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NOVEL FLUORESCENT SEQUENCE-RELATED AMPLIFIED POLYMORPHISM (FSRAP) MARKERS FOR THE CONSTRUCTION OF A GENETIC LINKAGE MAP OF WHEAT (*Triticum aestivum* L.)

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Novel fluorescent sequence-related amplified polymorphism (FSRAP) markers were developed based on the SRAP molecular marker. Then, the FSRAP markers were used to construct the genetic map of a wheat (*Triticum aestivum* L.) recombinant inbred line population derived from a Chuanmai 42 × Chuannong 16 cross. Reproducibility and polymorphism tests indicated that the FSRAP markers have repeatability and better reflect the polymorphism of wheat varieties compared with SRAP markers. A total of 430 polymorphic loci between Chuanmai 42 and Chuannong 16 were detected with 189 FSRAP primer combinations. A total of 281 FSARP markers and 39 SSR markers

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were classified into 20 linkage groups. The maps spanned a total length of 2499.3cM with an average distance of 7.81cM between markers. A total of 201 markers were mapped on the B genome and covered a distance of 1013cM. On the A genome, 84 markers were mapped and covered a distance of 849.6cM. On the D genome, however, only 35 markers were mapped and covered a distance of 636.7cM. No FSRAP markers were distributed on the 7D chromosome. The results of the present study revealed that the novel FSRAP markers can be used to generate dense, uniform genetic maps of wheat.

Keywords: genetic linkage map, FSRAP markers, RIL population, SRAP markers, wheat

INTRODUCTION

Common wheat (*Triticum aestivum* L.) is one of the most widely grown food crops in the world and is a part of the daily diet of over 70% of the world's population. Therefore, improving the yield potential of wheat is a primary objective of wheat-breeding scientists. The development of modern molecular biology and biotechnology has provided powerful tools, such as molecular markers, for crop breeding. Marker-assisted selection (MAS) is one of the most important techniques in modern plant breeding. MAS compensates for the shortcomings and inaccuracies of traditional selection techniques in crop breeding and accelerates the breeding process. Molecular markers and molecular linkage maps are basic tools for MAS and map-based gene cloning in wheat (SIMONS *et al*., 2006; UAUY *et al*., 2006) and in other crop species (HUANG *et al*., 2003; LIU *et al*., 2015). Wheat is an allohexaploid (2n = 6X = 42) species with A, B, and D genomes. Its large genome is approximately five times the size of the human genome and contains a high proportion of repetitive sequences. Therefore, compared with other crops such as corn and rice, the development of molecular markers for wheat is challenging.

Microsatellite (SSR) and single-nucleotide polymorphism (SNP) markers are currently widely used in the construction of genetic maps of wheat (SOMERS *et al*., 2004; CAVANAGH *et al*., 2013; LI *et al*., 2015). SSR markers are an attractive and valuable tool for population genetic research and genetic map construction because of their codominance and high levels of polymorphism (CERVERA *et al*., 2001; COLE, 2005). SNP markers are redundant in plant genomes and are widely used in crop genetic map construction (BRACHI *et al*., 2011; POLAND *et al*., 2012). Although dominant markers, such as amplified fragment length polymorphism (AFLP) and sequence-related amplified polymorphism (SRAP), are not as efficient as SSR and SNP for genetic map construction, these markers can enhance map density. SRAP markers were first reported by LI and QUIROS (2001) in *Brassica*. SRAP markers are arbitrarily designed to contain AT- and GC-rich motifs that anneal to introns and exons, respectively. Thus, SRAP markers amplify open reading frames (ORFs), including introns and promoter regions. SRAP is a simple and efficient marker system that can be adapted for various purposes in different crops, including map construction, gene tagging, genomic and complementary DNA fingerprinting, and map-based cloning. Moreover, SRAP has several advantages over other marker systems, including simplicity, a reasonable throughput rate, the disclosure of numerous codominant markers, and the easy isolation of bands for sequencing; most importantly, SRAP targets ORFs (LI and QUIROS, 2001). Nevertheless, SRAP products are visualized via silver staining, which is complex, time-consuming, expensive, inefficient, and not conducive for large-scale sample analysis.

