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Original articles

VALIDATION OF MARKER-TRAIT ASSOCIATIONS IN SIX-ROWED BARLEY LINES BRED IN KAZAKHSTAN

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ABSTRACT

Barley is an important cereal crop in Kazakhstan, mostly used for animal feeding, malting, and the food industry. The success of barley production is dependent on the genetic resources (cultivars) available, and local breeding programs that focus on the development of competitive cultivars. One way to develop new high-yielding cultivars and improve the efficiency of breeding programs is the application of modern molecular genetic and genomic tools. One such technology is genome-wide association study (GWAS), which has been successfully applied to identify the quantitative trait loci (QTLs) associated with the valuable traits. The identified single nucleotide polymorphisms (SNPs) based on GWAS can be converted to flexible and cost-effective KASP (Kompetitive Allele Specific PCR) assays and validated for use in future marker-assisted breeding projects. The purpose of this study was to genotype eleven promising six-rowed barley lines using twenty-one KASP assays associated with agronomic traits reported in previous GWAS. The genotyping results suggested that only seven out of twenty-one KASP markers were polymorphic in this group of barley accessions. The *t*-test output suggested that six out of nine agronomic traits were significantly associated with seven KASPs. Notably, two assays (*ipbb_hv_6, ipbb_hv_108*) affected both vegetation period (VP) and yield per m^2 (YM2) in conditions of Northern Kazakhstan, where barley is growing in more than 80% of total crop sowing areas of the country. The application of these highly informative KASP markers can help enhance the efficiency of local breeding projects in barley.

Key words: barley, marker-assisted selection, quantitative trait loci, agronomic trait, KASP markers.

INTRODUCTION

Barley is the fourth most important cereal crop after wheat, rice, and maize [1], and can successfully grow under drought and soil saline stress conditions [2]. In Kazakhstan barley is produced mainly for the livestock feeding and food industry, and exported to several of neighboring countries [3]. However, the average grain yield rarely exceeds 2 tons per hectare, which underlines the necessity for the development of new competitive cultivars for different regions of the country [4]. One of the ways to develop competitive new high-yield cultivars is the use of promising germplasm from other regions of the world, including six-rowed lines. The six-rowed barley is advantageous over two-rowed barley for feed due to the larger number of seeds per spike and the higher seed protein content. The growth of six-rowed barley is potentially important for breeding in agriculturally oriented countries, such as Kazakhstan [5]. Another way to construct new high-yield cultivars and improve the efficiency of breeding programs is the application of modern molecular genetic and genomic tools. Over the past three decades, different types of molecular markers including RFLP, RAPD, AFLP, SSR, and SNP markers have been developed and successfully used in a wide range of applications in barley genetics and breeding [6-12]. Among molecular markers, SNPs are the most abundant source of genetic variation loci in plant genomes, characterized by the highest density in the genome. Due to the high automatization process of genotyping, SNPs have a lower cost per sample compared with other types of DNA markers [8, 10-12].

Modern genomic technologies for searching the associations between DNA markers and complex quantitative agronomic traits, such as genome-wide association study (GWAS), have been successfully applied to identify the quantitative trait loci (QTLs) used in the breeding of crops for adaptation, productivity, and quality improvement [13]. In Kazakhstan, this approach was also successfully used in studies for productivity in barley [5, 14], bread wheat [15-16], durum wheat [17], and soybean [18]. Also, similar studies were performed for disease resistance in bread and durum wheat [19-20], barley [21], and soybean [22-23]. Hence, previous studies of QTL identification for valuable traits in crops generated abundant information about a wide range of genetic loci and SNPs associated with the key agronomic traits. However, the identified SNPs are required to be validated for use in future marker-assisted breeding programs. These SNPs can be converted to KASP (Kompetitive Allele Specific PCR) markers through different approaches and analyzed for association with studied traits by using different sets of the germplasm [24]. KASP technology is a flexible genotyping platform and cost-effective SNP genotyping method developed by LGC Limited [25-26].

