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PRODUCTION OF A PCR-BASED MARKER FOR DETECTING *PSATHYROSTACHYS HUASHANICA* KENG CHROMOSOMES IN A WHEAT BACKGROUND

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Abstract

In this study, we developed a genome-specific DNA sequence for detecting the incorporation of *Psathyrostachys huashanica* Keng chromatin into wheat. Random amplified polymorphic DNA (RAPD) analysis was used to identify genome-specific DNA sequences of *P. huashanica*, which were selected using 21 different plant species. A 716-bp diagnostic band specific to *P. huashanica* (pHs24) was cloned, sequenced, and converted into a sequence-characterized amplified region (SCAR) marker, designated as RHS107. The sequence of pHs24 had no significant homology with any sequences deposited in NCBI databases, which showed that it was a novel repetitive *P. huashanica* sequence. A primer pair flanking this specific sequence was designed and a genome-specific SCAR marker for *P. huashanica* was developed and characterized. We validated its specificity using 21 different plant species and a complete set of wheat-*P. huashanica* disomic addition lines (1Ns–7Ns, 2*n* = 44 = 22 II) that carried different *P. huashanica* chromosomes. Our results indicated that the SCAR marker targeted only the Ns genome of *P. huashanica* and it was present in all seven of the *P. huashanica* chromosomes. The SCAR marker developed in this study was a reliable and rapid method for large-scale screening of the introgression of *P. huashanica* chromosomes in wheat-*P. huashanica* derivatives.

Key words: *Psathyrostachys huashanica*, RAPD, addition lines, SCAR, wheat, Marker-assisted selection.

Introduction

Wild relatives of wheat (*Triticum aestivum* L.) in the tribe Triticeae possess many biotic and abiotic resistance genes that can be exploited as agronomically important traits via wide hybridization, which means they are a potentially valuable resource for wheat improvement (Graybosch, 2001; Friebe *et al*., 1996). In particular, *Psathyrostachys huashanica* Keng (2*n* = 2x = 14, NsNs) is a perennial cross-pollinating plant in the subfamily *Pooideae*, tribe *Triticeae*, family *Poaceae*, which is generally found only on the residual soil of mountainous braes and rocky slopes in the Huashan Mountains, Shaanxi Province, China (Baden, 1991; Kuo, 1987). This species has many excellent agronomic traits, including early maturity, disease resistance, and drought tolerance, so it is a useful resource for wheat improvement (Chen *et al*., 1991; Cao *et al*., 2008; Kang *et al*., 2009). Recently, we developed a complete set of wheat-*P. huashanica* disomic addition lines and we identified novel disease resistance and agronomic traits derived from *P. huashanica,* which were introduced into cultivated wheat (Du *et al*., 2013a; Du *et al*., 2013b).

P. huashanica is considered to be an excellent wild species with resistance to wheat stripe rust (Li *et al*., 2012; Du *et al*., 2013a), which can also improve the fecundity (Du *et al*. 2013b) and quality (Zhao *et al.* 2010) of cultivated wheat. In a previous study, our research team successfully produced an F_1 hybrid H881 (2*n* = 28, ABDNs) (Chen *et al*., 1991) and a heptaploid hybrid H8911 (2*n* = 49, AABBDDNs) (Chen *et al*., 1996) between common wheat cv. 7182 and *P. huashanica* via embryo culture, two rounds of backcrossing, and selfing, which were verified by cytology, genomic *in situ* hybridization (GISH), EST-SSR, EST-STS, glutenin, gliadin, and morphological analysis. We then developed a complete set of wheat-*P. huashanica* disomic addition lines related to *P. huashanica*, i.e., 1N (Du *et al*., 2014d), 2Ns (Du *et al*., 2014c), 3Ns (Du *et al*., 2014b), 4Ns (Du *et al*., 2014a), 5Ns (Du *et al*., 2013a), 6Ns (Du *et al*., 2013b), and 7Ns (Du *et al*., 2013c). Large populations of introgression lines were produced by the backcrossing and selfing with wheat to generate hybrids, which probably contained one or more exogenous chromosomes or fragments. Thus, suitable methods for the rapid and accurate identification of alien chromosomes in wheat and the detection of genetic modifications in a wheat background are essential before the use of these lines in breeding programs (Zhang *et al*., 2007).