In this study, the resolution and efficiency of SRAP markers were greatly improved by the addition of ALEXA 700, a fluorescent label to the 5′ end of the SRAP forward primer. The fluorescent-labeled SRAP markers were tentatively designated as fluorescent sequence-related amplified polymorphism (FSRAP) markers. The FSRAP markers were then used to construct the genetic linkage map of a recombinant inbred line (RIL) wheat population derived from a Chuanmai 42 × Chuannong 16 cross.

MATERIAL AND METHODS

Plant materials and DNA isolation

Mianyang 29 TP (MY29TP), a near-isogenic line (NIL) of the common wheat line Mianyang29 that carries the *Pis1* gene from the three-pistil (TP) mutant (YANG *et al*., 2011), and the recurrent parent *T. aestivum* cv. Mianyang29 (MY29) were used for reproducibility and polymorphism tests. A RIL population with 125 F_8 lines derived by single-seed descent from the F_2 by crossing Chuanmai 42 with Chuannong 16 was used as the mapping population for genetic linkage map construction (TANG *et al*., 2011). All plants were cultivated in the field at China West Normal University in Nanchong, China. The young leaves of MY29TP, MY29, Chuanmai 42, Chuannong 16, and RIL individuals were collected for DNA isolation. Samples were lyophilized and stored at -80 ℃ until use. Total genomic DNA was isolated using the Plant Genomics DNA Kit (TIANGEN Biotech, China) in accordance with the manufacturer's instructions. The concentration and quality of genomic DNA were tested by agarose gel electrophoresis and a ND-2000C spectrophotometer.

FSRAP and SRAP protocol

The primer sequence of FSRAP was consistent with that of SRAP, the only difference being that the forward FSRAP primer was coupled with a fluorescent label, ALEXA 700 at the 5′ end. A total of 1936 primer combinations were generated using 44 fluorescently labeled forward primers and 44 reverse primers (Table 1). A 10-μL volume of FSRAP reaction mixture contained 30 ng DNA, 5μL 2×Taq PCR Master Mix (TIANGEN Biotech, China), and 5μmol of each forward and reverse primer. PCR amplification was conducted in a T100 thermocycler (Bio-Rad, San Diego, USA) with the following cycling conditions: pre-denaturation at 94℃ for 5 min, followed by five cycles of 94℃ for 1 min, 35℃ for 30 s, and 72℃ for 1 min; 35 cycles of 94 ℃ for1 min, 54 ℃ for 1 min, 72℃ for 1 min; and a final extension at 72℃ for 10 min. A total of 0.5μL of stop solution was added to the PCR reaction mixtures, which were then heated at 95°C for 3 min to denature DNA and chilled immediately on ice. Banding patterns were visualized on a 0.3 mm-thick 8% polyacrylamide gel with a 64-tooth comb in a LI-COR 4300 DNA Analyzer (LI-COR Inc., NE, USA) ran at 1500 V with a scan speed of 2 for 3h. A DNA marker (50–700bp) was also loaded to determine the size of the fragments.

SRAP markers were used as a reference. The SRAP primer combinations were Me2-Em13, Me32-Em13, and Me32-Em14 (Table 1). The PCR reaction system and amplification program were the same as described above. The reaction products were mixed with 5μL loading buffer, denatured at 95°C for 3 min, and immediately cooled on ice. The amplification products were run on 8% (w/v) polyacrylamide gel at 200V for 3h in 1×TBE buffer and silver stained (BASSAM *et al*., 1991).

