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A novel PCR-based marker for identifying Ns chromosomes in wheat-*Psathyrostachys huashanica* Keng derivative lines

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Abstract

Psathyrostachys huashanica Keng is an endangered species that is endemic to China, which provides an important gene pool for wheat improvement. We developed a quick and reliable PCR-based diagnostic assay to accurately and efficiently detect *P. huashanica* DNA sequences from introgression lines, which was based on a species-specific marker derived from genomic DNA. The 900-bp PCR-amplified band used as a *P. huashanica*-specific RAPD marker was tested with 21 different plant species and was converted into a sequence-characterized amplified region (SCAR) marker by cloning and sequencing the selected fragments (pHs11). This SCAR marker, which was designated as RHS-23, could clearly distinguish the presence of *P. huashanica* DNA repetitive sequences in wheat-*P. huashanica* derivative lines. The specificity of the marker was validated using 21 different plant species and a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22II$). This specific sequence targeted the Ns genome of *P. huashanica* and it was present in all the seven *P. huashanica* chromosomes. Therefore, this SCAR marker is specific for *P. huashanica* chromosomes and may be used in the identification of alien repetitive sequences in large gene pools. This diagnostic PCR assay for screening the target genetic material may play a key role in marker-assisted selective breeding programs.

Additional key words: addition lines; marker-assisted selection; repetitive sequences; RAPD; SCAR.

Introduction

The genome of *Psathyrostachys huashanica* Keng ($2n = 2x = 14$, NsNs) contains many desirable characteristics, such as resistance to biotic and abiotic stresses, which make it suitable for wheat improvement (Kang *et al.,* 2009). Several *P. huashanica* genes have already been introgressed successfully into the wheat (*Triticum aestivum* L.) genome (Zhao *et al.,* 2010; Du *et al.,* 2013a,b). In the 1990s, we generated (Chen *et al.,* 1991, 1996) the F_1 hybrid H881 ($2n = 28$, ABDNs) and a derived heptaploid H8911 ($2n = 49$, AABBDDNs) of common wheat cv. 7182 and *P. huashanica* (GenBank Acc. No. 0503383) via embryo culture, two backcrosses, and selfing. As a result, we generated a large and complex range of wheat-*P. huashanica* offspring that carried different chromosome number or structure variants, *i.e.,* chromosome additions, deletions, and rearrangements. However, a major problem was the selection of recombinant plants derived from the large interbred population. After several years of screening and identification, a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22II$) was developed. This was a repetitive and difficult work. Although cytogenetic techniques proved to be very useful tools for alien identification, they had limitations because of the small population number, the complexity of the experimental techniques, and envi-

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Abbreviations used: AFLP (amplified fragment length polymorphism); EST (expressed sequence tag); GISH (genomic *in situ* hybridization); MAS (marker-assisted selection); RAPD (random amplified polymorphic DNA); SCAR (sequence-characterized amplified region); SSR (simple sequence repeat); STS (sequence tagged site).

ronmental effects. Therefore, an alternative approach was needed urgently to facilitate the rapid and accurate identification of alien chromatin from incorporated lines, which is essential before they can be utilized.

The choice of a DNA marker depends on the specific scale and the aims of identification. Some molecular markers techniques have already been applied successfully to identifying *P. huashanica* chromatin, *i.e.,* EST-SSR and EST-STS (Du *et al.,* 2013a,b), as well as for mapping *P. huashanica* resistance genes, *i.e.,* SSR (Li *et al.,* 2012) and AFLP (Cao *et al.,* 2008). In particular, random amplified polymorphic DNA (RAPD) markers are simple and cost-effective, while they require no previous sequence information and can be conducted using a small sample as the template DNA (Masojé *et al.,* 2001). Several RAPD markers have been developed for the effective screening of wheat lines containing *P. huashanica* chromatin (Kang *et al.,* 2008), However, the short primers and low annealing temperatures mean RAPD markers are extremely sensitive to the reaction conditions, none of which are suitable for large-scale selection because of their low reproducibility and specificity (Goulão *et al.,* 2001; Quian *et al.,* 2001). To overcome these problems, longer primers have been developed from RAPD fragment sequences to generate sequence-characterized amplified region (SCAR) markers, thereby allowing the amplification of a target DNA fragment by PCR.

