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IDENTIFICATION OF MFLP FINGERPRINT FOR HIGHER SEED ZINC ACCUMULATION IN BARLEY DH POPULATION

Behzad SADEGHZADEH[1*], Seyed Hossein JAMALI[2], Fatemeh VAFADAR-SHAMASBI[3]

[1]Dryland Agricultural Research Institute (DARI); Agricultural Research, Education and Extension Organization (AREEO), Maragheh, Iran
[2] Seed & Plant Certification & Registration Institute; Agricultural Research, Education and Extension Organization (AREEO), Karaj, Iran
[3]Department of Agricultural Biotechnology, Maragheh Branch Islamic Azad University, Maragheh, Iran

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Selection through molecular markers for seed Zn accumulation might be an efficient complementary breeding tool in barley breeding. To develop a specific molecular markers, 150 DH lines derived from a cross between Clipper (low-Zn-accumulator) and Sahara-3771 (high-Zn-accumulator) were screened under field and glasshouse conditions. Microsatellite-anchored fragment length polymorphism (MFLP) fingerprint generated by SSR-anchor primer MF128 in combination with AFLP primer MseI-AGA (5′-GATGAGTCCTGAGTAAAGA-3′) was identified as a candidate marker for tagging seed Zn accumulation gene. The sequencing of the band showed a marker of 369 bp with the sequence of SSR anchor primer MF128 and MseI-AGA at the two ends as expected. The MFLP marker associated with higher seed Zn accumulation has potential to be converted to a simple, sequence-specific, PCR-based, low-cost marker amenable to large populations, making it potentially viable for marker-assisted selection in barley breeding. This marker might be useful in the improvement of barley productivity and nutritional quality in Zn-deficient environments.

Key words: **b**arley, MFLP, molecular marker, seed zinc concentration

INTRODUCTION

Zinc is one of the nutrients being targeted in biofortification programs. Zinc deficiency in soils is a critical problem for cereal production, limiting the grain yield of crops in many parts

Corresponding author: Behzad Sadeghzadeh (Behzada4@yahoo.com), Dryland Agricultural Research Institute (DARI), North Ring-way, Maragheh, Iran, P.O. Box: 119. Phone: +98 421 228078, Fax: +98 421 2222069

of the world (SADEGHZADEH, 2013) as well as diminishing grain nutritional quality and human health (KNEZ and GRAHAM, 2013). Zinc deficiency declines the productivity of about 30% of cultivated lands around the world (ALLOWAY, 2008).

Genotypic variation for grain Zn concentration has been reported in crops such as wheat (CAKMAK, 2008; MCDONALD *et al*., 2008), barley (HUSSAIN *et al*., 2015) and rice (PINSON *et al*., 2015). Recent studies have shown that increased expression of six ZIP family and NAM genes can enhance Zn uptake and its root-to-shoot translocation, grain Zn and Fe concentrations (PINSON *et al*., 2015; UAUY *et al*., 2006). Producing genetically Zn-enriched cereals and improving its bioavailability are considered promising and cost-effective approaches for diminishing malnutrition (BOUIS, 2007; DISTELFELD *et al*., 2007; GHANDILYAN *et al*., 2006; POLETTI *et al*., 2004). Furthermore, micronutrient-dense seeds result in greater seedling vigour, bigger root system (SADEGHZADEH and RENGEL, 2011; WISSUWA *et al*., 2006) and higher crop yield when sowed to micronutrient-poor soils (WELCH, 1999; YILMAZ *et al*., 1998).

Screening for Zn-dense seed is one of the objectives in many breeding programs (SARDAR *et al*., 2015; SOREN *et al*., 2016). However, progress in developing mineral-dense seed has mainly relied upon conventional plant breeding approaches (BOUIS, 2007; CAKMAK, 2002; GHANDILYAN *et al*., 2006; POLETTI *et al*., 2004; WELCH and GRAHAM, 2004), a process which is labour-intensive and time-consuming. Hence, a comprehensive exploration of potential genetic resources and an in-depth understanding of Zn accumulation mechanisms are required.

