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RESEARCH ARTICLE

Significant association of the novel *Rf4*-targeted SNP marker with the restorer for WA-CMS in different rice backgrounds and its utilization in molecular screening

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Abstract

In the rice cytoplasmic-genetic male sterility (CMS) system, the combination of a CMS line, maintainer line and restorer line carrying the restorer gene to restore fertility, is indispensable for the development of hybrids. However, the process of screening for the trait of fertility restoration is laborious and time-consuming. In the present study, we analyzed the nucleotide sequence of the *Rf4* gene, which is the major locus controlling fertility restoration, to identify allele-specific variation. A single nucleotide polymorphism (SNP) A/C at +474 in the coding sequence (CDS) was found to be capable of strictly distinguishing groups of alleles *Rf4* (A) and *rf4* (C). Using KASP genotyping, this valuable SNP was converted to an allele-specific PCR marker. We evaluated and validated the marker among three-line parents with different backgrounds, and the results revealed a complete correlation between SNP alleles and the fertility restoration phenotype. Molecular screening was subsequently carried out for the presence of alleles of *Rf4* and *Rf3* among 328 diverse rice cultivars with worldwide distribution. The results demonstrate that this SNP marker could be the optimal choice for the molecular identification of potential restorers.

Keywords: hybrid rice, *Rf4*, SNP marker, fertility restorer

1. Introduction

Rice is one of the world's most important staple food crops and a primary source of food for more than half of the world's population due to its high calorific value. Hence, rice is the major source of food of many developing countries. Hybrid rice technology has contributed significantly to food security and provided rural employment in China for the last 30 years, and hybrid rice occupies approximately 50% of the total rice-planting area in China (El-Namaky *et al*. 2016). Cytoplasmic-genetic male sterility (CMS), combined with a fertility restoration system (consisting of fertility restorers, CMS lines and maintainers), has been found to be the most efficient genetic tool to exploit hybrid vigor on a commercial scale in rice, and three primary rice CMS systems — wild abortive (WA), boro type II (BT) and Honglian (HL) — have been characterized (Kiani 2015). WA is a widely used CMS source that at one time accounted for approximately 90% of the rice hybrids produced in China and 100% of the hybrids developed outside China (Kiani 2015). In the

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three-line breeding system, the CMS line is crossed with the restorer line (R line) to produce F_1 hybrid rice and with the maintainer line (B line) for self-reproduction. Identification of maintainers and restorers from elite breeding lines and landraces (through testcrossing) and their use in further breeding programs are the initial steps in three-line hybrid breeding (Singh *et al*. 2016).

The inheritance of fertility restoration in the WA-CMS system has been extensively investigated, and genetic analyses have made it clear that two major genes are generally involved: *Rf3* and *Rf4*. Bazrkar *et al*. (2008) found that the microsatellites (simple sequence repeats or SSRs) RM443 and RM315 flank the *Rf3* gene at genetic distances of 4.4 cM (LOD 10.29) and 20.7 cM (LOD 3.98) on chromosome 1, respectively. Qi *et al*. (2008) mapped *Rf3* to the genomic region between RM10338 and RM10376, which has a physical separation of 679.9 kb. In addition, Suresh *et al*. (2012) developed polymorphic markers to increase the resolution and narrow *Rf3* to a region of 392.5 kb. Recently, a new mitochondrial gene, *WA352*, was found to confer CMS in rice. WA352 interacts with the nuclear-encoded mitochondrial protein OsCOX11, which has a role in hydrogen peroxide degradation. The interaction between WA352 and OsCOX11 triggers an early reactive oxygen species (ROS) burst, which promotes the release of cytochrome c to the cytosol, causing premature programmed cell death (PCD) of the tapetum (Luo *et al*. 2013). Additionally, the *Rf4* locus encodes a mitochondrial-localized PPR protein that reduces WA352 transcripts (Kazama and Toriyama 2014; Tang *et al*. 2014). Despite the available knowledge regarding genetic locations, the applicability of markers associated with *Rf* loci for extensive and routine screening of restorer lines from previously uncharacterized rice germplasms has not yet been reported.

Breeders routinely identify restorers by testcrossing prospective lines with available CMS lines and evaluating F_1 progenies for pollen and spikelet fertility. Lines with progenies showing 80% pollen and spikelet fertility are then designated restorers (Sattari *et al*. 2007). Development of an MAS (marker-aided selection) procedure involving the two main restorer quantitative trait loci would significantly reduce the time and labor involved in making and evaluating testcrosses in an active hybrid rice-breeding program. In particular, the development of PCR-based markers would empower researchers in local agricultural research systems to apply the technology in local hybrid rice-breeding programs (Singh *et al*. 2014). To benefit from MAS for fertility restoration, it was therefore of great importance to develop and evaluate markers well suited for routine screening for hybrid rice breeders.

Exploitation of heterosis in hybrid rice technology has been contemplated as a potential strategy for yield enhancement in rice, and heterosis in rice has been acknowledged to be associated with the genetic divergence of the parents used in hybridization (Moreno *et al*. 2003). The restorer line is widely considered key to further improvement of the resistance, yield, quality, and heterosis of hybrid rice. However, previous research based on molecular tagging has suggested that the restorer lines used widely today have narrow genetic backgrounds (Li *et al*. 2012). Screening of new restorers from a broader range of sources will further promote the utilization of genetic component contributions and heterosis in high-yielding hybrid rice breeding.

Markers developed based on polymorphisms between functional and non-functional alleles can provide precise and accurate selection of target genotypes without the need for difficult, laborious, and time-consuming phenotyping (Tian *et al*. 2016). The present study was conducted with the objectives of identifying SNPs from the *Rf4* gene and discovering polymorphic variation between different alleles in order to develop an associated marker to distinguish a diagnostic potential restoration status for CMS. Using this novel marker, combined with a tightly linked marker for *Rf3*, we carried out allelic assessment and molecular screening of rice genotypes from diverse worldwide collections, identifying and verifying restorers for a hybrid rice-breeding program.

2. Materials and methods

2.1. Plant materials

The plant materials used in this study included two panels of diverse rice cultivars or breeding lines. The first panel of 92 rice lines was used for single nucleotide polymorphism (SNP) marker evaluation. This panel contained 22 restorer lines, 24 CMS or maintainer (A (male-sterile) or B (maintainer)) lines, and other breeding lines of unknown restoration status, mainly from China. The second panel of rice cultivars represented a diverse worldwide collection from more than 20 different countries and was obtained from the International Rice Research Institute and South China Agricultural University. For testcrossing, the CMS line of Tianfeng A was used as the female parent to generate F_1 hybrids.

2.2. Phenotyping of fertility

We used anthers collected from the spikelets 1–2 d before anthesis to determine pollen fertility. The anthers from each spikelet were smeared in a drop of 1% iodine-potassium iodide (I_2-KI) solution on a glass slide, and fertile and sterile pollen grains were counted in three randomly selected microscopic fields. Stained, well-filled and round pollen grains were counted as fertile, while unstained, shriveled,