Cytogenetic techniques, including chromosome banding and *in situ* hybridization, have been used to screen wheat lines containing alien chromatin, but they are limited because of their high technological demands and their low-scale screening applicability (Vaillancourt *et al*., 2008). By contrast, molecular markers based on the direct analysis of DNA sequence variations facilitate the rapid screening of large populations (Katto *et al*., 2004; Qamar *et al*., 2014). Recently, many studies have used random amplified polymorphic DNA (RAPD) analysis to develop sequence-characterized amplified region (SCAR) markers, which is an accurate and reliable technique (Turi *et al*., 2012). This technique can be used to develop markers based on specific PCR primers derived from RAPD fragments for testing whether wheat lines contain alien chromatin, e.g., from *Thinopyrum elongatum* (Host) D.R. Dewey (Xu *et al*., 2012), *Agropyron cristatum* (L.) Gaertn. (Wu *et al*., 2010), and *Thinopyrum intermedium* (Hu *et al*., 2012). In the present study, we analyzed RAPD patterns to develop a reliable SCAR marker for tracking *P. huashanica* chromatin inclusion in a wheat background. This SCAR marker could be a powerful tool for screening wheat and *P. huashanica* genetic material in a large genetic pool.

Materials and Methods

Plant materials: The RAPD polymorphic analysis used 21 distinct species (Table 1), which included common wheat cv. 7182 (AABBDD, 2*n* = 6*x* = 42) and *P. huashanica* Keng (NsNs, 2*n* = 2*x* = 14). These species were provided partly by the Center for Crop Germplasm Resources Research (CGRR) at the Institute of Crop Science, CAAS, Beijing, China. A complete set of wheat-*P. huashanica* disomic addition lines (1Ns–7Ns, 2*n* = 44 = 22 II) was produced by our own laboratory and used to validate the chromosomal location of the *P. huashanica*-specific SCAR markers, as shown in Table 2. These specimens were deposited at the Shaanxi Key Laboratory of Genetic Engineering for Plant Breeding, College of Agronomy, Northwest A&F University, Shaanxi, China.

Table 2. A complete set of wheat *P. huashanica* disomic addition lines (1Ns–7Ns) and its parents, common wheat cv. 7182 and *P. huashanica* of genetic constitution.

Plant code	2*n*	Chromosome composition (*P. huashanica* homoelogous pair)
P. huashanica	14	14 Ns
7182	42	42 W
12-3	44	42 W + 2 Ns (1)
3-6-4-1	44	42 W + 2 Ns (2)
22-2	44	42 W + 2 Ns (3)
24-6-3	44	42 W + 2 Ns (4)
3-8-10-2	44	42 W + 2 Ns (5)
59-11	44	42 W + 2 Ns (6)
2-1-6-3	44	42 W + 2 Ns (7)

Ns and W: *Psathyrostachys huashanica* and wheat chromosomes, respectively, which were determined by GISH using *P. huashanica* genomic DNA as a probe, EST-SSR and STS multiple-loci markers from seven wheat homoeologous groups, glutenin and gliadin

Table 1. List of the wild relatives of wheat used in this study.

	Species	Ploidy	Genome	Origin
Common wheat cultivars	*Triticum aestivum* L. (7182)	6×	AABBDD	Our research group
Rare species	*Psathyrostachys huashanica* Keng	2×	NsNs	
	Triticum monococcum L.	2×	AA	
	Triticum dicoccoides Korn.	4×	AABB	
	Triticum araraticum Jakubz.	4×	AAGG	
	Triticum zhukovskyi Men. et Er.	6×	AAAAGG	
Wild relative species	*Aegilops markgrafii* (Greuter) Hammer	2×	CC	The Chinese Academy of Agricultural Sciences
	Aegilops tauschii (Coss.) Schmal.	2×	DD	
	Thinopyrum elongatum	2×	EE	
	Hordeum violaceum	2×	HH	
	Hordeum vulgare L.	2×	II	
	Crithopsis delileana (Schult) Roshev	2×	KK	
	Aegilops comosa Sm. in Sibth. & Sm.	2×	MM	
	Agropyron cristatum Gaertn.	6×	PPPPPP	
	Eremopyrum orientale	4×	B′B′C′C′	
	Triticum timopheevii Zhuk.	4×	AtAtGG	
	Secale cereale L.	2×	RR	
	Aegilops speltoides Tausch	2×	SS	
	Roegneria ciliaris (Trin) Nevski	4×	SSYY	
	Elymus rectisetus	6×	SSYYWW	
	Pseudoroegneria strigosa A. Love	2×	StSt	