Modern biotechnology provides new tools that can facilitate development of trace-mineral-dense seeds in food crops (SHARMA *et al*., 2016; ZIMMERMAN and HURREL, 2002). Molecular markers have been used to identify the genomic regions controlling seed Zn content in plants including wheat (XU *et al*., 2012) and maize (QIN *et al*., 2012). In barley, a QTL increasing seed Zn content was mapped on the short arm of chromosome 2H (LONERGAN, 2001). SADEGHZADEH (2008) identified two regions on the long and short arm of 2H that were associated with seed Zn concentration and content of glasshouse- and field-grown barley plants. Two additional QTLs were also reported for seed Zn concentration in glasshouse-grown plants on 3HL and 4HS chromosomes. Furthermore, SADEGHZADEH *et al*. (2010) developed the molecular marker SZnR1 by using microsatellite-anchored fragment length polymorphism (MFLP) technique located on 2HS in barley. The developed sequence-specific PCR-based marker has the potential to be used for marker-assisted selection for higher seed Zn concentration and content in barley breeding.

Several methods are used for the construction of linkage maps and development of molecular markers for marker-assisted selection in crops (BOERSMA *et al*., 2007a; GUPTA *et al*., 1999). YANG *et al*. (2001) developed microsatellite-anchored fragment length polymorphism (MFLP) technique to amplify and detect simple sequence repeat (SSR) -related polymorphisms. MFLP method was derived from combination of SSR-anchor primers with the amplified fragment length polymorphism (AFLP) concept (SHAHIDUL *et al*., 2013). MFLP is capable of producing DNA markers in high-efficiency; each detected polymorphism is a SSR-*Mse*I fragment that contains a microsatellite motif sequence at one end and an AFLP primer sequence at the other end (YANG *et al*., 2004; 2002).

PCR-based markers such as AFLP amplify multiple loci in the genome and cannot be easily converted into sequence-specific PCR markers; but sequence-specific PCR markers are preferred for marker-assisted selection, because they are relatively inexpensive to run and can be used for screening a large number of samples (JIANG, 2013; JIANG, 2015). Conversion of an

identified marker from AFLP into a sequence-specific marker is often difficult and inefficient (BRUGMANS *et al*., 2003; GUPTA *et al*., 1999; SHAN *et al*., 1999).

Large numbers of microsatellite-related DNA polymorphisms can be produced by MFLP method (YANG *et al*., 2001). However, these markers cannot be used directly in marker-assisted selection because their implementation is expensive and time-consuming. Hence, there is necessity in converting the identified polymorphisms from MFLP into sequence-specific simple PCR-based markers, which are desirable for marker-assisted selection in the breeding programs (BOERSMA *et al*., 2007a; SADEGHZADEH *et al*., 2010; YANG *et al*., 2004). In this regards, the objectives of this study were: i) employment of MFLP technique to generate large numbers of DNA fingerprints, and ii) identify marker(s) linked to genomics regions involved in higher seed Zn accumulation. This procedure efficiently identifies markers linked to genes of interest, allowing their rapid placement on a genetic map. The identified marker also can be converted to sequence-specific PCR-based marker that has the potential to be used for marker-assisted selection for higher seed Zn concentration and content in barley breeding.

MATERIALS AND METHODS

Phenotyping

150 doubled-haploid (DH) lines were used for phenotyping and generating MFLP fingerprinting. The DH lines were derived from a cross between Clipper (low-Zn-accumulator) and Sahara-3771 (high-Zn-accumulator) barley genotypes. The population was produced by the *Hordeum bulbosum* method described by ISLAM and SHEPHERD (1981).

The parents and 150 DH lines were studied in two independent experiments, field and glasshouse. The field experiment was conducted under irrigated conditions in a completely randomised design with two replications at the University of Western Australia, Field Station, Shenton Park (31.9°S, 114.9°E). Each plot included 6 plants. The field management was done as per the standard practice. Soil was fertilized with 320 kg ha^{-1} Super Potash (P, 50 kg ha^{-1}; K, 95 kg ha^{-1}; S, 62 kg ha^{-1}) before sowing, and 50 kg/ha urea was applied every two weeks from week 6 to week 18 after sowing. Field soil properties in the experimental site were: $pH_{(water)}$ 6.8, organic matter 8.5 g kg^{-1} soil, P 43 mg kg^{-1} soil and DTPA (diethylenetriamine pentaacetic acid)-extractable Zn 1.0 mg kg^{-1} soil. At maturity, all ears were harvested by hand, air-dried to preserved seed viability, threshed and counted to obtain the 1000-kernel weight. Fifteen seeds from each individual DH line and the parents were oven dried at 70°C for 72 hours. After weighing, the seed samples were ashed at 550°C for 14 hours, solubilized in 10 mL 30% (v/v) hydrochloric acid (HCl) for 30 minutes at 50°C. Zinc concentration of seed was determined by inductively coupled plasma-mass spectrometry (ICP-MS). Measurements of minerals have been checked by using the certified values of the related minerals in the reference leaf and grain samples. Seed Zn content was calculated by multiplying seed dry weight with seed Zn concentration.