SCAR markers are generally simpler to use and more suited to high-throughput applications than RAPD markers (Chowdhury *et al.,* 2001; Bautista *et al.,* 2002). It has been used to develop molecular markers for genes that confer resistance to scald (Genger *et al.,* 2003), barley yellow dwarf (Zhang *et al.,* 2004), leaf rust (Gupta *et al.,* 2006), dwarf bunt (Gao *et al.,* 2010), and common bunt (Zhang *et al.,* 2012), as well as for specific genomes including *Secale africanum* Stapf. (Jia *et al.,* 2009), *Agropyron cristatum* (L.) Gaertn. (Wu *et al.,* 2010), *Thinopyrum elongatum* (Host) D.R. Dewey (Xu *et al.,* 2012), and *Thinopyrum intermedium* (Host) Barkworth and Dewey (Hu *et al.,* 2012). Thus, SCAR markers are the most practical method for screening large number of samples in a time-efficient and labor-saving manner because they are accurate, easy to use, and cost efficient (Kasai *et al.,* 2000). In this study, we isolated a new repetitive DNA sequence from *P. huashanica* by RAPD analysis and converted it into a stable Ns-chromosome-specific SCAR marker based on a comparative analysis. The SCAR marker was used to authenticate large-scale populations of wheat-*P. huashanica* derivatives and to detect Ns chromosomes in wheat-*P. huashanica* introgression lines. This diagnostic marker was designed to facilitate the tracing of *P. huashanica* genome sequences in a wheat background, which will improve the efficiency of targeting genetic material; it is suitable for high-throughput diagnosis.

Material and methods

Plant materials

We used genome samples of 21 different species (Table 1) for RAPD polymorphic analysis, including common wheat cv. 7182 (AABBDD, $2n = 6x = 42$) and *P. huashanica* Keng (NsNs, $2n = 2x = 14$). These species were provided partly by the Center for Crop Germplasm Resources Research (CGRR) at the Institute of Crop Science, CAAS, Beijing, China. We also used a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22$II) that carried different *P. huashanica* chromosomes, *i.e.,* each line included all 42 wheat chromosomes and a pair of alien *P. huashanica* chromosomes, 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.

DNA extraction and specific RAPD marker selection

The genomic DNA was extracted from leaf tissue using the CTAB method (Cota-Sanchez *et al.,* 2006) with a slight modification. To identify useful primers for this study, we used two-hundred 10-mer RAPD primers, which were synthesized by Sangon Biotech (Shanghai, China), to screen diagnostic markers of *P. huashanica* genome in 21 different species samples. The PCR amplification reaction mixture (20 µL) contained 2 µL 10 × PCR buffer, 5 µL primer (2.5 µmol mL^{-1}), 5 µL DNA template (50-100 µg μL^{-1}), 1.6 µL dNTPs (2.5 µmol mL^{-1}), 1.6 µL $MgCl_2$ (2.5 mmol mL^{-1}), 0.1 µL *Taq* polymerase (5 U μL^{-1}), 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×

Table 1. List of 21 different plant species used to screen the *P. huashanica*-specific RAPD marker

	Species	Ploidy	Genome	Origin
Common wheat cultivars	7182 (*Triticum aestivum* L.)	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 caudata L.	2×	CC	
	Aegilops tauschii (Coss.) Schmal.	2×	DD	
	Thinopyrum elongatum	2×	EE	
	Hordeum violaceum	2×	HH	
	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.	6×	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	

Table 2. The genetic constitution of the complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22II$) and its parents, common wheat cv. 7182 and *P. huashanica* (Acc. No. 0503383)

Plant code	2n	Chromosome composition[1] (*P. huashanica* homologous pair)
Parents		
P. huashanica	14	14 Ns
Wheat 7182	42	42 W
Addition lines		
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)

[1] Ns and W indicate *P. huashanica* and wheat chromosomes, respectively. The complete set of wheat-*P. huashanica* disomic addition lines were verified by morphology, cytology, genomic *in situ* hybridization (GISH), EST-SSR, EST-STS, glutenin and gliadin analyses.