DNA extraction and RAPD amplification: Genomic DNA was extracted from young leaf tissue using the cetyl trimethylammonium bromide (CTAB) method (Cota-Sánchez *et al*., 2006), with some modifications. Two-hundred 10-mer RAPD primers, which were synthesized by Sangon Biotech (Shanghai), were selected for preliminary amplification using samples from the 21 different species. The PCR amplification reaction mixture (20 µl) contained 2 µl 10× PCR buffer, 5 µl primer (2.5 µmol/ml), 5 µl DNA template (50–100 µg/µl), 1.6 µl dNTPs (2.5 µmol/ml), 1.6 µl $MgCl_2$ (2.5 mmol/ml), 0.1 µl *Taq* polymerase (5 U/µl), and 4.7 µl ddH_2O. The PCR program comprised 4 min at 94°C; 45 cycles at 94°C for 30 s, 45 s at 34°C, and 1.5 min at 72°C; and a final 10-min extension at 72°C. The PCR products were separated on 1% agarose gel in 1× TAE buffer and stained with ethidium bromide (EB), before they were visualized using an automatic gel imaging analysis system.

Cloning and sequencing of the specific RAPD product: PCR bands were amplified (716 bp) by the random primer OPBB05 (ACGCAGGCAC, annealing temperature 34ºC) were excised from the 1% agarose gel and extracted using a gel extraction kit (TaKaRa). The recovered DNA fragments were cloned into the pMD19-T vector and transformed into *Escherichia coli* DH5a-competent cells by heat shock transformation. Positive colonies were selected by blue/white screening. Plasmids were extracted from randomly selected white colonies using a plasmid kit. Nucleotide sequencing was performed at Sangon Biotech (Shanghai). The BLAST program in the GenBank database was used to search for similar DNA sequences via BLASTn and BLASTx.

Design of specific primers and amplification of the target fragments: Based on the cloned sequence of the RAPD products, a pair of specific PCR primers (F: GGGCCGAACAAAGCCGACAGGAC, R: GGGCCG AACATGCGAAAACGTAA, annealing temperature 74ºC) i.e., RHS107 was designed using Primer 5.0 and Oligo 6.0, and synthesized by Sangon Biotech (Shanghai). The primer pairs were used to amplify *P. huashanica* DNA to identify a species-specific SCAR marker. The PCR reaction mixture (20 µl) contained 2 µl 10× PCR buffer, 2 µl primer (2.5 µmol/ml), 2 µl DNA template (50–100 ng/µl), 1.6 µl dNTPs (2.5 µmol/ml), 1.6 µl $MgCl_2$ (2.5 mmol/ml), 0.2 µl *Taq* polymerase (5 U/µl), and 10.6 µl ddH_2O. The amplification procedure comprised an initial denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 50 s, 74°C for 50 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The annealing temperature was optimized empirically for each primer pair. The amplified products were resolved by electrophoresis on 1% agarose gels, as described previously.

Tracking *P. huashanica* chromatin in wheat-*P. huashanica* derivatives: Species-specific amplification was performed using the SCAR primer pair RHS107 to test the genomic DNA of the complete set of disomic addition lines (1Ns–7Ns, $2n = 44 = 22$ II) and the parents, common wheat cv. 7182 and *P. huashanica*. The amplification, electrophoresis and imaging were conducted as described previously.

Results

RAPD marker: Of the 200 primers screened, 30 produced clear and distinct patterns in all samples (data not shown). Of these, the primer OPBB05 generated a polymorphic band that distinguished *P. huashanica* from the other plant species (Fig. 1). This result indicated that the OPBB05 marker was sufficient to differentiate between *P. huashanica* and the other species tested so it was suitable for identifying *P. huashanica* chromatin.