In the glasshouse experiment, the same set of experimental materials was studied as used in the field experiment. Seeds of 150 DH lines and the parents were hand sorted to a uniform size, surface sterilized and pre-germinated on filter paper in Petri dishes. Sandy Lancelin soil was collected from the virgin site about 150 km north of Perth, Western Australia (31.56° S, 115.20° E), air-dried and sieved (2mm); 1.5 kg of soil was placed into plastic-bag-lined cartons (70 × 70 × 200 mm). The soil used for glasshouse experiment had $pH_{(water)}$ 6.1, organic matter 12 g kg^{-1}, P 3.3 mg kg^{-1} soil and DTPA-extractable Zn 0.1 mg kg^{-1} soil. Basal nutrients (in mg

kg^{-1} of dry soil) 91 KH_2PO_4, 145 K_2SO_4, 147 $CaCl_2.2H_2O$, 21 $MgSO_4.7H_2O$, 2 $CuSO_4.5H_2O$, 15 $MnSO_4.H_2O$, 0.7 H_3BO_3, 0.2 $Na_2MoO_4.2H_2O$ and 93 NH_4NO_3 together with 0.8 mg Zn kg^{-1} soil as $ZnSO_4.7H_2O$ were applied to the soil. Nine pre-germinated seeds of each genotype were sown in each pot in a randomised completely block design with three replications. Seedlings were thinned for uniformity to 4 plants per pot at the three-leaf stage. Pots were rotated within a block daily to minimize the effect of microenvironments. Plants were grown in a glasshouse, and watered with deionized water daily by weight, keeping water content at 90% of the field capacity, which was determined to be 10% (w/w). Harvests were performed at maturity.

Marker development

The DH population was first divided into two subpopulations (two-rowed *vs* six-rowed) based on the QTL on chromosome 2HL associated with the two/six rowed phenotype (SADEGHZADEH *et al*., 2008). Thus, only the QTL from the chromosome 2HS was segregated in each sub-population. Ten DH lines (five each of high-Zn-accumulator and low-Zn-accumulator) were selected from the two-rowed sub-population based on the two RFLP markers Xbcd175 and Xpsr108 flanking the chromosome 2HS QTL for seed Zn accumulation.

Leaf tissues were used for DNA extraction according to the Mini-prep DNA extraction method. The ten selected doubled haploid lines (see above) and two parents were used to generate MFLP fingerprints. DNA from each twelve representative plants was digested by the restriction enzyme *Tru*9I (Roche Diagnostics, Australian National University), and its isoschizomer *Mse*I. The *Mse*I-adaptor was ligated onto the restriction fragments using T4 DNA-ligase. To optimize and reduce the number of DNA bands on the sequencing gel, and to decrease the amplification of *Mse*I-*Mse*I fragments, the template DNA was further digested with a 'frequent cutter', *Hae*III (Roche Diagnostics). This was achieved by precipitating the DNA in the 10-µL restriction-ligation mix, digesting the DNA in 50 µL of *Hae*III digestion mix containing 2.5 units *Hae*III, 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl and 1 mM dithiothreitol, and incubating at 37°C for 2 h. The reaction mix was diluted by adding 150 µL TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8) and used as a template for pre-selective amplification. Detailed methodology for MFLP has been described by YANG *et al*. (2001).

Pre-selective MFLP reactions were run by using 4 SSR-anchor primers (Table 1) each in combination with an AFLP primer (*Mse*I-A). The structure of the *Mse*I primers was the same as in AFLP (VOS *et al*., 1995), with one selective nucleotide A at the 3′ end (5′-GATGAGTCCTGAGTAA**A**-3′). MFLP pre-selective amplifications were set up by incorporating 1.5 µL of the template DNA (described above) into 8.5 µL PCR mix containing 1 unit of Taq polymerase (Fisher Biotec, Perth, Australia), 5 pmol SSR-anchor primer, 5 pmol *Mse*I primer, 67 mM Tris-HCl pH 8.8, 2 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 0.45% (v/v) Triton X-100, 4 µg gelatin and 0.2 mM dNTPs. Pre-selective PCR was performed with a Hybrid DNA Express thermocycler for 25 cycles each at 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min. Of the PCR mix, 10 µL was diluted by adding 290 µL TE buffer, and used as the template DNA for selective amplifications (YANG *et al*., 2002).

