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SSR based Heat Stress Related Primers Optimization for Heat Tolerant and Susceptible Genotypes of Wheat (*Triticum aestivum* L.)

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ABSTRACT: PCR has been extensively utilized for the amplification of DNA sequences. In our study, we aimed to identify the optimal amplification conditions for detecting specific SSR polymorphisms associated with heat stress in wheat. Leaf samples from young seedlings were collected, and DNA was isolated from 30 genotypes, representing both heat-tolerant and heat-susceptible types. The extracted DNA from these genotypes underwent PCR optimization. The optimized reaction conditions included a combination of 5 X PCR buffer (3.0 µl), 10 mM $MgCl_2$ (1.2 µl), 200 µM dNTP (3 µl), forward primer (1 µl), reverse primer (1 µl), template DNA (1.5 µl), and 1 unit/ml Taq polymerase (0.5 µl). The PCR protocol comprised an initial denaturation at 95 °C for 7 minutes, followed by 35 cycles of denaturation for 45 seconds at 94°C, annealing for 45 seconds at 48 to 65 °C, extension for 45 seconds at 72°C, and a final extension for 10 minutes at 72 °C. Employing 32 designed SSR primer pairs, the optimized PCR conditions produced well-defined bands for the molecular characterization of heat-tolerant and susceptible wheat genotypes.

Keywords: PCR, SSR Primers, Optimization, Wheat, Heat Tolerant and Susceptible.

INTRODUCTION

Wheat (*Triticum aestivum* L.) serves as a crucial staple crop supporting a substantial portion of the global population. However, its productivity faces increasing challenges from environmental stresses, particularly heat stress, intensified by climate change (Lesk *et al.,* 2016). Elevated temperatures present a significant threat to wheat production, given the crop's high sensitivity to increased temperatures during critical growth stages. Understanding the molecular basis of heat tolerance is essential for developing strategies to cultivate resilient wheat varieties capable of withstanding climate change challenges (Maulana *et al.,* 2018). Polymerase Chain Reaction (PCR) plays a pivotal role in molecular biology research, enabling precise amplification of designated DNA segments. Since its discovery by Saiki *et al*. (1988). PCR has evolved into a crucial instrument in modern molecular biology investigations. Its applications span various fields, including population genetic analysis, food quality testing, varietal selection, genetic resource management, and genomic-assisted crop breeding programs. PCR's reputation for dependability, precision, sensitivity, and rapidity requires fine-tuning under specific conditions for amplifying templates with distinctive traits, such as AT or GC richness or a high abundance of tandem repeats. Optimization is imperative to achieve selective amplification, especially in analytical contexts where suboptimal outcomes may occur during the initial setup involving new template DNA, primers, or the Taq polymerase enzyme. The complexity of the wheat genome and the intricate genetic mechanisms governing heat stress responses have been widely recognized in various studies. Simple Sequence Repeats (SSRs) or microsatellites emerge as valuable molecular markers for studying genetic diversity and mapping important traits in plant species (Varshney *et al.,* 2005). SSRs, with attributes such as high polymorphism, co-dominant inheritance, and reproducibility, serve as an ideal tool for investigating the genetic basis of complex traits, including heat tolerance. This paper focuses on fine-tuning the $MgCl_2$ concentration, DNA template quantity, primer pair concentration, Taq polymerase concentration, and annealing temperature to optimize the amplification of SSR primers specific to heat stress in wheat genotypes. By targeting specific genes associated with the wheat heat stress response and examining both heat-tolerant and susceptible genotypes, the study aims to unravel genetic variations contributing to divergent heat stress responses. The use of SSR markers provides a fine-scale resolution, enabling the identification of allelic variations linked to heat tolerance. This research introduces an economically efficient and targeted amplification procedure, addressing the optimization of $MgCl_2$, template DNA, primers, Taq polymerase, and reaction volume (Kumar *et al.,* 2016). Ultimately, it contributes to advancing our understanding of the molecular aspects of heat tolerance in wheat, offering valuable insights for the development of robust crop varieties (Frona *et al.,* 2019).