Sequence analysis: The *P. huashanica* genome-specific RAPD band was cloned and sequenced. Its full length was 716 bp and it was designated pHs24 (GenBank accession no. HR614223). The BLASTn and BLASTx searches showed that this sequence shared no homology with sequences deposited in public databases. Thus, we hypothesized that pHs24 was a new repetitive DNA sequence.

Validation of the SCAR primer RHS107: Based on the nucleotide sequence of pHs24, a pair of SCAR primers, RHS107, was designed to test the validity of the molecular marker. The SCAR primer RHS107 was tested to determine whether it could amplify a *P. huashanica* genome-specific band from 21 different plant species, i.e., Ns, ABD, A, AB, AG, AAG, C, D, E, H, I, K, M, PPP, B′C′, AtG, R, S, SY, SYW, and St in Table 1. The PCR amplification results showed that the target band of 716-bp was only present in *P. huashanica* because it was not amplified from the other genomes (Fig. 2). This suggested that the marker RHS107 may be an Ns genome-specific SCAR marker.

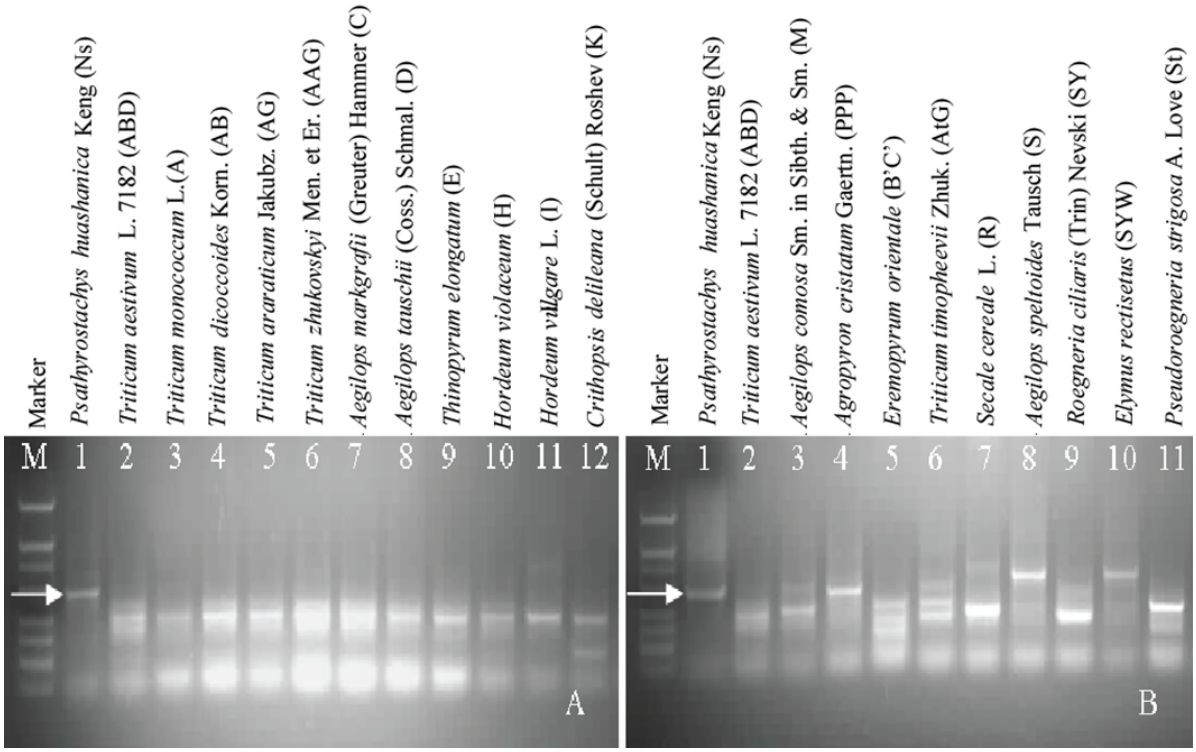

Fig. 1. The RAPD marker $OPBB05_{716}$ was linked to the *P. huashanica* genome. (A) M, marker;1, Ns; 2, ABD; 3, A; 4, AB; 5, AG; 6, AAG; 7, C; 8, D; 9, E; 10, H; 11, I and 12, K genomes. (B) M, marker; 1, Ns; 2, ABD; 3, M; 4, PPP; 5, B′C′; 6, AtG; 7, R; 8, S; 9, SY; 10, SYW; and 11, St genomes. The arrow indicates the species-specific diagnostic band of *P. huashanica*. The full species names are listed in Table 1.