This study aimed to genotype eleven spring promising six-rowed barley lines using 21 KASP markers associated with agronomic traits [5, 14], and to validate the efficiency of these KASPs based on the field performance of the analyzed samples. The application of these highly informative KASP markers can help enhance the efficiency of breeding programs in barley.

MATERIAL AND METHOD

Evaluation of the breeding lines for variation in agronomic traits

The subject of the study was 11 spring six-rowed barley lines (hereafter referred to as KBL) bred at the Karabalyk

Agricultural Experimental Station (KAES, Kostanay region, north of Kazakhstan). Eleven barley lines have been planted on the experimental plots of the Kazakh Research Institute of Agriculture and Plant Industry (KRIAPI, Almaty region, southeast of Kazakhstan) and KAES during the 2021 vegetation season. The climate conditions recorded during the trials are shown in Figure 1 [27].

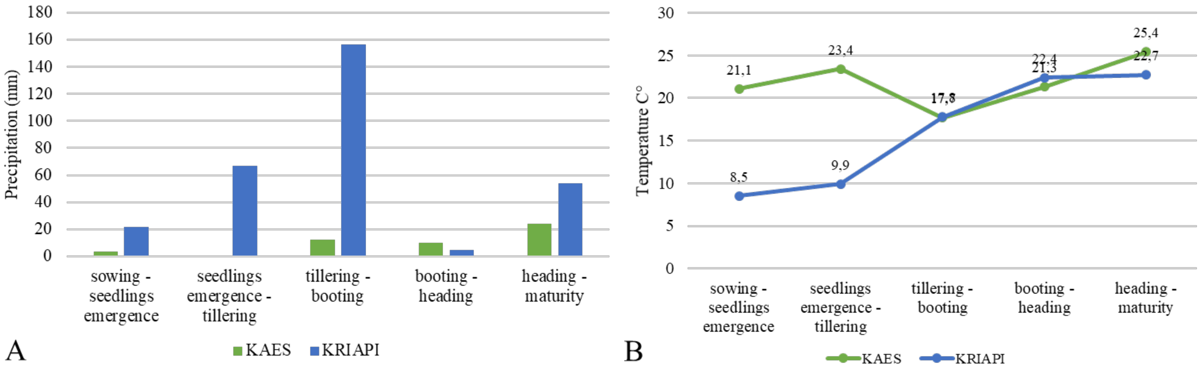


Figure 1 – Temperature (A) and precipitation (B) data in the KRIAPI and KAES in 2021 [27]

The rows were spaced 15 cm apart, and the distance between plants within a row was 5 cm. In total, the data for mean values of nine agronomic traits for the eleven KBLs harvested in the 2021 trials were analyzed. The studied traits are divided into two groups: 1) plant adaptation-related traits and 2) yield components. The first group included the following traits: heading time (HT, days), seed maturation time (SMT, days), vegetation period (VP, days), plant height (PH, cm), and peduncle length (PL, cm). The group of yield components consisted of the following traits: spikes length (SL, cm), the number of kernels per spike (NKS, pcs), thousand kernels weight (TKW, g), and yield per m^2 (YM2, g/m^2).

DNA extraction and KASP Assay

Genomic DNA of 11 KBLs was isolated from single seedlings in three replications of each line using the Delaporta protocol with modification [28]. The DNA concentration for each sample was adjusted to 30 ng/ µL.

Twenty-one KASP markers were developed by using SNPs associated with agronomic traits (Table 1). Most of these markers were reported by Genievskaya with co-authors (2018) [14] and Almerekova with co-authors (2019) [5]. The KASP reaction was performed in a volume of 10 µL with 5 µL DNA and 5 µL of the prepared genotyping mix (2× KASP master mix and primer mix). A total of twenty-one representative SNPs were used for the development of KASP assays by LGC Genomics Company [25]. KASP amplification was performed using the following thermal cycling profile, starting with 15 min at 94 °C, followed by 40 cycles of 94°C for 20 s and 60 °C for 1 min.