Table 1. FSRAP or SRAP primers used in this study

No.	Forward primer	No.	Reverse primer
Me1	TGAGTCCAAACCGGATA	Em1	GACTGCGTACGAATTAAT
Me2	TGAGTCCAA ACCGGAGC	Em2	GACTGCGTACGAATTTGC
Me3	TGAGTCCAA ACCGGAAT	Em3	GACTGCGTACGAATTGAC
Me4	TGAGTCCAA ACCGGACC	Em4	GACTGCGTACGAATTTGA
Me5	TGAGTCCAA ACCGGAAG	Em5	GACTGCGTACGAATTAAC
Me6	TGAGTCCAA ACCGGTAA	Em6	GACTGCGTACGAATTGCA
Me8	TGAGTCCAA ACCGGTGC	Em8	GACTGCGTACGAATTCTG
Me7	TGAGTCCAA ACCGGTCC	Em7	GACTGCGTACGAATTCCA
Me9	TGAGTCCAA ACCGGAAC	Em9	GACTGCGTACGAATTCGA
Me0	TGAGTCCAA ACCGGATG	Em10	GACTGCGTACGAATTCAG
Me11	TGAGTCCAA ACCGGAGA	Em11	GACTGCGTACGAATTCCA
Me12	TGAGTCCAA ACCGGAGT	Em12	GACTGCGTACGAATTATT
Me13	TGAGTCCAA ACCGGAGG	Em13	GACTGCGTACGAATTACG
Me14	TGAGTCCAA ACCGGACA	Em14	GACTGCGTACGAATTATG
Me15	TGAGTCCAA ACCGGACT	Em15	GACTGCGTACGAATTCGG
Me16	TGAGTCCAA ACCGGACG	Em16	GACTGCGTACGAATTGAT
Me17	TGAGTCCAA ACCGGTAG	Em17	GACTGCGTACGAATTAAG
Me18	TGAGTCCAA ACCGGTAC	Em18	GACTGCGTACGAATTATA
Me19	TGAGTCCAA ACCGGTTG	Em19	GACTGCGTACGAATTATC
Me20	TGAGTCCAA ACCGGTGA	Em20	GACTGCGTACGAATTAGC
Me21	TGAGTCCAA ACCGGTGT	Em21	GACTGCGTACGAATTACT
Me22	TGAGTCCAA ACCGGTCA	Em22	GACTGCGTACGAATTACC
Me23	TGAGTCCAA ACCGGTCT	Em23	GACTGCGTACGAATTTAA
Me24	TGAGTCCAA ACCGGTCG	Em24	GACTGCGTACGAATTTAG
Me25	TGAGTCCAA ACCGGGAA	Em25	GACTGCGTACGAATTTTG
Me26	TGAGTCCAA ACCGGGAT	Em26	GACTGCGTACGAATTTGT
Me27	TGAGTCCAA ACCGGGAG	Em27	GACTGCGTACGAATTTGG
Me28	TGAGTCCAA ACCGGGAC	Em28	GACTGCGTACGAATTTCA
Me29	TGAGTCCAA ACCGGGTA	Em29	GACTGCGTACGAATTTCG
Me30	TGAGTCCAA ACCGGGGT	Em30	GACTGCGTACGAATTTCA
Me31	TGAGTCCAA ACCGGGCA	Em31	GACTGCGTACGAATTGAA
Me32	TGAGTCCAA ACCGGGCT	Em32	GACTGCGTACGAATTGAG
Me33	TGAGTCCAA ACCGGGCG	Em33	GACTGCGTACGAATTGTA
Me34	TGAGTCCAA ACCGGGCC	Em34	GACTGCGTACGAATTGTG
Me35	TGAGTCCAA ACCGGCAA	Em35	GACTGCGTACGAATTGTC

Table 1 continued. FSRAP or SRAP primers used in this study

Me36	TGAGTCCAA ACCGGCAT	Em36	GACTGCGTACGAATTGGA
Me37	TGAGTCCAA ACCGGCAG	Em37	GACTGCGTACGAATTGGT
Me38	TGAGTCCAA ACCGGCAC	Em38	GACTGCGTACGAATTGGC
Me39	TGAGTCCAA ACCGGCTA	Em39	GACTGCGTACGAATTGCT
Me40	TGAGTCCAA ACCGGCTT	Em40	GACTGCGTACGAATTGCG
Me41	TGAGTCCAA ACCGGCTG	Em41	GACTGCGTACGAATTGCC
Me42	TGAGTCCAA ACCGGCTC	Em42	GACTGCGTACGAATTCAT
Me43	TGAGTCCAA ACCGGCCA	Em43	GACTGCGTACGAATTCTC
Me44	TGAGTCCAA ACCGGCCG	Em44	GACTGCGTACGAATTCGC

