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Development of SCAR Markers for Identification of Perennial and Annual Types in the Asian Wild Rice

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ABSTRACT

Asian wild rice, *Oryza rufipogon* Griff., is recognized as wild progenitor of Asian cultivated rice (*O. sativa*). This species tends to differentiate into annual and perennial types. In order to help establish a basis for the identification of the two types of this species, the SCAR markers have been developed. A total of 20 random primers were screened among 60 individuals of each type using random amplified polymorphic DNAs (RAPDs). One diagnostic PCR product for each type was isolated, sequenced and converted to sequence characterized amplified region (SCAR). The SCAR primer pairs (OPAM10-900F/R) were able to amplify the specific DNA fragments from the DNA of the perennial type but not from the DNA of the annual type. The amplified fragment operated by that was specify to perennial type could be used as a rapid means of identifying the perennial type from annual type of *O. rufipogon.*

The other single unique fragment occurring in both types of *O. rufipogon* and *O. sativa* was generated by the second SCAR primer pairs (OPAM10-800F/R). This evidence supports the idea that the progenitor of the cultivated species is assumed to be *O. rufipogon*. Furthermore, the nucleotide sequences from the fragment may be useful to elucidate the evolutionary relationships within A genome of *Oryza* species.

Key words: *Oryza rufipogon*, RAPDs, SCAR markers

INTRODUCTION

The common Asian wild rice with AA genome, *O. rufipogon* Griff. is recognized as the progenitor of the Asian cultivated rice (*O. sativa)*. *O. rufipogon* has shown ecotypic differentiation particularly in their growth habits and habitat conditions. It tends to differentiate into annual and perennial types. The annual and perennial types of this species are sometimes classified as two different species, *O. nivara* and *O. rufipogon*, respectively (Chang, 1976). Recently, Lu (1999) proposed that the perennial type and annual type were treated as *O. rufipogon* and *O. nivara*, respectively. However, Morishima *et al*. (1984) treated both perennial and annual types under *O. rufipogon.*

From an interesting study of wild rice in Thailand, Morishima *et al.*(1984) reported that habitat condition of *O. rufipogon* populations were in deep swamp for perennial populations, while annual populations were in shallower or drier habitats. Furthermore, Barbier (1989) described that several populations of *O. rufipogon* distributed in the suburbs of Bangkok were clearly differentiated into annual populations reproducing exclusively by seeds, whereas perennial populations characterized by a predominant to exclusive use of asexual reproduction.

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To date, the species status of *O. rufipogon* and *O. nivara* is generally accepted. The method of identifying these two species still relied on morphological characters e.g., spikelets and stoloniferous. *O. rufipogon* is found as small or large populations with a great morphological variation in a wide range of environments (Lu, 1999). Some authors have recognized *O. nivara* as an ecotypic differentiation of *O. rufipogon* (Barbier and Ishihama, 1990). Consequently, large overlaps exist, which make it difficult to identify samples from the field unambiguously. In addition, no sound genetic data have been provided to support this designation. From the previous molecular study, Sano and Sano (1990) demonstrated that the spacer-length variations were detected among individuals of *O. rufipogon*. It is therefore necessary to adopt more reliable and powerful methods at the DNA level to investigate the genomic variation in the two forms of this species.

Molecular markers have been developed and were used extensively in assessing genetic diversity in rice (Cao and Oard, 1997). In rice, several workers have successfully classified cultivars using RAPD markers (Mackill, 1995; Cao and Oard,1997). However, the reliability of RAPD markers has been subjected to controversy.

Recently, a more reliable and specific PCR-based marker known as Sequence Characterized Amplified Region (SCAR) was developed by Paran and Michelmore (1993). SCAR primers are longer than RAPD primers and highly stringent annealing temperature can be employed that prevents mismatching in the priming site during DNA amplification (Paran and Michelmore, 1993). In addition, SCAR primers can amplify a single locus that appears as a single scored band in agarose gels (Melotto *et al.*, 1996).

The present study was initiated to develop SCAR primers to discriminate annual and perennial types of a wild rice, *O. rufipogon*, that were collected from northeastern Thailand and to test the robustness of the SCAR primers developed with different heat-stable DNA polymerases.