Table 1 – List of KASP markers associated with agronomic traits of barley

KASP ID	**Chromosome**	**Position, cM**	**Trait**
ipbb_hv_5	1H	64.9	NKS [14]
ipbb_hv_6	3H	79.1	HT/SMT/PH/NKS/PL [14]
ipbb_hv_10	2H	157.4	NKS [5]
ipbb_hv_11	7H	146.0	TKW [5]
ipbb_hv_101	4H	51.3	HT/PH/YM2/TKW [14]
ipbb_hv_102	5H	60.74	TKW [29]
ipbb_hv_105	4H	103.11	NKS/PL[unpublished]
ipbb_hv_106	7H	91.79	NKS [29]
ipbb_hv_107	Un	0.0	NKS [unpublished]
ipbb_hv_108	2H	6.45	SL [unpublished]
ipbb_hv_109	5H	137.16	YM2 [14]
ipbb_hv_110	Un	0.0	SL [29]
ipbb_hv_111	6H	52.75	NKS [unpublished]
ipbb_hv_113	Un	0.0	TKW[14]
ipbb_hv_114	5H	46.23	YM2 [unpublished]
ipbb_hv_115	1H	66.7	NKS/SL/YM2 [unpublished]
ipbb_hv_116	1H	50.60	HT/SMT/PH/PL/TKW/YM2 [14]
ipbb_hv_128	Un (7H)	0.0	TKW/YM2/PL/SMT/NKS [14]
ipbb_hv_129	2H	67.89	NPS [5]

ipbb_hv_130	3H	58.31	SL/PL [5]
ipbb_hv_132	1H	48.12	HT [5]
Note: Un – unknown chromosome, HT– heading time, SMT – seed maturation time, PH – plant height, PL – peduncle length, SL – spikes length, NPS – number of productive spikes, NKS – number of kernels per spike, TKW – thousand kernels weight, YM2 – yield per m^2.			

Statistical data analysis

t-test of studied traits was assessed using Statistical Package for the Social Sciences Version 16.0 [30]. The descriptive statistics of all traits were done using MS Excel. The correlation analysis was calculated using the R studio software [31] Genetic diversity was assessed via Shannon's information index, unbiased diversity, and percentage of polymorphic loci using the GenAlEx ver.6.5 program [32].

RESULTS

The field evaluation of eleven KBLs was conducted in two locations (KRIAPI and KAES) in 2021. High phenotypic variability of studied traits has been registered between the two regions. For instance, the earliest HT was noted in KAES (35.7±0.6 days), and the latest was in KRIAPI (45.7±0.9 days). For SMT it was determined the earliest seed maturation was registered in Almaty (35.0±1.4 days) and the latest in Karabalyk (41.2±0.8 days). The YM2 was higher in Northern Kazakhstan in comparison to yield performance in South-east Kazakhstan (Table 2).