MATERIAL AND METHODS

DNA extraction was carried out on 30 wheat genotypes (refer to Table 1). The DNA samples extracted from these 30 genotypes were utilized for PCR optimization. The experiment took place in the Plant Genomics laboratory within the Division of Plant Biotechnology at Sardar Vallabhbhai Patel University of Agriculture and Technology in Meerut, Uttar Pradesh.

A. DNA Isolation

Genomic DNA extraction from bulked leaf samples was conducted utilizing the CTAB method. The process involved the use of Cetyl-trimethyl-ammonium bromide (CTAB), an effective cationic detergent, which aided in membrane solubilization and formed a complex with DNA. Following cell disruption and incubation with hot CTAB isolation buffer, proteins were removed using chloroform: isoamyl alcohol, and the CTAB-DNA complex was precipitated with isopropanol. The resulting DNA pellet, obtained through centrifugation, underwent a series of steps, including washing, drying, and redissolving. To eliminate RNA and certain polysaccharides, the protocol incorporated RNase treatment and sodium acetate precipitation. The genomic DNA extraction process involved the following steps: 300 mg of frozen leaves from each genotype were crushed in liquid nitrogen using a mortar and pestle, and 1 ml of pre-warmed (65°C) extraction buffer was added. The samples were homogenized by inverting the tubes and then subjected to a 1-hour incubation in a shaking water bath at 65°C. After cooling at room temperature, 800 µl of chloroform: iso-amyl alcohol (24:1) was gently added, and the tubes were inverted for 15-20 minutes. Subsequent centrifugation at 10,000 rpm for 10 minutes at 4°C separated the aqueous phase (supernatant), which was transferred to a fresh Eppendorf tube. The supernatant was mixed with 3-5 µl of (10 mg/ml) RNase and incubated for 30 minutes. After incubation, 600 µl of isopropanol was added, gently mixed for 3-5 minutes, and then centrifuged at 8,000 rpm for 10 minutes. The supernatant was discarded, and the pellet was washed with 70% ethanol before being dried in a laminar airflow. Finally, the pellet was re-suspended in 100 µl of TE buffer and stored at 4°C for immediate use. For long-term storage, double the volume of absolute ethanol was added, and the samples were stored at (-80°C). This streamlined extraction protocol ensures the preservation of high-quality genomic DNA from frozen leaf samples. The DNA was then prepared for qualitative and quantitative analysis using agarose gel electrophoresis and a Nanodrop Spectrophotometer, respectively.

Table 1: Information regarding the wheat genotypes employed in the current investigation.