SSR protocol

To generate a linkage map for wheat using FSRAP markers, 42 SSR markers from the map constructed by TANG *et al*. (2011) for the RIL population of a Chuanmai 42 × Chuannong 16 cross were selected for conjoint analysis with FSRAP markers. The PCR reaction was performed using a T100 thermocycler (Bio-Rad, San Diego, USA) with a 10-μL reaction mixture that contained 50 ng DNA, 5μL of 2×Taq PCR Master Mix (TIANGEN Biotech, China), and 10μmol of each forward and reverse primer. The PCR program for SSR primers included an initial denaturation step at 94°C for 5min; followed by 35 cycles of 94°C for 1 min, 50–60°C for 30 s, and 72°C for 1 min; and a final extension step of 72°C for 10min. Amplicons were separated on 8% (w/v) denaturing polyacrylamide gels and visualized by silver staining (BASSAM *et al*., 1991).

Data analysis and linkage mapping

The presence or absence of polymorphic markers in the mapping population of 125 RIL individuals was analyzed. Unreliable markers were considered missing data. JoinMap version 4.0 was utilized to construct a linkage map with FSARP and SSR data (VAN OOIJEN *et al*., 2006). Map construction excluded markers with obvious segregation distortion from the expected Mendelian segregation ratios 1:1 ($p<0.001$, χ^2 test). Markers were positioned on linkage groups based on the independence LOD threshold values of 2.0–12.0. Linkage analysis and marker ordering were performed using a regression mapping algorithm. Recombination fractions between markers were converted to map distances in cM with the Kosambi mapping function (KOSAMBI *et al*., 1994). Linkage maps were drawn using MapChart 2.2 (VOORRIPS *et al*., 2002).

RESULTS

Reproducibility and polymorphism of FSRAP markers

The Me2-Em13, Me32-Em13, and Me32-Em14 primer combinations were randomly selected for the reproducibility and polymorphism test. Using the same primer combinations, SRAP and FSRAP markers were utilized to amplify DNA from the NIL MY29TP and its recurrent parent MY29. Each primer combination had three biological replicates. For SRAP, the primer combination Me2-Em13, Me32-Em13, and Me32-Em14 amplified two, five, and two bands, respectively, in MY29TP and MY29 (Figure 1a). Furthermore, the three primer

combinations did not amplify different bands between MY29TP and MY29. In MY29TP and MY29, 31, 27, and 27 bands were amplified by the FSRAP marker combinations Me2-Em13, Me32-Em13, and Me32-Em14, respectively. Moreover, Me2-Em13 and Me32-Em13 amplified two different bands, whereas Me32-Em14 amplified one different band (Figure 1b).These results indicated that the FSRAP marker can better reflect the polymorphism of wheat varieties compared with the SRAP marker. The reproducibility test result indicated that three biological replicates showed identical profiles for each primer combination (Figure 1b). Therefore, the FSRAP markers have repeatability and can be used to construct genetic maps of wheat.

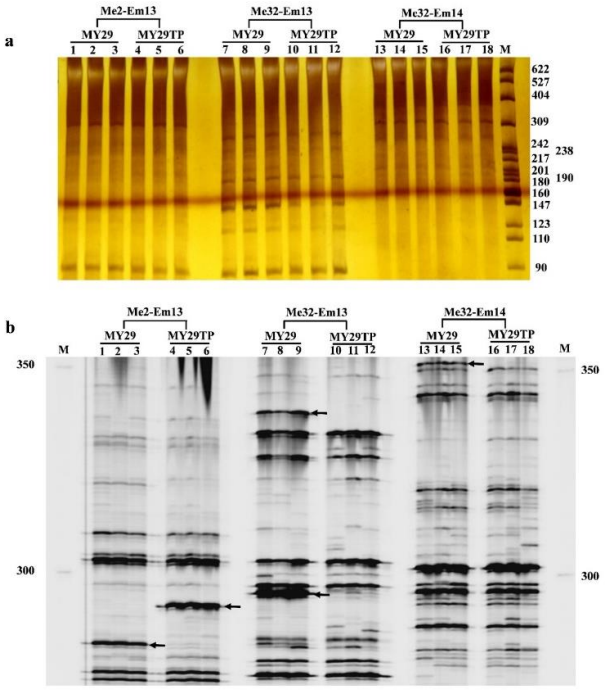

Fig. 1 Amplification results of primer Me2-Em13, Me32-Em13 and Me32-Em14 using SRAP and FSRAP in MY29 and MY29TP. Amplification results using SRAP (a); Amplification results using FSRAP (b). M: DNA marker. The differential bands are indicated by arrows.