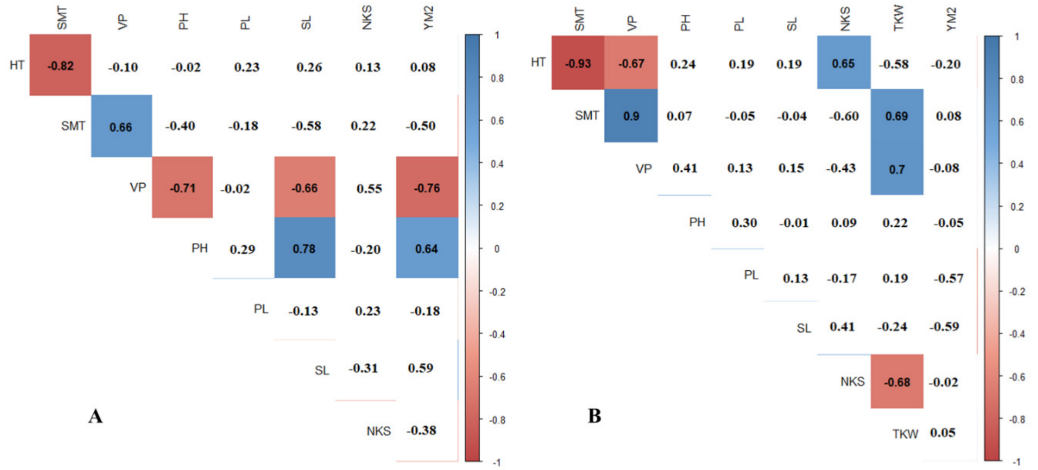
The assessment of correlations among the traits suggested that in the Northern region the YM2 was significantly correlated with VP and PH, while in the South-east region there were no obvious correlations of YM2 with other studied traits (Fig. 2). However, TKW has positively correlated with SMT and VP.

The results of field trials were taken for the validation of marker-trait associations (MTA) listed in Table 1. In the first step of the study, twenty-one SNPs associated with nine agronomic traits were converted to KASP arrays (Table 1). In the second step, eleven KBL were genotyped using 21 KASP arrays (Figure 2). The results of genotype scoring showed that only seven KASPs were polymorphic out of 21 assays in this group of samples. The effective number of alleles was 1.838±0.51, unbiased Nei's genetic diversity index was 0.499±0.017 (Table 3), and the percentage of polymorphic loci was 100 %.

The significance of each KASP assay for a particular trait was assessed using the *t*-test. As it was mentioned above the studied traits can be divided into two groups: 1) plant adap-

Table 2 – Average values of agronomic traits in the collection of barley lines in two regions of Kazakhstan.

Traits	**Karabalyk**	**Almaty**
	KBL (mean ± SE)	**KBL (mean ± SE)**
Heading time (HT, days)	35.7±0.6	45.7±0.9
Seed maturation time, (SMT, days)	41.2±0.8	35.0±1.4
Vegetation period (VP, days)	76.9±0.5	80.7±0.7
Plant height (PH, cm)	51.4±1.8	49.6±0.9
Peduncle length (PL, cm)	13.4±0.8	12.7±0.4
Spike length (SL, cm)	5.8±0.6	8.3±0.3
Number kernel per spike (NKS, pcs)	26.2±3.1	41.8±2.4
Thousand kernel weight (TKW, g)	NA	36.5±1.0
Yield per m^2 (YM2, g/m^2)	146.3±9.9	111.7±10.6



Note: Correlations with $P < 0.05$ are highlighted in color. The color indicates either positive (blue) or negative (red) correlation.

Figure 2 – Pearson's correlation index among means of studied traits in the KAES (A) and KRIAPI (B) in the 2021 growing season

tation-related traits and 2) yield-related traits. The first group included five traits: HT, SMT, VP, PH, and PL. The second group of yield components consisted of SL, NKS, TKW, and YM2.

The results of the *t*-test suggested that all seven polymorphic KASP arrays were significant both in KAES and KRIAPI. Four KASP assays (*ipbb_hv_6, ipbb_hv_108, ipbb_hv_128* and *ipbb_hv_130)* were significant for both studied groups of traits, and three assays (*ipbb_hv_102, ipbb_hv_105,* and *ipbb_hv_114)* for yield-related traits (Table 3). Notably, five KASP assays were significant at the KAES site, while all seven assays were found to be efficient at the KRIAPI site. Nevertheless, all five assays at the KAES site were associated with YM2, while none of the assays were associated with YM2 at the KRIAPI site (Table 3).

The analysis of the phenotypic effect of KASPs in KAES suggested that two assays (*ipbb_hv_6, ipbb_hv_108*) affected both VP and YM2 (Table 3 & 4). Interestingly, the largest effect on YM2 showed *ipbb_hv_108* (61.2 g), which is also associated with shorter VP duration. Similarly, *ipbb_hv_6* has positively influenced YM2, and negatively VP (Table 4).