MATERIALS AND METHODS

Plant materials

During 1999-2000, several extensive collecting trips for the wild rice species were conducted in northeastern Thailand. The nomenclature for identifying *O. rufipogon* in this paper is fundamentally based on Morishima *et al.*(1984). Thus, both annual and perennial types were treated under *O. rufipogon*. Both types were collected in the form of living plants from natural populations. For the perennial type, the samples were collected from several populations maintained by vegetative propagation, whereas, the seed samples of annual type were collected from living plant in different populations, and grown in the green house for harvesting young leaves and examining their habits.

Fresh young leaves of two types of *O. rufipogon* were harvested and placed in an ice box. Sixty individuals were sampled from each type and brought back to laboratory, then stored in a freezer (-20°C) until use.

DNA isolation

Total genomic DNA of an individual was extracted using a CTAB method similar to that of Doyle and Doyle (1987), as described in Prathepha and Baimai (1999).

Screening of RAPD markers

A total of 20 decamer primers (OPAM1-20) from Operon Technologies were used for amplification of genomic DNA of *O. rufipogon*. RAPD-PCR amplification was run on a Hybaid Thermal Cycler. PCR was carried out in a total reaction volume of 20 μL containing 1XPCR buffer (Promega), 200 μM of each dNTP, 3mM $MgCl_2$, 10 pM of primer, 100 ng DNA and 0.5 unit of *Taq* DNA polymerase (Promega). The PCR profile was performed as follows: initial 1 min 94°C

denaturation; 45 cycles of 1 min 94°C, 1 min 36°C, 2 min 72°C; and 5 min 72°C extension. PCR products were loaded onto 1.4% agarose gels using 0.5X TBE buffer and separated for 3 h at 75 volts, stained with ethidium bromide, and photographed under UV light using Gel Documentation System, GDS8000 (UVP, Inc., California, USA). Molecular weights were estimated using a 100 bp ladder DNA (Gibco, BRL).

Development of SCAR markers

RAPD-PCR profiles operated by primer OPAM-10 were chosen for designing longer primers. The OPAM-10 primer generated a marker band of approximately 800 bp, which was present in all 60 individuals examined for the annual type of *O. rufipogon*, while a similar result was observed in perennial type that revealed a marker band of approximately 900 bp. These two RAPD bands observed in this study were used for developing SCAR primers.

The SCAR fragments were recovered from the gel slices by using the GENECLEAN Kit (Promega). Then, the purified PCR fragments were sequenced directly on a ABI 373 Automated Sequencer. The DNA sequences obtained from this experiment were used for designing SCAR primers. These primers were synthesized by BIOSERVICE (NSTDA, Bangkok, Thailand). The SCAR primers have none of the original decamer due to ambiguity in DNA sequences. Each PCR reaction (20 μL total volume) consisted of 1X PCR buffer (50mM Tris-HCl (pH 9.0), 50mM KCl, 0.1%Triton®X-100), 1.5 mM $MgCl_2$, 100mM each of dNTPs, 10 pM each of the SCAR primers, 0.5 units of *Taq* DNA polymerase (Promega), DNA extracts (2 μL). For the PCR amplification, the cycling parameters were 1 cycle of 94°C for 1 min; 35 cycles of 94°C for 1 min, 60°C for 1 or 1.5 min, and 72°C for 1.5 min; and 1 cycle of 72°C for 5 min. After amplification, 10 μL of each reaction product was electrophoresed in 2% agarose gels using 1X TBE buffer, and the agarose gels were stained with ethidium bromide and viewed on an ultraviolet transilluminator. The reproducibility of the SCAR markers was confirmed with *Taq* DNA polymerase (BRL Gibco) using the same amplification protocol.

RESULTS AND DISCUSSION

Two specific SCAR primer pairs (Table 1) were synthesized based on the DNA sequence amplified by the OPAM-10 primer. These SCAR primers contained 24-27 bp without the original RAPD primers.

The SCAR primers, OPAM10-900F/R, could amplify fragments of ca. 750 bp from the DNA of all individuals of the perennial type of *O. rufipogon* as illustrated in Figure 1, while, the amplification using same primer pairs for the DNA

Table 1 Sequence-specific primer pairs derived from RAPD markers for discrimination between perennial and annual types of *O. rufipogon*.