Selective MFLP amplifications were performed by PCR (ingredients for PCR were the same as used for pre-selective MFLP with the exception of the primers) with one SSR-anchor primer and one *Mse*I primer. A total of 64 sets of selective MFLP fingerprints were produced by using four SSR-anchor primers in combination with 16 AFLP primers having two additional selective nucleotides at the 3′ end (*Mse*I-ANN). The SSR-anchor primer used in selective

amplification was the same as that used in the corresponding pre-selective amplification, but was labelled with γ-^{33}P as described by vos *et al.* (1995). Selective PCR was performed on a thermocycler with each cycle comprising 30 s at 94 °C, 30 s at the annealing temperature (see below), and 1 min at 72 °C. The annealing temperature of the first cycle was 60 °C, and decreased by 0.7 °C in each subsequent cycle until the temperature reached 54 °C. The final 25 cycles used an annealing temperature of 54 °C. The selective MFLP amplification products were separated on a 5% (w/v) polyacrylamide denaturing sequencing gel (7 M urea) in a Sequi-Gen GT sequencing cell (BioRad, Hercules, USA). After electrophoresis at 55 W for about 2.5 h, the gel was dried on a gel drier (Model 583, BioRad). Marker bands were detected by autoradiography with overnight exposure of an X-ray film (Kodak X-Inat Blue XB-1) on the dried gel (YANG *et al.*, 2002; YOU *et al.*, 2005). MFLP polymorphisms showing evidence of correlation with seed Zn accumulation were considered as candidate markers linked to the Zn accumulation gene (YANG *et al.*, 2004).

RESULTS AND DISCUSSION

To identify molecular marker involved in regulating higher seed Zn accumulation in barley, 150 DH lines and their parents grown in the field and glasshouse conditions were analysed for seed Zn concentration and content. The DH population varied considerably in both mean values and ranges for Zn in seed. The distribution of the progeny mean values was normal for all measured traits. Seed Zn concentration in DHs showed a range of 22-61 mg kg^{-1} dry matter in the field study, and 16-48 mg kg^{-1} in the glasshouse experiment. In both experiments, the presence of DH lines with higher mean values compared with parents evidenced occurrence of transgressive segregation.

The number of polymorphic markers detected for individual primer pair ranged from 9 to 28, with the average of 18 fragments per primer combination. Based on linkage analysis, a MFLP fingerprint generated by SSR-anchor primer MF128 (Table 1) in combination with AFLP primer *Mse*I-AGA (5′-GATGAGTCCTGAGTAAAGA-3′) was identified as a candidate marker for tagging seed Zn accumulation gene. This marker was specific to high-Zn-accumulating parent (Sahara-3771) and all five high-Zn-accumulating DH lines, and this marker was not amplified in low-Zn-accumulating parent (Clipper) and all five low-Zn-accumulating DH lines (Figure 1). The sequencing of the band showed a marker of 369 bp with the sequence of SSR anchor primer MF128 and *Mse*I-AGA at the two ends as expected (Table 2). Based on BLASTn search of the NCBI database, the marker sequence did show similarity with any reported gene or significant homology with any genome based (http://www.ncbi.nlm.nih.gov/).

Table 1. DNA sequence of SSR-anchor primers used in MFLP

Primer name	**Sequence (5'-3')**
MF 128	AGTAGCAGTTCTCTCTCTCTCTC
MF 129	ACTAGCACTTGTGTGTGTGTGTG
MF 01	GTCCGAGAGAGAGAGA
MF 02	GGCATGTGTGTGTGTG