DISCUSSION

The development of new molecular tools can be potentially very efficient for breeding activities towards the construction of new highly productive cultivars. Latest achievements using GWAS provided plenty of opportunities to identify important genes and QTL that may potentially accelerate and optimize the breeding processes in cereals, including barley [11, 13, 33-35]. One of the important parts of the application of these tools in breeding is the validation of identified significant associations between DNA markers and agronomic traits using alternative groups of germplasm in different environmental conditions [5, 14]. Therefore, in this work, we attempted to validate SNP markers with the highest significance in marker-traits associations (MTA) identified using previous barley GWAS based on evaluation of local six-rowed promising lines [5, 14, 29]. Hence, selected 21 SNP markers from previously identified MTA were converted to 21 KASP assays using LGC protocols. Further, the group of eleven promising six-rowed barley lines was tested in two different regions, including the Kostanay region (KAES, North Kazakhstan) and the Almaty region (KRIAPI, South-east Kazakhstan) in 2021. The field performance of the samples suggested that eleven KBL showed higher YM2 in Northern Kazakhstan in comparison with Southeast Kazakhstan. Nevertheless, the SL and NKS, which are one of the main components of grain yield, showed high values in Southeast Kazakhstan (Table 2). The correlation analysis showed that YM2 was negatively correlated with VP, and positively correlated with PH (Fig. 2). In contrast, in the Almaty region, YM2 showed none of the significant correlation with studied traits. However, the TKW, one of the major yield components, has positively correlated with SMT and VP (Fig. 2), which is suggesting that the performances of the studied group of six-rowed barley lines have

Table 3 – The *t*-test results in the identification of the relations between KASP markers and studied traits in two regions in the 2021 growing season.

KASP	Almaty (KRIAPI)				Karabalyk (KAES)	
	HT	MT	NKS	TKW	VP	YM2
ipbb_hv_6	0.001***	0.007**	0.244	0.351	0.027*	0.007**
ipbb_hv_102	0.228	0.120	0.002**	0.043*	0.894	0.071
ipbb_hv_105	0.529	0.237	0.010 *	0.088	0.612	0.134
ipbb_hv_108	0.000***	0.000***	0.003**	0.007**	0.015*	0.000***
ipbb_hv_114	0.233	0.079	0.013*	0.088	0.612	0.016*
ipbb_hv_128	0.003**	0.012*	0.365	0.245	0.375	0.006**
ipbb_hv_130	0.016*	0.013*	0.104	0.025*	0.079	0.021*
Note: The p-values are provided with a significance level indicated by the asterisks. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$.						

Table 4 – The effects of *ipbb_hv_6, ipbb_hv_108* on agronomic trait “yield per square meter” in the Karabalyk (KAES) site in 2021.

Trait	Region	Allele	N	Mean	SD	Phenotype effect
ipbb_hv_6						
Vegetation period (VP, days)	Karabalyk	A	5.00	78.00	1.41	-2.00 days
		G	6.00	76.00	1.10	+2.00 days
Yield per m^2 (YM2, g/m^2)	Karabalyk	A	5.00	120.20	10.21	+47.86 g
		G	6.00	168.06	28.92	-47.86 g
ipbb_hv_108						
Vegetation period (VP, days)	Karabalyk	A	4.00	75.50	0.58	+2.21 days
		G	7.00	77.71	1.38	-2.21 days
Yield per m^2 (YM2, g/m^2)	Karabalyk	A	4.00	185.25	8.65	-61.20 g
		G	7.00	124.05	13.46	+61.20 g

significantly depended on environmental conditions. Therefore, it was expected that the validation of studied KASP assays would have a different outcome for two sets of generated field data.