Sr. No.	Genotypes	Pedigree	Origin
1.	HI8713	HD-4672/PDW-233	IARI, Regional Station, Indore
2.	DBW14	RAJ3765/PBW343	IIWBR, Karnal
3.	DBW90	HUW-468/WH-730	IIWBR, Karnal
4.	DBW93	WHEAR/TUKURU//WHEAR	IIWBR, Karnal
5.	HD2329	HD-2252/UP-262	IARI, New Delhi
6.	HD2864	K7537/HD2160/HD2278//L24/K4.14	IARI, New Delhi
7.	HD2888	STAMPALLI/C-306//DL-153-2/HW-2003	IARI, New Delhi
8.	HD2932	KAUZ/STAR/HD 2643	IARI, New Delhi
9.	HD2967	ALD/CUC//URES/HD2160/HD2278	IARI, New Delhi
10.	HD2985	PBW 343/ PASTOR	IARI, New Delhi
11.	HD3086	DBW14/HD2733//HUW468	IARI, New Delhi
12.	HS375	BLUEBIRD/GALLO//CAJEME-71/3/TR.AE//KALYANSONA/BLUEBIRD	IARI Regional Station, Tutikandi, Shimla
13.	HS 490	HS-364/HPW-114//HS-240/HS-346	IARI, RRS, Shimla
14.	HS507	KAUZ/MYNA/VULTURE//BUCKBUCK/FLICKER/4/MILAN	IARI, RRS, Shimla
15.	HUW234	HUW-12*2/CPAN-1666//HUW-12	BHU, Varanasi
16.	PBW 343	ND/VG1944//KAL//BB/3/YACO's/4/VEE5's'	PAU, Ludhiana
17.	PBW644	PBW-175/HD-2643	PAU, Ludhiana
18.	RAJ4083	PBW-343/UP-2442//WR-258/UP-2425	RAU, Durgapura
19.	WH1021	NY0T95/SONAK	CCS HAU, Hisar
20.	WH1080	PRL/2*PASTOR	CCS HAU, Hisar
21.	WH1124	MUNIA/CHTO/AMSEL	CCS HAU, Hisar
22.	WH147	E4870-C303/S339-PV18	CCS HAU, Hisar
23.	DBW71	PRINIA/UP2425	IIWBR, Karnal
24.	WH1142	CHENAEGILOPSSQUARROSA(TA US)//FCT/3/2*WE AVER	CCS HAU, Hisar
25.	PBW723	PBW343+Lr57/Yr40+Lr37/Yr17	PAU, Ludhiana
26.	HD4728	ALTAR84/STINT// SILVER 453/ SOMAT 3.1/4/ GREEN14/YAV10 /AUK	IARI, New Delhi
27.	WB02	DICOCCON CI9309/AE. SQUARROSA(409)/3/MILAN/S87230/ / BAV92/4/2*MILAN/S8732 0//BA	IIWBR, Karnal
28.	PBW757	PBW550/YR15/6*AVOCET/3/2*PBW550/4/ PBW568+YR36/3*PBW550	PAU, Ludhiana
29.	HD3226	GRACKLE/HD2894	IARI, New Delhi
30.	DBW303	WBLL1*2/BRAMBLING/4/BABAX/LR42//BABAX*2/3/SHAMA*2/5/PBW343*2/ KUKUNA*2//FRTL/PIFED	IIWBR, Karnal

B. Analysis for Quality and Quantity

The quality of the isolated DNA samples was assessed through 0.8% agarose gel electrophoresis. The positioning of DNA within the gel was determined by staining with a low concentration of ethidium bromide, serving as a fluorescent intercalating dye. DNA concentrations were quantified using the Nanodrop Spectrophotometer, with 1 µl of each DNA sample loaded onto the Nanodrop for analysis. The purity of the recovered total DNA was verified by measuring the absorbance ratio at (260-280) nm. A sample with an absorbance (A260/A280) ratio within the range of 1.85-1.95 was considered of the highest quality. The final DNA concentration was adjusted by dilution using TE buffer

C. Optimization of the PCR Master Mix

The concentrations of PCR components, including $MgCl_2$, Taq polymerase, DNA template, and primers, were fine-tuned (refer to Table 2) with a total reaction volume set at 15 µl for each.

D. Designing Primers and Optimizing PCR

A set of 32 SSR markers was designed for optimization using the BatchPrimer3 web-based application with 30 wheat genotypes (Pachauri *et al.,* 2014). These SSR markers are located on chromosome 2B, 7B, and 7D, contributing to their identification and characterization for heat stress response in wheat (Patidar *et al.,* 2015). To create a working solution, the primers were dissolved in an appropriate amount of distilled water, resulting in a concentration of approximately 10 µg/µl. For the identification of the optimal PCR amplification temperature, DNA templates were randomly selected from five genotypes. Eight different annealing temperatures, ranging from 50 to 65.5 ºC, were applied to all primers. The selective amplification of the targeted genomic region was carried out using a standardized polymerase chain reaction (PCR) protocol optimized for laboratory conditions. The PCR reaction mixture for a 15 µl volume was prepared as follows: 3.8 µl of nuclease-free water, 3.0 µl of 5X PCR buffers, 1.2 µl of 2mM MgCl2, 3 µl of 10mM dNTP mix, 1 µl of 10µM forward primer, 1 µl of 10µM reverse primer, 1.5 µl of DNA template (40-80 ng/µl), and 0.5 µl of Taq polymerase. The PCR amplification was carried out using a thermal cycler with a program standardized for this purpose, involving an initial denaturation for 7 minutes at 95 °C, followed by 35 cycles of denaturation for 45 seconds at 94 °C, annealing for 45 seconds at 50 to 65.5 °C, extension for 45 seconds at 72 °C, and a final extension for 10 minutes at 72 °C.