TAE buffer, stained with ethidium bromide, and visualized using an automatic gel imaging analysis system.

Cloning and sequencing of the RAPD product

The putative marker OPL05 amplified (≈900 bp) by the random primer (ACGCAGGCAC, annealing temperature 34°C) was excised from 1% agarose gel, then purified and extracted using a TaKaRa Agarose Gel DNA Purification Kit (Takara, Japan). The selected DNA fragments were ligated into the pMD19-T vector and transformed into *Escherichia coli* DH5α-competent cells by heat shock transformation. Positive colonies were determined by blue/white screening. The white colonies were picked from LB-ampicillin plates and the recombinant DNA was extracted using a plasmid kit for each overnight cultured colony. Both ends of each DNA insert were sequenced by Sangon Biotech (Shanghai, China). The DNA sequence was submitted to GenBank (Acc. No. HR614210). Homology searches were performed in the GenBank nonredundant database using BLASTn and BLASTx via the National Center for Biotechnology Information (NCBI) website.

Specificity and sensitivity of the SCAR marker

Based on the sequence of the cloned fragments, we designed a pair of oligonucleotide primers named RHS23 (F: ACGCAGGCACGTTCTGATGACTACT, R: ACGCAGGCACCAAATAACAATTATT, annealing temperature 69°C). These primers were used to amplify 21 different species samples to confirm the specificity of the *P. huashanica* SCAR marker. The PCR reaction mixture (20 μL) contained 2 μL 10× PCR buffer, 2 μL primer (2.5 μmol mL^{-1}), 2 μL DNA template (50-100 ng μL^{-1}), 1.6 μL dNTPs (2.5 μmol mL^{-1}), 1.6 μL $MgCl_2$ (2.5 mmol mL^{-1}), 0.2 μl *Taq* polymerase (5 U μL^{-1}), 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, 72°C for 1 min, and a final extension at 72°C for 10 min. The annealing temperature was optimized empirically for the primer pair. The amplified products were resolved by electrophoresis on 1% agarose gels, as described previously.

Detection of *P. huashanica* genome sequences in addition lines

The corresponding SCAR marker primer pair of *P. huashanica* was designed based on the sequence of the RAPD marker. This was evaluated as a tool to test the validity of the molecular marker in a complete set of disomic addition lines (1Ns – 7Ns, $2n = 44 = 22$ II) and their parents, common wheat cv. 7182 and *P. huashanica*. The PCR reactions, electrophoresis, and imaging were performed as reported previously.

Results

Identification of a RAPD marker for *P. huashanica*

Two-hundred primers with arbitrary sequences were screened and 30 primers produced distinct, reproducible amplification profiles with all of the screened DNA samples (data not shown). Of these, the primer OPL05 consistently amplified a single, intense band of approximately 900-bp that was specific to *P. huashanica*, but absent from the other 20 species (Fig. 1). This band (pHs11) was selected as a putative *P. huashanica*-specific marker and used to develop the SCAR marker.

Sequence analysis

The cloned DNA fragments of interest were sequenced and the length of pHs11 was found to be 900-bp (Fig. 2; Suppl. Fig. 1[pdf]). A BLAST search showed that the pHs11 sequence did not have homology with any sequence deposited in public databases.

Validation of the SCAR primer RHS23

A pair of specific SCAR primers, RHS23, was designed based on the nucleotide sequence of pHs11 to test its specificity and efficiency. The primer pair was designed to produce the full-length RAPD fragment. In some cases, the SCAR was shorter than the initial RAPD fragments because the reverse primer was in-