The genotyping of eleven six-rowed barley accessions has allowed determining that only seven out of twenty-one KASP assays were polymorphic (Table 3). The KASP genotyping resulted in the separation of the accessions in two groups of samples with different allele statuses for each of the seven polymorphic assays. The *t*-test was applied to assess the associations of seven KASPs with five plant adaptation-related traits and four yield-related traits (Table 3). The results suggested that six out of nine agronomic traits were significantly associated with seven KASPs. Particularly, four KASPs were associated with HT, SMT, NKS, and YM2, three KASPs with TKW, and two assays showed significance with VP. Notably, the *ipbb_hv_102* and *ipbb_hv_114* were associated only with yield-related traits (NKS, TKW, and YM2). Five KASP assays were associated both with yield-related traits (NKS, TKW, and YM2) and plant adaptation-related traits (HT, MT, and VP). Interestingly, two assays (*ipbb_hv_6, ipbb_hv_108*) affected both VP and YM2 (Table 3 & 4) in conditions of North Kazakhstan, where Kazakhstan has over 80% of barley growing area [3]. The phenotypic effects for both cases (*ipbb_hv_6, ipbb_hv_108*) showed that when the vegetation period was shorter for 2.0 and 2.2 days, the yield was higher for 47.9 g and 61.2 g, respectively (Table 4).

Thus, the study validated the importance of at least seven KASPs that were polymorphic for this group of six-rowed barley samples tested in Northern and South-eastern Kazakhstan. Therefore, the application of KASP assays can be efficiently used in the pyramiding of favorable genes [36] and successfully introduced into local breeding projects for the development of new competitive six-rowed barley cultivars.

CONCLUSIONS

The eleven spring six-rowed barley lines bred at the KAES were analyzed using nine agronomic traits using experimental plots of the KRIAPI and KAES during the 2021 season. The correlation analysis showed that YM2 in Northern Kazakhstan was negatively correlated with VP, and positively correlated with PH. In contrast, in the Almaty region TKW, one of the major yield components, has positively correlated with SMT and VP, which is suggesting that the performances of the studied group of six-rowed barley lines have significantly depended on environmental conditions. The genotyping of eleven six-rowed barley accessions has allowed determining that only seven out of twenty-one KASP assays were polymorphic. The *t*-test results suggested that six out of nine agronomic traits were significantly associated with all seven polymorphic KASPs. Notably, two assays (*ipbb_hv_6, ipbb_hv_108*) affected both VP and YM2 in conditions of North Kazakhstan. The phenotypic effects of *ipbb_hv_6* and *ipbb_hv_108* showed that when the vegetation period was shorter, the yield was higher for 47.9 g and 61.2 g per square meter, respectively. Thus, the study validated the importance of at least seven KASPs that were polymorphic for this group of six-rowed barley accessions tested in Northern and South-eastern Kazakhstan.

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ВАЛИДАЦИЯ АССОЦИАЦИЙ МАРКЕР-ПРИЗНАК В СЕЛЕКЦИОННЫХ ЛИНИЯХ ШЕСТИРЯДНОГО ЯЧМЕНЯ В КАЗАХСТАНЕ