Habit	Primer pairs	Sequence (5'-3')	Annealing temp(°C)
Annual	OPAM10-800F	GTA CCG TTA GGT AGG CCG TCT CCG	60°C
	OPAM10-800R	ATT ACT TTG ATT AGA TTC ATG AAC	
Perennial	OPAM10-900F	ACT AAT TTA TCC GGA GGT AAG AGC CTT	60°C
	OPAM10-900R	CTA ATT TAT CCG GAG GTA AGA GCC TTC	

from all individuals of the annual type resulted in absence of the diagnostic fragment. These results suggested that sequence-specific primer derived from a RAPD marker, OPAM10-900 could be used in differentiating between the perennial and annual types of Asian wild rice, *O. rufipogon*.

The other SCAR primers, OPAM10-800 F/R, could generate the DNA fragment of ca. 350 bp from the DNA of the annual type (Figure 2). However, this fragment was also observed in the perennial type of *O. rufipogon* and cultivated rice, *O. sativa* cv. KDML105 (Figure 2). The result from this study supports the idea that the progenitor of the cultivated species, *O. sativa*, is assumed to be *O. rufipogon*.

Fundamentally, both types of *O. rufipogon*, are morphologically similar. They are distinguishable from one another by the different type of habit, i.e. annual and perennial types. Moreover, these two types generally belong to the same biological species as cultivated species, *O. sativa* since they interbreed and produce viable hybrids when they coexist (Harlan and De Wet, 1971). As a result, *O. sativa-O. rufipogon* complex naturally occur in rice. Sano and Morishima (1982) reported that the *O. sativa- O. rufipogon* complex was extremely complicated, since *O. rufipogon* tends to be differentiated into perennial and annual types. The present result can show distinct difference between the two types of *O. rufipogon*. In addition, the DNA sequences obtained from the SCAR fragments may be useful to elucidate the evolutionary relationships within *O. sativa- O. rufipogon* complex.

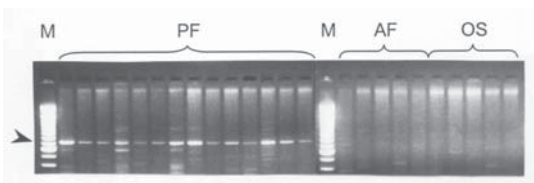

Figure 1 PCR analysis with primer OPAM10-900 F/R. The diagnostic band was present only in perennial form (PF)(indicated by arrow) of *O. rufipogon* . While this band was not produced in annual form (AF) of *O. rufipogon* and *O. sativa* cv. KDML105 (OS). M = 100 bp ladder (Gibco, BRL).

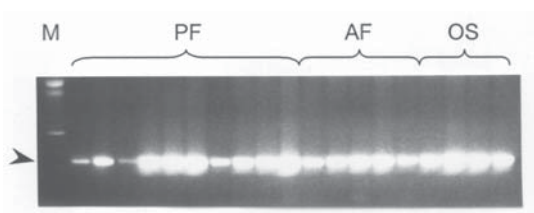

Figure 2 PCR analysis with primer OPAM10-800 F/R among both forms of *O. rufipogon* and *O. sativa* cv. KDML105. M = 100 bp ladder (Gibco, BRL)

To determine the robustness of these SCAR primers, further analyses were conducted with the same PCR reaction conditions using two sources of *Taq* DNA polymerase (Promega and Gibco, BRL). The same results were obtained from the experiment. The DNA amplified fragment of the same size were generated (data not shown) and the SCAR primer pairs also gave single band without any interference in the DNA amplification. This meant that the presence or absence of the SCAR marker was strongly corresponded to the presence or absence of the DNA of annual or perennial types of *O. rufipogon*. Annual and perennial types of *O. rufipogon* could also be differentiated by having a retrotransposon, p-SINE1-r2 at the Waxy locus as reported by Yamanaka *et al*. (http://www.carleton.ca/~bgordon/Rice/papers/yama20.htm). They demonstrated that most annual type of *O. rufipogon* possessed p-SINE1-r2, while most perennial type did not. Previously, Hirano *et al.* (1994) concluded that p-SINE1-r2 polymorphisms had no tendency to associate with annual-perennial types of *O. rufipogon*.

In summary, the SCAR primer pairs, OPAM10-900 F/R can be used in discrimination of annual and perennial types of Asian common wild rice, *O. rufipogon* collecting from northeastern Thailand.

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