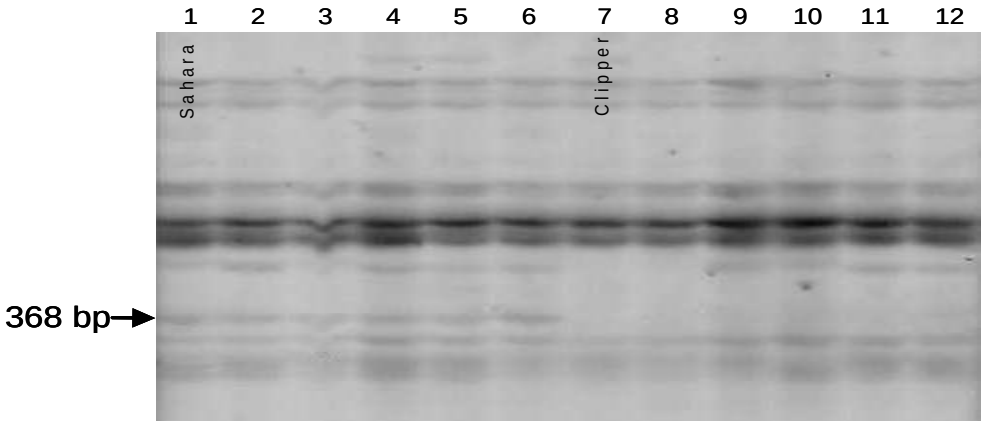

Figure 1. Identification of DNA polymorphisms linked to the gene for seed Zn accumulation in barley by MFLP fingerprinting generated using SSR primer MF128 and AFLP primer *Mse*I-AGA. Twelve genotypes were tested, including high-Zn-accumulating parent Sahara-3771 (Lane 1), five high-Zn-accumulating DH lines (Lanes 2-6), low-Zn-accumulating parent Clipper (Lane 7), and five low-Zn-accumulating DH lines (Lanes 8-12). An arrow indicates the candidate MFLP marker associated with the gene regulating higher seed Zn accumulation.

Identification of DNA polymorphisms linked to the gene for higher seed Zn accumulation in barley by MFLP fingerprinting in this study illustrates the advantage of the technique for developing markers suitable for marker assisted selection. MFLP, similar to AFLP, generates a larger number of polymorphic DNA bands compared with RFLP and RAPD, and each amplified fragment in MFLP contains a microsatellite motif sequence (YANG *et al*., 2002). Microsatellites are highly polymorphic and abundant in plant genome (DONINI *et al*., 1998), and MFLP can detect microsatellite-originated DNA polymorphisms without construction of genomic library (YANG *et al*., 2001).

Table 2. DNA sequence of the MFLP marker showing primers (see the footnote).

Position	Sequence (5'-3')
1	*GGGTCTCTCTCTCTCTC*[1]GATGACGACAAACGAAATGACCAGAATGTGGGG
51	CAACAACCTCGAAGCATGTGTCCGCACATATGGGAATCCGTCAAAAGATA
101	ATTTGTCGTAACAAGATATCTCTGCCAGAATCTCCAACAATCAAGTCAGC
151	ATATATATAAACAGATGAAAGTTAGAGTAACATATGAGGATCCGGTGGAA
201	CAAACCACCCTGAAACCAGATAGTCAGTCAGCAAAGATGTTATCGGAAGT
251	ATTATTCTAAGGAAAGAAGGAAACCCGATTTTGAAACAGTTTCCTCAAGA
301	AAGGATACTTATGTTGAGCCTTGAGCAATCAAATCTCGAGGACGAGATT
350	**TCTTTACTCAGGACTCATC**[2]

[1]: Italic are shows SSR-anchor primer MF128 (5'-GGGTCTCTCTCTCTCTC-3')

[2]: Bold are shows the annealing site of AFLP primer *Mse*I-AGA (5'-GATGAGTCCTGAGTAAAGA-3')

Although, this marker system requires small amount of DNA template, it cannot be used directly in marker-assisted selection because its implementation is time-consuming and expensive. However, using MFLP for fingerprinting is advantageous as many MFLP polymorphisms could easily be converted into simple PCR based markers desirable for routine marker implementation in marker-assisted selection (BOERSMA *et al*., 2007a; SADEGHZADEH *et al.*, 2010; SOREN *et al*., 2016; YANG *et al.*, 2004). MFLP identifies, theoretically, four types of polymorphisms: (i) microsatellite polymorphisms arising from variation in the number of repeat units within SSRs targeted by the SSR-anchor primers, which are co-dominant; (ii) polymorphisms originating from the annealing sites variation for the SSR-anchor primer, which are dominant; (iii) polymorphisms resulting from insertions/deletions outside the SSR region within the SSR-*Mse*I fragments, which are co-dominant; and (iv) polymorphisms arising from variation in restriction sites. YANG *et al*. (2001) confirmed that the first three types of MFLP polymorphisms can be converted easily into sequence-specific PCR markers. The first two types of MFLP polymorphisms could be converted into simple PCR-based markers using SSR-anchor primer employed in MFLP and a sequence-specific primer for screening of the converted marker.