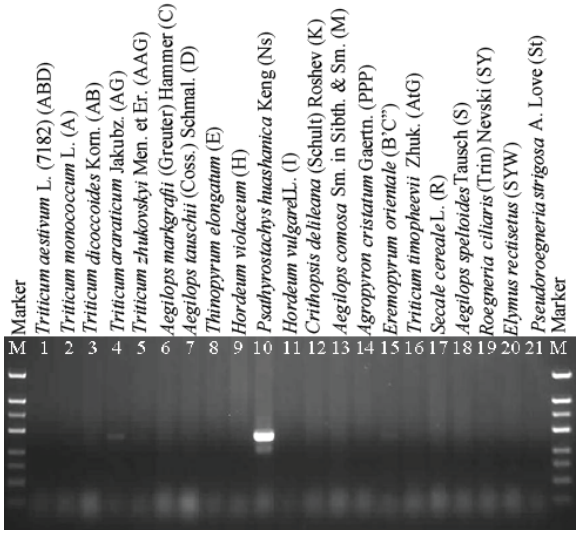

Fig. 2. *P. huashanica* chromatin identification using the SCAR primer RHS107 based on Triticeae species. M, marker; 1, ABD; 2, A; 3, AB; 4, AG; 5, AAG; 6, C; 7, D; 8, E; 9, H; 10, Ns; 11, I; 12, K; 13, M; 14, PPP;15, B′C′;16, AtG; 17, R; 18, S; 19, SY; 20, SYW; and 21, St genomes. The final band indicates the *P. huashanica* genome-specific markers. The full species names are listed in Table 1.

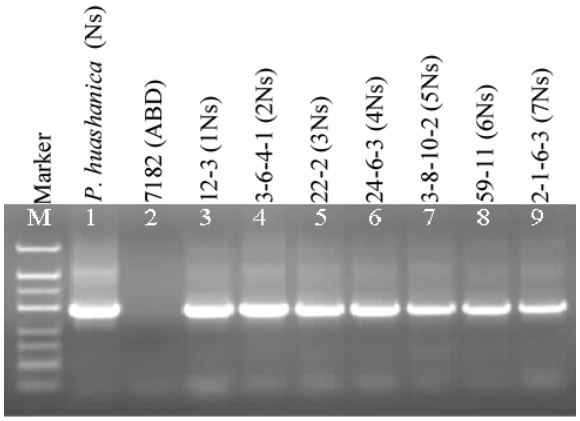

Fig. 3. Amplification patterns of a complete set of wheat-*P. huashanica* disomic addition lines (1Ns–7Ns, 2*n* = 44 = 22II), which were generated using the specific SCAR primer RHS107. M, marker; 1, *P. huashanica*; 2, 7182; 3, 12-3 1Ns; 4, 3-6-4-1 2Ns; 5, 22-2 3Ns; 6, 24-6-3 4Ns; 7, 3-8-10-2 5Ns; 8, 59-11 6Ns; 9, 2-1-6-3 7Ns.

Specific amplification using wheat-*P. huashanica* addition lines: We used the SCAR primer RHS107 to test a complete set of wheat-*P. huashanica* disomic addition lines (1Ns–7Ns, 2*n* = 44 = 22 II). The amplification results indicated that the target band of 716-bp was present in *P. huashanica* and all of the addition lines, whereas it was absent from common wheat cv. 7182 (Fig. 3). The results showed that the target DNA band was present in all seven lines, which indicated that pHs24 had amplification sites in all of the *P. huashanica* chromosomes so it could be used as a marker-assisted selection (MAS) tool for tracking *P. huashanica* chromatin.