E. Electrophoretic Separation of Amplified Products on Agarose Gel

To create a 2% agarose gel, 2g of agarose was dissolved in 100 ml of 1X TAE using a volumetric flask. The solution underwent microwave heating with intermittent mixing until complete agarose melting occurred, resulting in a clear solution. After cooling to 60 °C, ethidium bromide (2-3µl) was added. The liquid gel was then carefully poured into the gel casting plate of the gel casting unit to avoid bubble formation and allowed to solidify at room temperature for 20-30 minutes. Following solidification, the comb was gently removed to create wells. The gel was placed in the migration chamber connected to the power pack, and 1X TAE running buffer was added to submerge the gel. For monitoring DNA movement, a gel loading dye (composed of 40% sucrose and 0.25% bromophenol blue) was added to the amplified products. Samples, along with a 100 bp DNA ladder for molecular size determination, were loaded into the wells using a micropipette and micro-tips. Electrophoresis was conducted by applying 80V for 45 minutes. The amplified products were subsequently visualized indirectly under UV light (332 nm) using the gel documentation system from Bio-Rad.

Findings and Discussion

The DNA purity across all samples consistently exhibited an absorption ratio within the specified range. These findings are in agreement with prior research (Ferdous *et al.,* 2012), suggesting that a satisfactory DNA purity falls within the absorption ratio (A260/A280) range of 1.80 to 2.0, and the absorption ratio at A260/230 is 2.0-2.22 for impurity-free DNA (Arruda *et al.,* 2017). The quantity of DNA in the genomic DNA samples obtained from 30 wheat genotypes was evaluated by measuring the optical density at 260/280 nm. Details of the optical density of DNA samples from various wheat genotypes can be found in Table 2. The data revealed the minimum DNA amount in the HS507 sample (177.66 ng/µl), while the maximum DNA amount was present in the RAJ4083 sample (346.51 ng/µl). Variations in the extracted DNA quantity were attributed to measurement errors and differential grinding of leaf samples from different varieties. In most instances, the absorbance ratio (the ratio of absorbance at 260 and 280 nm) fell within the range of 1.80 to 1.95, indicating the superior quality of DNA in samples extracted from leaves at the seedling stage across different genotypes in the current investigation. A graphical representation of the absorbance of DNA samples, their absorbance ratio at 260 and 280 nm, and the quantity of DNA (ng/µl) is presented in Fig. 1 for enhanced comparison.

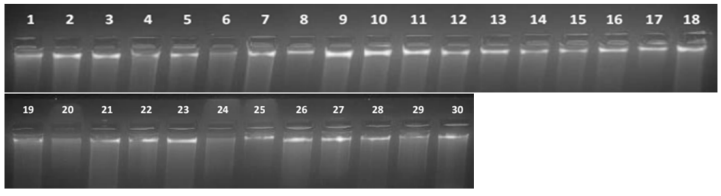

Fig. 1. The genomic DNA obtained from the leaves during the seedling stage of the thirty wheat genotypes utilized in this study.

Table 2: The absorbance levels of DNA samples and the quantity of DNA in samples derived from thirty wheat genotypes employed in the study.