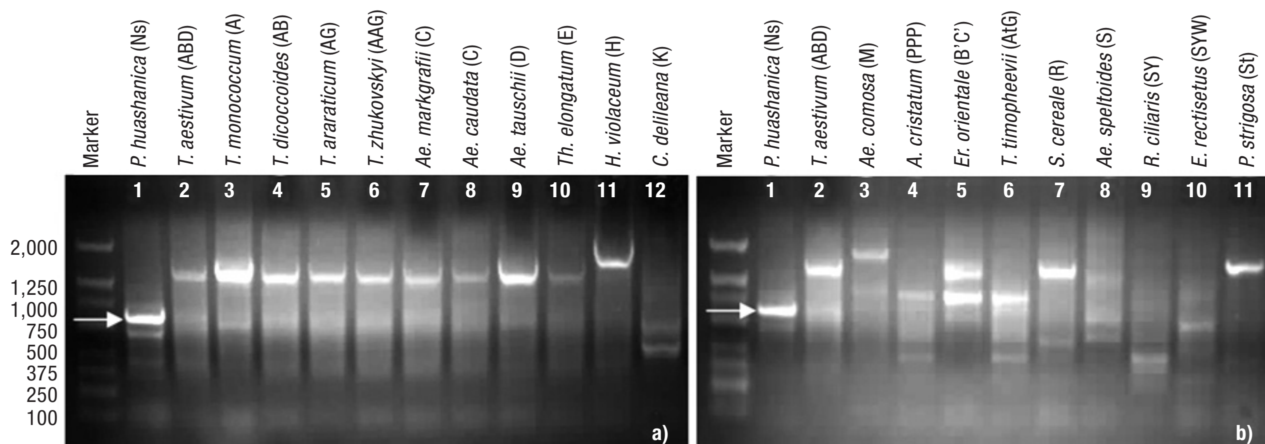

Figure 1. RAPD patterns of 21 different plant species (a) and (b) generated using the primer $OPL05_{900}$. The arrow indicates the species-specific diagnostic band of *P. huashanica.* The full species names are listed in Table 1.

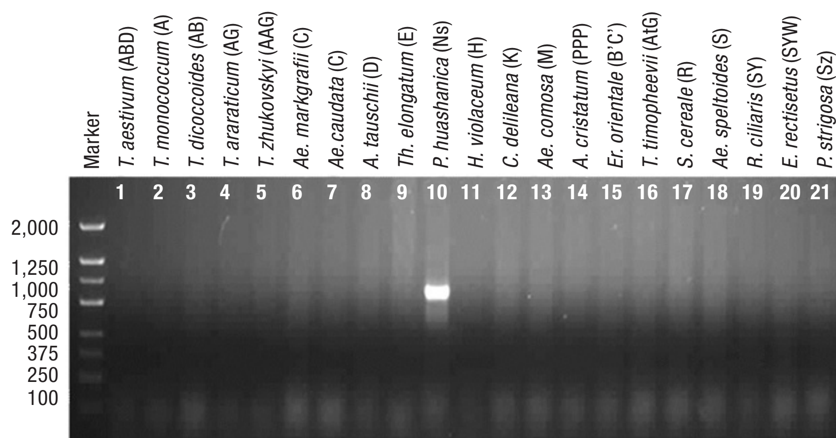

Figure 2. Amplification patterns for *P. huashanica* generated using the specific SCAR primer RHS23. The observable band indicates the *P. huashanica* genome-specific marker. The full species names are listed in Table 1.

ternal to the RAPD fragments so the annealing temperature was optimized. The PCR primer pair was further validated using 21 different plant species. The PCR amplification product with primer pair RHS23 had high specificity and it clearly distinguished the expected 900-bp specific band present in the *P. huashanica* genome, which was absent from the ABD, A, AB, AG, AAG, C, C, D, E, H, K, M, PPP, B'C', AtG, R, S, SY, SYW, and St genomes. This confirmed that the RHS23 marker was a novel, Ns-genome-specific DNA marker for *P. huashanica*, and the species specificity of the marker.

SCAR marker for identifying *P. huashanica* genome sequences

The practical applicability of the marker was tested by detecting the presence of *P. huashanica* in wheat-*P. huashanica* addition lines. The primer pair designed to produce the genome-specific SCAR marker was used to amplify the genomic DNA of a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22II$) and their parents, common wheat cv. 7182 and *P. huashanica*, to determine the chromosomal locations of the SCAR marker sequence. This showed that RHS23 produced a very intense marker at 900-bp (see Fig. 3), which was present in *P. huashanica* and in all the seven wheat-*P. huashanica* addition lines (1Ns to 7Ns), whereas it was absent from the maternal parent (the common wheat cv. 7182). Being the marker present on all the seven *P. huashanica* chromosomes, it could be used as a marker-assisted selection (MAS) tool for tracking *P. huashanica* chromatin.