Discussion

The introgression of alien genes from tertiary gene pool species could increase the genetic diversity and agronomic performance of cultivated wheat (Able *et al*., 2007). Distant hybridization has played an important role in the introduction of exogenous genes from wheat-related species into wheat (Jiang *et al*., 1994). *P. huashanica* is a novel germplasm resource that has been transferred successfully into common wheat via wide hybridization. After several years of screening and identification, a complete set of wheat-*P. huashanica* disomic addition lines (1Ns–7Ns, 2*n* = 44 = 22 II) was developed. However, it is time-consuming and difficult to isolate each homoeologous group addition line and to clarify the relationships in a large and chaotic genetic pool. Traditional cytogenetic techniques have been used to screen wheat lines containing alien chromatin but they are not suitable for breeding programs, which require the rapid screening of large numbers of genotypes (Vaillancourt *et al*., 2008). Thus, it is important to develop rapid and accurate methods for identifying *P. huashanica* chromatin before its utilization. DNA molecular marker techniques based on the sequence variation in specific genomic regions are powerful identification tools because they are rapid, require less labor, and are more efficient (Dongre & Parkhi, 2005; Garg *et al*., 2006; Sultan *et al*., 2013).

PCR-based markers are simple and rapid methods for tracking genetic material from alien species in a wheat background, particularly specific molecular markers related to unique, single-copy segments of the genome that can be considered co-dominant and can be used in closely related species (Shan *et al*., 1999). RAPD markers have many advantages, such as high speed, low cost, high polymorphism, and a requirement for only tiny amounts of plant material (Williams *et al*., 1990; Zeb *et al*., 2011). RAPD is a widely used technique in plant genome analysis but its weaknesses are its poor reproducibility and sensitivity. In practical applications, researchers usually produce SCAR markers from the longer primers used for RAPD sequences. This approach has significantly improved the reproducibility and reliability of PCR when tracking genetic material derived form *Sorghum bicolor* (L.) Moench (Singh *et al*., 2006), *Secale africanum* (Jia *et al*., 2009), *Hordeum chilense* (Hernández *et al.* 1999), Sesame (Akbar *et al*., 2011) and *Aegilops cylindrica* Host. (Schoenenberger *et al*., 2005). In the present study, we analyzed the RAPD patterns to develop a reliable SCAR marker for discriminating *P. huashanica* chromatin in *P. huashanica*-wheat derivatives. The results showed that our SCAR marker could be a rapid and convenient method for detecting *P. huashanica* chromatin during the large-scale screening of samples.

Plant breeders may use MAS in conventional breeding programs because molecular markers have several advantages compared with traditional phenotypic markers, i.e., no environmental impact and they can be used for detection at all stages of plant growth (Cao *et al*., 2001; Jan *et al*., 2011). The use of RAPD markers is a simple and inexpensive technique, which facilitates more rapid and simpler detection compared with restriction

fragment length polymorphism (RFLP) (Williams *et al*., 1990; Pervaiz *et al*., 2010). Many RAPD markers have been successfully converted into SCAR markers linked to desirable resistance genes or agronomic traits for use in MAS (Srivastava *et al*., 2012; Gupta *et al*., 2006). Amplified fragment length polymorphism (AFLP) is a very powerful tool for DNA fingerprinting but the development of specific sequence primers from AFLP markers for use in SCAR markers is not an efficient process in wheat (Shan *et al*., 1999). The SCAR marker developed in the present study for detecting *P. huashanica* chromatin was based on the sequence of the RAPD marker OPBB05. The sequence amplified was the same size as that using the RAPD marker OPBB05 but it was expressed more intensely and was easier to recognize, while the use of the SCAR marker also overcome the poor reproducibility of RAPD markers. This method will be very useful for tracking *P. huashanica* chromatin in early wide crosses and is should help breeders to improve the efficiency of selection.

Conclusion

RAPD technique was used to develop a SCAR marker for indentifying *P. huashanica* chromatin in a wheat background. This SCAR marker could be used as a MAS tool to facilitate the screening of *P. huashanica* chromatin in large gene pools. This SCAR marker should be suitable for authenticating homoeologous group plants containing introduced *P. huashanica* chromatin. We employed a simple and reliable approach for generating effective and specific molecular markers to assist *P. huashanica* chromosome testing.

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