Screening of polymorphic markers in the parents

Polymorphisms between the parental lines Chuanmai 42 and Chuannong 16 were screened with 1936 primer combinations, which were generated with 44 fluorescently labeled forward primers and 44 reverse primers. A total of 189 primer combinations identified 430 polymorphic loci between parents. The average polymorphism for each primer combination was 2.28. Among these marker combinations, the primer pairs Me24-Em21, Me25-Em8, and Me32-Em10 produced 10 polymorphic loci; 76 primer pairs produced a single polymorphic locus. Each polymorphic locus was scored as a dominant marker.

Linkage map construction

A genetic linkage map was constructed using 472 markers (42 SSR markers and 430 FSRAP markers), among which 320 markers (39 SSRs and 281 FSRAPs) were mapped into 20 linkage groups (Figure 2). These linkage maps spanned a total length of 2499.3cM with the length of individual chromosomes ranging from 53.7cM (4D) to 269.5cM (4B) (Table 2). The average distance between markers was 7.81cM. The number of FSRAP markers in the different chromosomes ranged from 2 (1D and 6D) to 46 (1B) and were unevenly distributed on each chromosome. A total of 84 markers (71 FSRAP markers and 13 SSR markers) were mapped on the A genome and covered genetic distances of 849.6cM. On the B genome, 201 markers (184 FSRAP markers and 17 SSR markers) were mapped and covered distances of 1013cM. Only 35 markers were mapped on the D genome and covered distances of 636.7cM. No FSRAP markers were distributed on the 7D chromosome. The 1B chromosome contained the majority of markers and had 48 markers, as well as exhibited the highest marker density. On the 1B chromosome, the average genetic distance between the two markers was 1.5cM. The 1D and 4D chromosomes contained the fewest markers and had only two FSRAP markers and one SSR marker. The 4D chromosome had the shortest genetic distance of only 53.7cM. By contrast, the 4B chromosome had the longest genetic distance, with 36 markers that covered distances of 269.5cM.

Table 2 Distribution of the mapped FSRAP markers on 20 chromosomes in wheat

Genome	Chromosome	Length (cM)	Total No.loci	No. loci of FSRAP	No. loci of SSR	Average distance (cM)
A	1A	78.7	12	10	2	6.56
	2A	137.1	11	9	2	12.46
	3A	123.2	8	6	2	15.4
	4A	59.6	5	4	1	11.92
	5A	193.6	27	25	2	7.17
	6A	109.6	16	14	2	6.85
	7A	147.8	5	3	2	29.56
	Subtotal	849.6	84	71	13	10.11
B	1B	72.2	48	46	2	1.5
	2B	172	10	8	2	17.2
	3B	93.9	38	36	2	2.47
	4B	269.5	36	31	5	7.49
	5B	142.1	23	21	2	6.19
	6B	135.2	35	33	2	3.86
	7B	128.1	11	9	2	11.65
	Subtotal	1013.0	201	184	17	5.04
D	1D	92.3	3	2	1	30.77
	2D	184.8	7	5	2	26.4
	3D	112	13	11	2	8.62
	4D	53.7	3	2	1	17.9
	5D	92	5	4	1	18.4
	6D	101.9	4	2	2	25.48
	Subtotal	636.7	35	26	9	18.19