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АННОТАЦИЯ

Ячмень является важной сельскохозяйственной культурой в Казахстане, в основном используемой для корма для животных, солодовенной и пищевой промышленности. Успех производства ячменя зависит от имеющихся в наличии генетических ресурсов (сортов) и местных селекционных программ, направленных на создание новых конкурентно способных сортов. Одним из способов создания новых высокоурожайных сортов и повышения эффективности селекционных программ является применение современных инструментов молекулярной генетики и геномики. Одной из таких технологий является полногеномный анализ ассоциаций (GWAS), который успешно применяется для идентификации локусов количественных признаков (QTL), связанных с ценными признаками. Идентифицированные SNP (однонуклеотидные полиморфизмы), основанные на GWAS, могут быть преобразованы в удобные и экономически эффективные маркеры KASP (конкурентная аллель-специфическая ПЦР) и подтверждены для дальнейшего использования в маркер-опосредованной селекции. Целью данного исследования было генотипирование одиннадцати перспективных линий шестирядного ячменя с использованием двадцати одного KASP маркера, связанного с агрономическими признаками, опубликованных в предыдущих работах по GWAS. Результаты генотипирования показали, что только семь из двадцати одного KASP маркера были полиморфными для данной группы образцов. Результаты *t*-теста показали, что шесть из девяти агрономических признаков оказались статистически значимыми для данной коллекции образцов и связаны с семью KASP маркерами. Примечательно, что два маркера (*ipbb_hv_6, ipbb_hv_108*) ассоциированы как с вегетационным периодом (VP), так и с урожайностью на м^2 (YM2) в условиях Северного Казахстана, где ячмень выращивается более, чем на 80% всех посевных площадей страны для данной культуры. Применение этих высокоинформативных KASP маркеров может помочь повысить эффективность местных селекционных проектов ячменя.

Ключевые слова: ячмень, маркер-опосредованная селекция, локусы количественных признаков, агрономические признаки, KASP маркеры.

ҚАЗАҚСТАНДАҒЫ АЛТЫ ҚАТЫРЛЫ АРПАНЫҢ СЕЛЕКЦИЯЛЫҚ ЛИНИЯЛАРЫНДАҒЫ МАРКЕР-БЕЛГІ АССОЦИАЦИЯЛАРЫН ВАЛИДАЦИЯЛАУ

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ТҮЙІН

Арпа-мал азығы, уыт және тамақ өнеркәсібі үшін пайдаланылатын Қазақстандағы маңызды ауылшаруашылық дақыл. Арпа өндірісінің жетістігі қолда бар генетикалық ресурстарға (сорттарға) және жаңа бәсекеге қабілетті сорттарды құруға бағытталған жергілікті селекциялық бағдарламаларға байланысты. Жаңа жоғары өнімді сорттарды құрудың және селекциялық бағдарламалардың тиімділігін арттырудың бір әдісі-молекулалық генетика мен геномиканың заманауи құралдарын қолдану. Осындай технологиялардың бірі ассоциацияларды толық геномдық талдау (GWAS) болып табылады, ол құнды белгілермен байланысты сандық белгі локустарын (QTL) анықтау үшін сәтті қолданылады. GWAS негізделіп анықталған SNP (бір нуклеотидті полиморфизмдер) ыңғайлы және үнемді KASP маркерлеріне (аллель-спецификалық бәсеке ПТР) түрлендіріліп, одан әрі маркер-жанама селекциясы үшін расталуы мүмкін. Зерттеу жұмысының мақсаты алдыңғы GWAS еңбектерінде жарияланған агрономиялық белгілермен байланысты жиырма бір KASP маркерлерін қолдана отырып, алты қатарлы арпаның он бір перспективалық линияларын генотиптеу. Генотиптеу нәтижелері жиырма бір KASP маркерінің жетеуі ғана белгілі бір үлгілер тобы үшін полиморфты екенін көрсетті. *t*-тест нәтижелері тоғыз агрономиялық белгілердің алтауы осы үлгілер коллекциясы үшін статистикалық маңызды болып табылатындығын және жеті KASP маркерлерімен байланысты екенін көрсетті. Бір қызығы, екі маркер (*ipbb_hv_6, ipbb_hv_108*) арпа дақылы үшін елдің барлық егістік алқаптарының 80% - дан астамы өсетін Солтүстік Қазақстан жағдайында вегетациялық кезеңмен (VP) және м^2 өнімділігімен (YM2) ассоцияцияланған. Осы жоғары ақпараттық KASP маркерлерін қолдану жергілікті арпа селекциясы жобаларының тиімділігін арттыруға көмектеседі.

Кілт сөздер: арпа, маркер-жанама селекциясы, сандық белгілер локустары, агрономиялық белгілер, KASP маркерлер