Sr. No.	Genotypes	Concentration ng/µl	O.D. (A260/280)
1.	HS 490	232.93	1.82
2.	HS507	276.83	1.79
3.	DBW303	250.54	1.77
4.	DBW93	206.70	1.71
5.	HD2329	182.68	1.78
6.	HD2967	238.97	1.78
7.	HD2868	241.83	1.82
8.	HD3086	355.75	1.83
9.	HD2932	334.05	1.86
10.	HD4728	262.25	1.87
11.	HD2864	251.19	1.83
12.	HD2932	188.26	1.88
13.	HD2985	185.08	1.81
14.	HS507	177.66	1.72
15.	HUW234	182.21	1.83
16.	PBW343	197.61	1.84
17.	PBW644	210.69	1.76
18.	HI8713	181.39	1.85
19.	RAJ4083	346.51	1.82
20.	WH1021	232.45	1.86
21.	WH1124	252.75	1.81
22.	PBW71	312.79	1.84
23.	DBW14	221.71	1.83
24.	DBW90	212.81	1.73
25.	PBW757	279.64	1.82
26.	WH1080	288.43	1.83
27.	WH147	264.35	1.87
28.	PBW723	220.13	1.84
29.	WB02	242.31	1.83
30.	WH1142	320.23	1.81

To achieve successful PCR amplification and select polymorphic bands that can be reliably reproduced, specific concentrations of various reagents were crucial. The optimization process focused on $MgCl_2$, Taq polymerase, DNA template, and primer concentrations, while keeping buffer concentration and dNTPs constant. Following established PCR protocols (Padmalatha and Prasad 2006; Anerao *et al.,* 2016; Kumar *et al.,* 2018; Subedee *et al.,* 2020), the buffer and dNTPs concentrations were maintained at 3 µl in all optimized conditions (see Table 3). Magnesium ions play a crucial role as a cofactor for Taq polymerase activity, and their concentration was tested at various levels (0, 1.0, 1.2, 1.5, 2.0, and 3 µl). Optimal results were achieved with 1.2 µl of $MgCl_2$ per PCR Master Mix (15 µl), showing precise and reproducible amplification with clear bands bands (Batra *et al.,* 2006) (Fig. 2). Taq polymerase optimization involved testing different concentrations (0, 0.1, 0.2, 0.3, 0.6, and 1.0 µl). Gel electrophoresis revealed that all concentrations produced PCR product amplification, but clear bands were observed with 0.1 µl of Taq polymerase (Fig. 2). Template concentrations (0, 0.5, 1, 1.5, 2.0, and 3.0 µl) were optimized, indicating that concentrations between 0.5 and 2 µl reproduced amplified PCR products. However, band clarity decreased with 0.5 µl template DNA compared to higher concentrations (3.0 µl), which either did not amplify or resulted in an almost invisible smear (Fig. 2). Forward and reverse primer concentrations (0, 0.2, 0.5, 1.0, 1.5, and 2.0 µl) were individually optimized, with a concentration of 1.0 µl for both forward and reverse primers demonstrating desirable band clarity in the PCR amplified products (Fig. 2). PCR reaction buffers were chosen, and 3 µl resulted in precise PCR amplification (Shahzad *et al.,* 2020). The concentration of dNTPs, serving as substrates for the PCR reaction, was set at 3 µl of 200 µM dNTPs. Ultimately, the optimized PCR reagents, determined to produce sharp bands, were 5X PCR buffer (3.0 µl), 10 mM MgCl2 (1.2 µl), 200 µM dNTPs (3 µl), forward primer (1.2 µl), reverse primer (1.2 µl), template DNA (1.5 µl), and 1 unit/ml Taq polymerase (0.5 µl) (Table 2 and Fig. 2). The optimal annealing temperature for the PCR setup was 49- 60°C, but it is emphasized that achieving the optimal annealing temperature is crucial for obtaining high-quality amplified products in the form of distinct bands. Higher annealing temperature may lead to a lack of amplification, while too low annealing temperature increases the chances of non-specific binding, potentially causing primer dimer formation (Vestheim and Jarman 2008). Optimizing the annealing temperature can help diminish the likelihood of primer dimer formation (Ekman, 1999). The optimal annealing temperature for the chosen primers, as determined in this study, distinctly demonstrates that the 32 SSR primers based on heat stress-related candidate genes exhibited positive amplification at eight different optimal temperatures (Moraga *et al.,* 2012). Utilizing the primer pair of Barc128 in polymerase chain reaction, successful amplification was achieved in all genotypes (McPherson and Moller 2006; Lorenz, 2012). A single polymorphic band was visualized among the genotypes representing the amplified