In the present study, MFLP marker linked to seed Zn accumulation was DNA sequence internal to the SSR-*Mse*I fragment and easily could be converted into a simple sequence-specific PCR-based marker (YANG *et al*., 2002). In contrast to MFLP, the internal sequence of AFLP fragments used in designing primers for PCR rarely generates polymorphic bands (GUPTA *et al*., 1999; SHAN *et al*., 1999). The developed marker has potential for marker-assisted selection of higher seed Zn concentration and content in barley breeding. A molecular marker would be ideal for marker-assisted selection in practical plant breeding when the marker is closely linked to the gene of interest, in which case the linkage between marker and the gene is almost unbreakable (ELLIS *et al*., 2002). However, developing perfect markers is very difficult; most of applied markers in plant breeding are positioned at a certain genetic distance from the gene of interest (BOERSMA *et al*., 2007b; GUPTA *et al*., 1999; YOU *et al*., 2005).

The efficient selection of elite recombinants for any important trait, with an aim to minimize the number of lines from large populations, is one of the challenges in plant breeding programs. Therefore, any technique helping breeders to screen and select in early generations is important. One of the aims of marker-assisted selection is to increase the frequency of desirable alleles in the early generation breeding populations by removing lines that are poor for a trait of interest. The use of MFLP fingerprints associated with the seed Zn accumulation and their conversion to PCR-based markers might allow this to be achieved in barley breeding. The relatively high proportion of co-dominant markers in MFLP is an advantage when converted into sequence-specific, simple PCR based markers for MAS, as they are able to identify homozygous and heterozygous individuals (YANG *et al.*, 2001).

In conclusion, identified marker for higher seed Zn accumulation, described in this study, has the potential to be converted to a simple, sequence-specific, PCR-based, low-cost marker amenable to large numbers of plant samples, making it potentially viable for marker-assisted selection (MAS) in practical barley breeding programs. With the development of PCR primers for this marker, it will be possible to breed barley varieties with much greater seed Zn without the need for lengthy phenotypic screening procedures. The marker SZnR1 negatively correlated with higher seed Zn accumulation gene was already being developed by SADEGHZADEH *et al*. (2010). With further development of new sequence-specific PCR-based

markers and the identification of QTLs for higher seed Zn, it is anticipated that the breeding of barley using MAS will be greatly enhanced, resulting in the release of superior cultivars over a reduced time scale.

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IDENTIFIKACIJA MFLP FINGERPRINTA ZA VISOKU AKUMULACIJU CINKA U SEMENU KOD DH POPULACIJA JEČMA

Behzad SADEGHZADEH[1*], Seyed-Hossein JAMALI[2], Fatemeh VAFADAR-SHAMASBI[3]

[1] Poljoprivredno istraživački institut za sušu (DARI), Organizacija za istraživanja I edukaciju u poljoprivredi (AREEO), Maragheh, Iran
[2] Institut za oplemenjivanje biljaka, Univerzitet Sidnej, NSW, Australija
[3] Odeljenje za poljoprivrednu biotehnologiju, Maragheh ogranak, Islamski Azad Universitet Maragheh, Iran

Izvod

Selekcija pomoću molekularnih markera za akumulaciju cinka u semenu može biti efikasno komplementarsno sredstvo u oplemnejivanju ječma.Za razvoj specifičnih molekularnih markera 150 DH linija dobijenih iz ukrštanja Clipper (niska-Zn-akumulacija) i Sa+hara 3771 (visoka-Zn-akumulacija) su ispitane u polju i staklari. MFLP fingerprint dobije sa SSR-anchor primerom MF128 u kombinaciji sa AFLP primerom MseI-AGA (5´-GATGAGTCCTGAGTAAAGA-3´) je identifikovan kao kandidat marker za tagging gena za akumulaciju Zn. Sekvencioniranje trakepokazalo je marker od 369 bp sa sekvencom SSR anchor primera MF128 i MseI-AGA na dva kraja. MFLP marker povezan sa visokom akumulacijom Zn u semenu ima potencijal da se konvertuje u jednostavan, sekvenca-specifičan, PCR-zasnovan, jefitn marker primenjiv za veću populaciju, pogodan za MAS ječma. Ovaj marker može biit koristan u poboljšanju produktivnosti ječma I nutritivnog kvaliteta u sredinama sa manje Zn.

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