Discussion

Although genetic diversity in wheat was reduced during its domestication, some diversity can be restored by introgression from its progenitors or from more distant wild relatives (Dubcovsky & Dvorak, 2007). The introgression of genetic material from wild relatives or distantly related species into wheat germplasm is a classical and effective approach for broadening the genetic basis of this crop (Hernández *et al.,* 1999). In previous studies, we successfully transferred the Ns genome of *P. huashanica* into wheat using wide hybridization methods and we generated a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22II$). However, a rapid and accurate method for identifying *P. huashanica* chromosomes and chro-

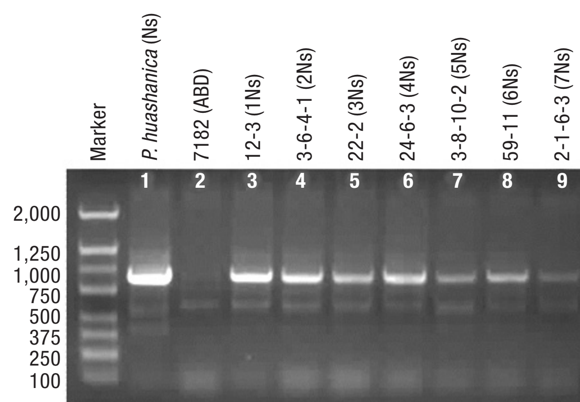

Figure 3. The specific fragment amplified with the SCAR primer RHS23 using a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, $2n = 44 = 22II$) and its parents, common wheat cv. 7182 and *P. huashanica*.

mosomes fragments in derived lines is required before they can be utilized effectively (Wu *et al.,* 2010). Molecular markers, particularly genome-specific markers, are useful for identifying the genome constitution of unknown species and they also provide efficient tools for confirming the transfer of target alien genes to wheat (Wang *et al.,* 2010).

In the present study, we developed a SCAR marker known as RHS23 by directly sequencing RAPD products and it was verified by wide screening of a complete set of wheat-*P. huashanica* disomic addition lines (1Ns – 7Ns, 2*n* = 44 = 22II) that carried different *P. huashanica* chromosomes. The result demonstrated that this novel SCAR marker is suitable for distinguishing *P. huashanica* genome in a wheat background. This is the first report of the development of a SCAR marker for the detection of *P. huashanica* in wheat-*P. huashanica* derivative lines.

Marker-assisted selection (MAS) has great potential for increasing the efficiency of the breeding process by increasing the number of traits that can be selected in one population and by improving the precision of genotype selection (Wang *et al.,* 2012). A SCAR marker with high reproducibility and reliability can be used easily in a MAS program and this is an attractive technique for indentifying exogenous chromatin from introgression lines. To ensure that the Ns-chromosome-specific marker delivered consistent performance with homologous groups, the complete set of wheat-*P. huashanica* disomic addition lines with different Ns chromosomes were used to test the SCAR marker. All homologous groups of *P. huashanica* chromosomes could be located in the addition lines, which show that our primer pair can amplify the Ns chromosome-specific sequence in all the different homologous groups. The development of a SCAR marker from specific repetitive DNA bands provides a practical tool for determining introgression, which supports MAS in wheat. Our results suggest that this SCAR marker could be used to discriminate derivative lines from wheat-*P. huashanica* addition lines and for detecting alien *P. huashanica* DNA fragments in wheat. Further diagnostic markers for *P. huashanica* chromatin are also required for the fine mapping of genes and for future genomic studies.

In summary, this PCR-based assay can be rapidly applied to monitor the presence of *P. huashanica* in a wheat background during the early stages of development. Thus, breeders could obtain early information about the presence of *P. huashanica* chromatin, which may contribute to making decisions about the retention or elimination of lines. This rapid and efficient method has high specificity and reproducibility so it could be used for screening the targeted genetic material.

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