product This primer pair revealed the amplified products differential molecular weight, which varied between 170-180 bp, Genotypes HS 490, HS507, DBW303, HI8713, PBW757, HS375, WH1021, WH147 and WH1124 shows amplified band at 172.21 bp. Genotypes namely, HD2329 HD2967, HD2868, HD3086, DBW93, HD4728, DBW90, PBW71, PBW 343, WH1080, WH1142 and HD3236 shows amplified band at 175.58 bp and RAJ4083, DBW14, HD2864, HD2985, HD2932, PBW644,WB02, HUW234 and PBW723 showed amplified band at 178.23 bp (Fig. 2).

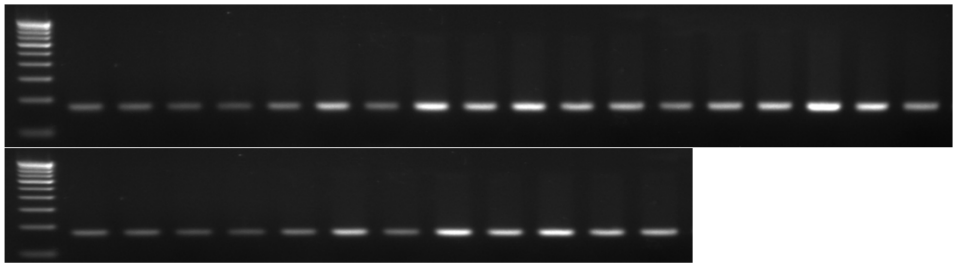

Fig. 2. Amplification Profile of Targeted Genomic Region in Thirty Wheat Genotypes Using Xbarc128 Primers.

Table 3: PCR Optimization with Primer Barc128 at 60°C using Different Reagent Volumes.

Reagent	**$MgCl_2$**						**Reagent**	**Taq**					
H_2O	5	4	3.8	3.5	3	2	H_2O	4.3	4.2	4.1	4	3.7	3.3
$MgCl_2$	0	1	1.2	1.5	2	3	$MgCl_2$	1.2	1.2	1.2	1.2	1.2	1.2
PCR Buffer	3	3	3	3	3	3	PCR Buffer	3	3	3	3	3	3
dNTP	3	3	3	3	3	3	dNTP	3	3	3	3	3	3
F-Primer	1	1	1	1	1	1	F-Primer	1	1	1	1	1	1
R-Primer	1	1	1	1	1	1	R-Primer	1	1	1	1	1	1
DNA Template	1.5	1.5	1.5	1.5	1.5	1.5	DNA Template	1.5	1.5	1.5	1.5	1.5	1.5
Taq	0.5	0.5	0.5	0.5	0.5	0.5	Taq	0	0.1	0.2	0.3	0.6	1
Reagent	**DNA Template**						**Reagent**	**Primers**					
H_2O	5.3	4.8	4.3	3.8	3.1	2.3	H_2O	5.8	5.4	4.8	3.8	2.8	1.8
$MgCl_2$	1.2	1.2	1.2	1.2	1.2	1.2	$MgCl_2$	1.2	1.2	1.2	1.2	1.2	1.2
PCR Buffer	3	3	3	3	3	3	PCR Buffer	3	3	3	3	3	3
dNTP	3	3	3	3	3	3	dNTP	3	3	3	3	3	3
F-Primer	1	1	1	1	1	1	F-Primer	0	0.2	0.5	1	1.5	2
R-Primer	1	1	1	1	1	1	R-Primer	0	0.2	0.5	1	1.5	2
DNA Template	0	0.5	1	1.5	2	3	DNA Template	1.5	1.5	1.5	1.5	1.5	1.5
Taq	0.5	0.5	0.5	0.5	0.5	0.5	Taq	0.5	0.5	0.5	0.5	0.5	0.5

Table 4: Concentration of optimized PCR components utilized in master mix.