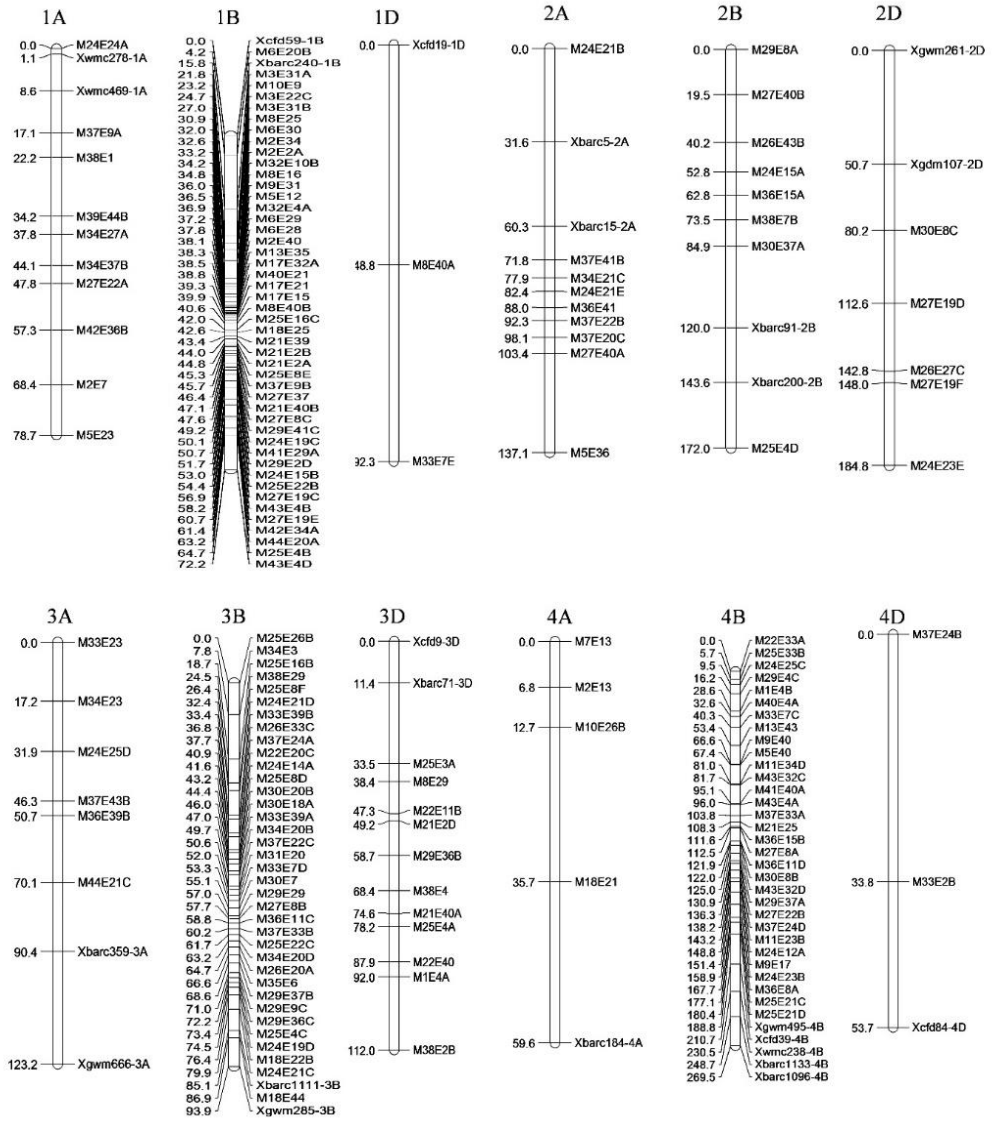

Fig. 2 The genetic linkage map of wheat based on FSRAP markers and SSR markers.

