0.2756	0.0049**	12.72	6.73	16.69
	PSP3071-6A	0.0686	0.0263*	0.0232*	17.69	20.46	20.58
	WMC48-6D	0.0109*	0.0052**	0.2048	17.11	20.31	8.99
SL	WMC89-4A	0.0155*	0.0286*	0.0087**	19.96	18.99	23.16
	PSP3071-6A	0.0426*	0.0692	0.0193**	18.74	17.53	21.92

R (%) – total phenotypic variation of HT (heading time), FT (flowering time), SH (stem height) and SL (spike length); *$P < 0.05$ and **$P < 0.01$

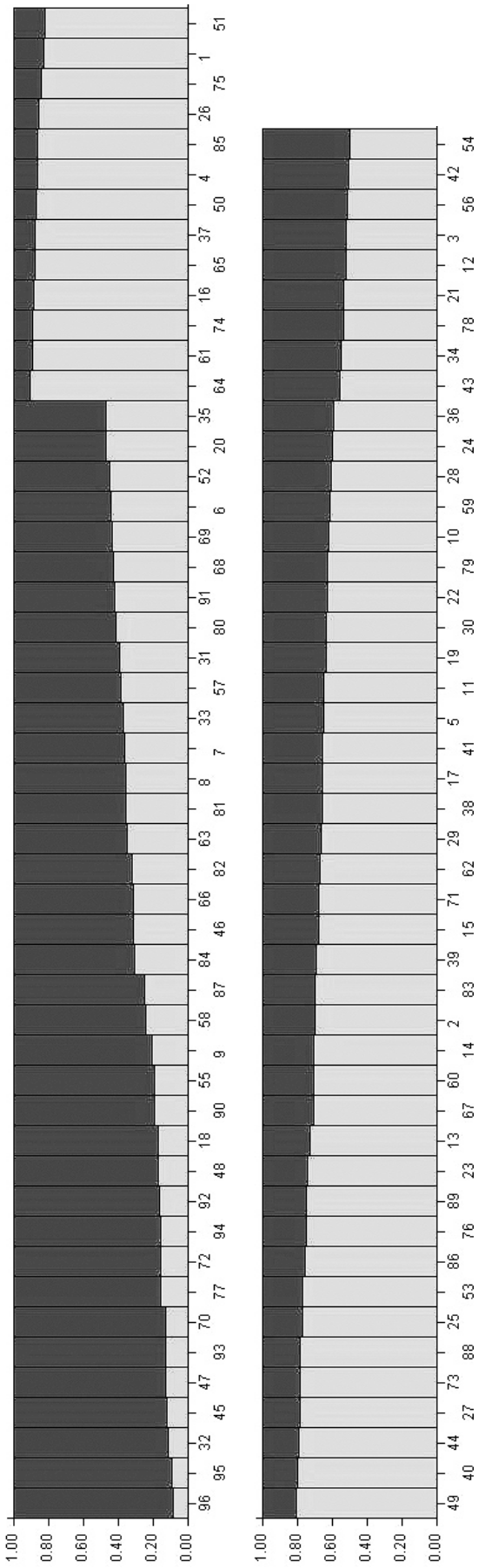

Figure 1. Distributions of 96 genotypes in two groups based on molecular data processed in Structure 2.2 program

the chosen genotypes was determined using a Bayesian clustering approach implemented in the Structure program. The genotypes were clustered into 5 subpopulations according to an admixture model used by the software. Evanno *et al*. (2005) proposed the correction of data necessary to obtain actual number of clusters. The final number of subpopulations was two (Figure 1). The first cluster consisted of 59 genotypes (out of 96) whereas the second cluster is composed of 37 genotypes.

Association analysis

The results of significant marker-trait associations in three analysed years are shown in Table 4. The highest number of associations was detected for heading and flowering time. Four markers (*gwm*261, *gwm*11, *gwm*337 and *wmc*48) showed significant associations with QTL for heading and flowering date in all years analysed, whereas the markers *gwm*18 and *wmc*89 showed association with the same traits only in two years (Table 4). The QTL located near the *gwm*18 marker explained about 23% of the total variability for plant height in three growing seasons. Two regions on chromosomes 4A and 6A were associated with spike length. The QTL near the *wmc*89 marker showed the greatest stability over three seasons with phenotypic variability ranging from 18.99% to 23.16%.

DISCUSSION

SSR markers have a high level of PIC in comparison with other types of molecular markers (Gupta *et al*. 2008). PIC values (from 0.318 to 0.886) and a significant number of allelic forms at all loci indicate a wide genetic variation accumulated in the breeding material. Considerable variability at the analysed loci is a result of the high resolution of the ABI 3130 detection system. Although the lowest level of polymorphism was detected on homoelogous group 4 chromosomes (Huang *et al*. 2002), a number of allelic forms at loci *wmc*89 and *psp*3130 was found in our material.