Reagents	**Final Concentration**	**Volume (15µl)**
Nuclease free water	—	3.8
5X PCR buffer	1X	3.0
2mM Mgcl2	0.2 mM	1.2
10 mM dNTPs mix	0.2 mM	3.0
10µM forward primer	0.5 µM	1.0
10µM reverse primer	0.5 µM	1.0
Template DNA	50-90 ng/ µl	1.5
Taq Polymerase	0.1 unit/ µl	0.5

Table 5: Optimal annealing temperature for Heat stress related 32 SSR primers.

Sr. No.	**Annealing Temperature (°C)**	**Total primers**	**Name of the SSR primer**
1.	57	1	Barc159
2.	58	5	Xbarc128, Wms0935, Xgwm148, Xgwm374, Xbarc123
3.	53	6	Gwm257, Xgwm526, Xgwm131, Xgwm577, Gwm111.2, Xgwm294
4.	59	8	Cfa2278, Xgwm614, Wmc517, Xgwm297, Xgwm130, Xgwm121, Wmc597, Xgwm400
5.	49	2	Barc101, Barc70
6.	63	4	Xwmc311, Xwmc557, Xbarc87, Xbarc111
7.	61	2	Xwmc500, Xgwm350
8.	62	2	Xwmc473, Xcfd41

Table 6: Summary of information for heat stress related 32 SSR primers-based PCR optimization located on 2B, 7B and 7D chromosome.

Sr No.	Primer Name	Primer Sequence (5′-3′)	Chromosome Location	Ta (°C)	Good amplification	Moderate amplification	No amplification	Product Size
1	Barc159	F-CGCAATTTATTATCGGTTTTAGGAA R-CGCCCGATAGTTTTTCTAATTTCTGA	2B	57	✓			240
2	Xbarc128	F-GCGGGTAGCATTTATGTTGA R-CAAACCAGGCAAGAGTCTGA	2B, 7D	58	✓			180
3	Gwm257	F-AGAGTGCATGGTGGGACG R-CCAAGACGATGCTGAAGTCA	2B	53	✓			350
4	Cfa2278	F-GCCTCTGCAAGTCTTTACCG R-AAGTCGGCCATCTTCTTCCT	2B	59	✓			200
5	Wms0935	F-GTCCGCCACCTCCTCTG R-GAGCACACCCTGTTGCG	2B	58	✓			175
6	Xgwm614	F-GATCACATGCATGCGTCATG R-TTTTACCGTTCCGGCCTT	2B	59		✓		230
7	Wmc597	F-AACACACCTTGCTTCTCTGGGA R-GACTAGGGTTTCGGTTGTTGGC	2B	59	✓			190
8	Barc101	F-GCTCCTCTCACGATCACGCAAAG R-GCGAGTCGATCACACTATGAGCCAATG	2B	49	✓			120
9	Xgwm526	F-CAATAGTTCTGTGAGAGCTGCG R-CCAACCCAAATACACATTCTCA	2B	53		✓		320
10	Xgwm148	F-GTGAGGCAGCAAGAGAGAAA R-CAAAGCTTGACTCAGACCAAA	2B	58	✓			240
11	Xgwm374	F-ATAGTGTGTTGCATGCTGTGTG R-TCTAATTAGCGTTGGCTGCC	2B	58	✓			170
12	Xgwm131	F-AATCCCCACCGATTCTTCTC R-AGTTCGTGGGTCTCTGATGG	7B	53	✓			182
13	Xgwm577	F-ATGGCATAATTTGGTGAAATTG R-TGTTTCAAGCCCAACTTCTATT	7B	53		✓		235
14	Wmc517	F-ATCCTGACGTTACACGCACC R-ACCTGGAACACCACGAC	7B	59	✓			217