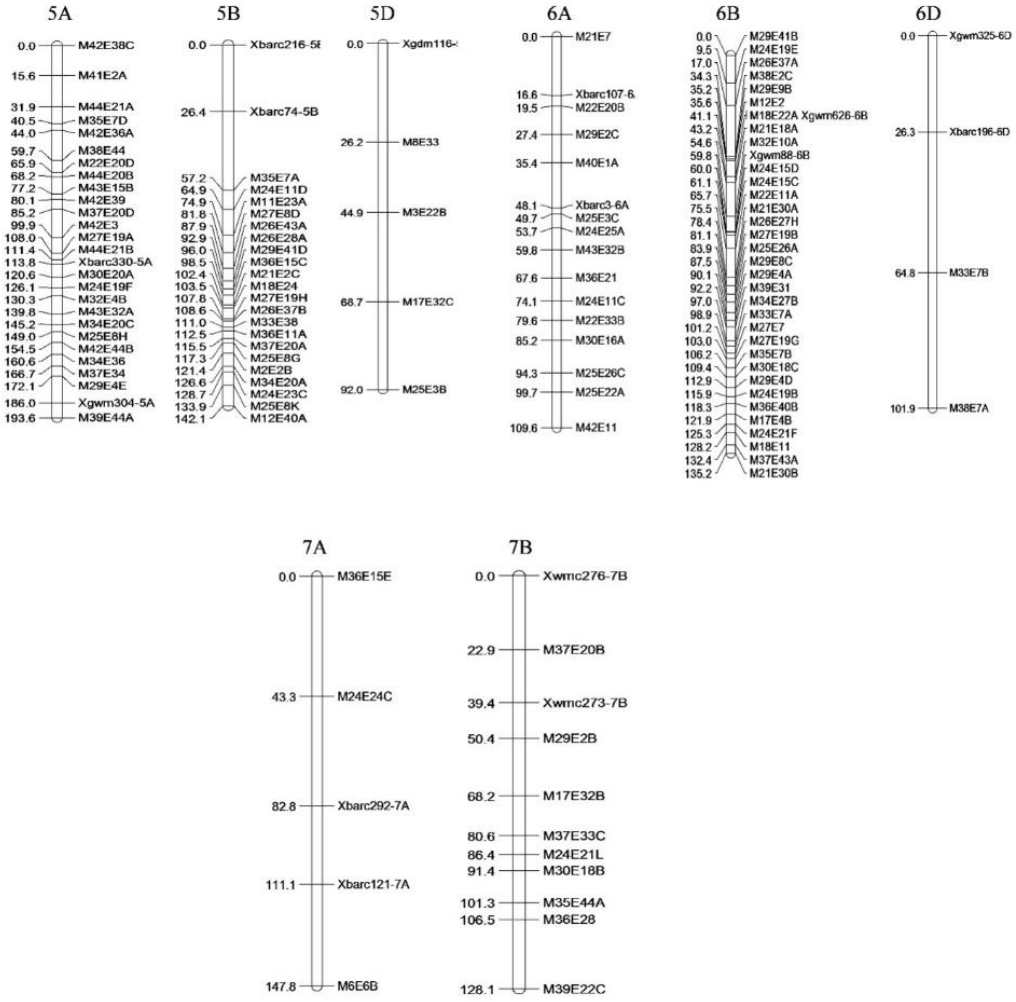

Fig. 2 (Continued from preceding page)

DISCUSSION

Currently, SSR and SNP markers are widely used for the construction of linkage maps in wheat (SOMERS *et al*., 2004; WANG *et al*., 2014; LI *et al*., 2015). Although SSRs are highly informative, polymorphic, and codominant Mendelian markers (POWELL *et al*., 1996), the number of SSR markers in wheat are limited, thus hindering the construction of an ultradense SSR-based linkage map of wheat (YANG *et al*., 2017). In recent years, SNP markers have been rapidly developed and widely used in the construction of linkage maps (SPINDEL *et al*., 2013; LIU *et al*., 2014). However, SNP marker development is very expensive given its requirement for next-generation sequencing technology. Therefore, the development of a simple, efficient, and

inexpensive molecular marker for wheat is crucial. SRAP is a useful marker that is characterized by simplicity, convenience, high repeatability, and ability to amplify ORF regions. Since this system was first reported by LI and QUIROS (2001), SRAP has been successfully used in genetic diversity analysis, comparative genetics, genetic map construction, and other fields (RIAZ *et al*., 2004; SUN *et al*., 2007; LIU *et al*., 2011; GUO *et al*., 2014;). TANG *et al*. (2011) constructed a linkage map of wheat using 184 SSR markers and 34 SRAP markers. Nevertheless, SRAP bands need to be visualized by silver staining, which is complex, time-consuming, expensive, inefficient, and not conducive for large-scale sample analysis. In this study, a fluorescent label, ALEXA 700, was added at the 5′ end of the SRAP forward primer to improve the resolution and efficiency of SRAP markers.