An important prerequisite for association analysis is the existence of a certain level of population structure to reduce linkage disequilibrium (LD) between appropriate loci (Yu *et al*. 2006). To eliminate false positive associations, genotypes were carefully chosen based on a wide range of

phenotypic diversity. Most varieties created in Serbia are distributed to the same subgroups indicating similar selective pressures during the breeding process.

Association analysis

Modern breeding exploits genetic variability of genes which control flowering time and sensitivity to photoperiod as crucial traits for wheat adaptation in certain regions. Many QTLs for flowering time were revealed in different regions of individual chromosomes, especially on chromosomes of groups 2 and 5 near the *Ppd* and *Vrn* genes (Lin *et al*. 2008). In this study, the detected QTLs were found in the same chromosome regions as in previous studies (Kirwigi *et al*. 2007; Kuchel *et al*. 2007). The region on 1B chromosome near the *gwm*11 marker had the largest effects on total variability of heading and flowering time. Kuchel *et al*. (2007) detected the same marker *gwm*11 near a QTL which explained phenotypic variation for grain yield with consistent and stable associations in most of the environments investigated (LOD ≥ 3). Neumann *et al*. (2011) detected associations between flowering time and regions on the 1BL and 1D chromosomes indicating new potential loci for flowering time. Ma *et al*. (2001) identified two loci on the 1B chromosome near *gwm*11 and *gwm*18 closely linked to a *Yr* (yellow rust) gene which was placed 1.9 cM distally to these markers. Kirwigi *et al*. (2007) detected QTL in the proximal region of 4A chromosome for grain yield, grain fill rate, spike density, grains per m^2, biomass production, biomass production rate and drought susceptibility index (DSI). The associations between the *wmc*89 marker and all major QTLs covered 7.7 cM and also explained the greatest proportion of phenotype variability. This region was important and responsible for phenotypic variation for heading, flowering time and spike length. Liu *et al*. (2010) detected the same marker *wmc*89 near QTL for grains per spike, which may explain that the correlated characteristics often have a similar position on the genetic map (Paterson *et al*. 1991).

Kobiljski *et al*. (2002) identified a QTL for earliness and short stature which was associated with *gwm*337 marker on 1B chromosome. The same QTL did not have a correlation with stem height in our breeding material. Quarrie *et al*. (2003) identified a QTL with significant associations with flowering time located near the *psp*3200 marker on the short arm of 6D chromosome. In this study, the *psp*3200 marker explained from 12.72% (2006/07) to 16.69% (2008/2009) of variability in stem height. Since there were no associations with other traits, this could be a new QTL for this trait under Serbian climatic conditions. Chaturvedi and Gupta (1995) pointed out the direct positive effects of plant height on grain yield, so direct identification of genomic regions which affect expression of stem height is important for the breeding process. In this study, four QTLs for stem height were determined on chromosomes 1B, 6A, 6D and 7D. According to Pestsova and Röder (2002), 21 genes have been reported, or are known, to have great effects on plant height, known as *Rht* (reduced plant height) genes. *Rht*8 is the most important gene for decreasing stem height, introduced together with the *Ppd* D1 gene (Korzun *et al*. 1998) in our genotypes. In this case, no significant associations were detected between the *gwm*261 marker and stem height, which is situated close to *Rht*8 (0.6 cM) on 2D chromosome. However, associations between this marker and heading and flowering date can be explained by the proximity of the *Ppd*-D1 gene on the genetic map. The significance of SSR locus *gwm*261 on the 2DS chromosome for improving the bread wheat yield potential was confirmed by its association with heading and flowering time in a specially designed double haploid population (Trkulja *et al*. 2011). Confirmation of marker trait associations in our elite diverse material increases the possibility of using this candidate marker for MAS. A new QTL affecting variability in spike length was detected on 6A (*psp3071*). This can be caused by the appearance of new alleles since different allelic forms at the analysed locus may increase the effects of QTL on phenotypic expression. Macaferri *et al*. (2008) confirmed that only stable QTL expression in a wider range of agro-meteorological conditions is one of the determining factors in breeding high-yielding varieties adapted to different environmental conditions.

According to our results, stable marker-trait associations were identified during three growing seasons, making the markers *gwm*261, *gwm*11, *gmw*337, *wmc*48, *gwm*18, *wmc*89 potentially significant for MAS in the Serbian agro-climate region. The genomic regions of other marker trait associations (*psp*3071, *psp*3200, *wmc*89, *gwm*18), which

were not significant in all environments, could be the target of further validation and genetic dissection in relevant segregating populations or other breeding collections. The significant associations detected in this research are mostly in the regions where QTLs were previously reported. Additionally, new QTLs were detected in our study, suggesting that the association analysis shows a higher resolution than previous biparental mapping, so it could be more valuable for use in MAS.

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