		AAA						
15	Xgwm400	F-GTGCTGCCACCACTTGC R-TGTAGGCACTGCTTGGGAG	7B	59	✓			145
16	Xbarc123	F-GGCCGAATTGAAAAAGCC R-CCTGCCGTGTGCCGACTA	7B	58		✓		222
17	Xgwm297	F-ATCGTCACGTATTTTGCAATG R-TGCGTAAGTCTAGCATTTTCTG	7B	59	✓			290
18	Xwmc311	F-GGGCCTGCATTTCTCCTTTCTT R-CTGAACTTGCTAGACGTTCCGA	7B	63	✓			275
19	Xwmc500	F-ATAGCATGTTGGAACAGAGCAC R-CTTAGATGCAACTCTATGCGGT	7B	61	✓			165
20	Xwmc273	F-AGTTATGTATTCTCTCGAGCCTG R-GGTAACCACTAGAGTATGTCCTT	7B	60		✓		320
21	Xwmc557	F-GGTGCTTGTTCATACGGGCT R-AGGTCCTCGATCCGCTCAT	7B	63	✓			310
22	Gwm111.2	F-TCTGTAGGCTCTCTCCGACTG R-ACCTGATCAGATCCCACTCG	7D	53	✓			265
23	Xgwm294	F-GGAGTTAAGAGAGAACCG R-GCAGAGTGATCAATGCCAGAGGATT	7D	53	✓			310
24	Xgwm428	F-AGCGTTCTTGGGAATTAGAGA R-CCAATCAGCCTGCAACAAC	7D	58	✓			250
25	Xgwm130	F-AGCTCTGCTTCACGAGGAAG R-CTCCTCTTTATATCGCGTCCC	7D	59	✓			315
26	Barc70	F-GCGAAAAACGATGCGACTCAAAG R-GCGCCATATAATTCAGACCCACAAAA	7D	49	✓			190
27	Xgwm121	F-TCCTCTACAAACAAACACCAC R-CTCGCAACTAGAGGTGTATG	7D	59	✓			195
28	Xcfd41	F-TAAAGTCTCAGGCGACCCAC R-AGTGATAGACGGATGGCACC	7D	62	✓			280
29	Xgwm350	F-ACCTCATCCACATGTTCTACG	7D	61		✓		360

		R-GCATGGATAGGACGCCC						
30	Xwmc473	F-TCTGTTGCGCGAAACAGAATAG R-CCCATTGGACAACACTTTCACC	7D	62	✓			440
31	Xbarc87	F-GCTCACCGGGCATTGGGATCA R-GCGATGACGAGATAAAGGTGGAGAAC	7D	63		✓		275
32	Xbarc111	F-GCGGTCACCAGTAGTTCAACA R-GCGTATCCCATTGCTCTTCTTCACTAAC	7D	63	✓			175
F- Forward Primer R- Reverse Primer Ta- Annealing Temperature, PS-Amplified Product Size								

CONCLUSIONS

In conclusion, our study demonstrated consistent DNA purity across all samples, aligning with established absorption ratio ranges for satisfactory DNA quality. The quantity of DNA varied among 30 wheat genotypes, with the HS507 sample exhibiting the minimum and the RAJ4083 sample displaying the maximum DNA amount. Variations in DNA quantity were attributed to measurement errors and differential grinding of leaf samples. The absorbance ratios mostly fell within the range of 1.80 to 1.95, indicating superior DNA quality in samples from seedling stage leaves across diverse genotypes. To ensure successful PCR amplification and reproducible polymorphic bands, we optimized concentrations of various reagents. The PCR setup included specific concentrations for $MgCl_2$, Taq polymerase, DNA template, and primers, while maintaining constant buffer and dNTPs concentrations. Optimal results were achieved with 1.2 µl of $MgCl_2$, 0.1 µl of Taq polymerase, template DNA concentrations between 0.5 and 2 µl, and 1.0 µl for both forward and reverse primers. The chosen PCR reagent concentrations produced sharp bands, and the optimal annealing temperature for successful amplification of SSR primers based on heat stress-related candidate genes was determined to be 49-60°C. The primer pair Barc128 exhibited positive amplification in all genotypes, revealing polymorphic bands with distinct molecular weights. This comprehensive optimization process enhances the reliability of PCR results and contributes to the robustness of genomic studies in wheat genomics.

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Conflict of Interest. None.

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