The reproducibility and polymorphism of FSRAP markers were tested using the NIL MY29TP and the recurrent parent MY29. MY29TP and MY29 have similar phenotypes and genetic background except for the TP phenotype (YANG *et al*., 2012). The polymorphic bands were not amplified when used available SSR and AFLP markers (data not given in this study). Using the three SRAP primer combinations, a nonpolymorphic band was amplified between MY29TP and MY29 (Figure 1a). Using the same FSRAP primer combinations amplified polymorphic bands (Figure 1b). In addition, FSRAP markers amplified considerably more bands than SRAP. Therefore, FSRAP has higher resolution than SRAP. There are two main reasons for the high resolution of FSRAP. Firstly, the thickness of the polyacrylamide gel affects resolution: FSRAP products were resolved on 0.3-mm-thick gels, whereas SRAP products were resolved on 1-mm-thick gels. Previous studies have shown that thinner gels provide better resolution than thicker gels (MARSHALL *et al*., 1981). Secondly, the 5′ end of the FSRAP forward primer contains the fluorescent label ALEXA 700, which fluoresces when excited by far-infrared rays of 650–700nm. Therefore, the resolution and detection sensitivity of this visualization method are higher than those of traditional silver staining. Moreover, the electrophoresis results of FSRAP can be detected in real time.

Of the 1936 FSRAP primer combinations generated from 44 fluorescently labeled forward primers and 44 reverse primers, 1760 primer combinations (90.9%) yielded PCR products. Only 189 primer combinations (10.7%), however, yielded 430 polymorphic loci, among which 281 FSRAP markers were mapped into 20 linkage groups. Nevertheless, our results were higher than those reported by previous studies. For example, TANG *et al*. (2011) used 233 SRAP primer combinations to construct a linkage map for the RIL population of a Chuanmai 42 × Chuannong 16 cross. They reported that 34 SRAP markers were mapped into 8 linkage groups (TANG *et al*., 2011). GUO *et al*. (2016) used 612 SRAP primer combinations to construct a linkage map of the F_2 population of a Xinong 981 × Shanmai 159 cross. They reported that only 55 markers were mapped into 8 linkage groups. In this work, 233 SRAP makers and 39 SSR markers were mapped into 20 linkage groups and spanned a total length of 2499.3cM. The B genome exhibited the highest distribution of FSRAP markers with184 markers, followed by the A genome with 71 FSRAP markers. The D genome exhibited the lowest distribution of FSRAP markers with only 35. No FSRAP markers were distributed on the 7D chromosome. This results further confirmed that there were the highest genetic diversity in the B genome, followed by the A genome, and the D genome have the lowest genetic diversity (CAVANAGH *et al*., 2013; WANG *et al*., 2014; YANG *et al*., 2017).The results of the present study revealed that the novel FSRAP markers can be used to generate dense, uniform genetic maps of wheat.

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NOVE FLUORESCENTNE SEKVENCE-VEZANI AMPLIFIRAJUĆI POLIMORFIZAM MARKERI (FSRAP) ZA KONSTRUKCIJUGENETIČKE MAPE PŠENICE (*Triticum aestivum* L.)

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Izvod

Na osnovu SRAP molekularnih markera razvijeni su markeri nove fluorescentne sekvence vezani amplifirajući polimorfizam marker (FSRAP). FSRAP markeri su korišćeni za izgradnju genetske mape populacije rekombinantne inbred linije pšenice (Triticum aestivum L.) izvedene iz ukrštanja Chuanmai 42 × Chuannong 16. Testovi za reprodukciju i polimorfizam pokazali su da FSRAP markeri imaju ponovljivost i bolje odražavaju polimorfizam sorti pšenice u poređenju sa SRAP markerima. Ukupno 430 polimorfnih lokusa između Chuanmai 42 i Chuannong 16 otkriveno je sa 189 kombinacija prajmera FSRAP. Ukupno 281 FSARP markera i 39 SSR markera podeljeno je u 20 grupa povezivanja. Mape su obuhvatile ukupnu dužinu od 2499.3cM sa prosečnom rastojanjem od 7.81cM između markera. Ukupno 201 marker je mapirano na B genomu i pokrivalo je rastojanje od 1013cM. Na A genomu, 84 markera su mapirani i pokrivali su rastojanje od 849.6cM. Na genome D, međutim, mapirano je samo 35 markera i pokrivalo rastojanje od 636.7cM. Nijedan FSRAP marker nije bio distribuiran na 7D hromozomu. Rezultati ove studije pokazali su da se novi FSRAP markeri mogu koristiti za stvaranje gustih, uniformnih genetskih mapa pšenice.

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