Breeding Science 66: 768–775 (2016)
doi:10.1270/jsbbs.16044

Research Paper

Development of a platform for breeding by design of CMS restorer lines based on an SSSL library in rice (*Oryza sativa* L.)

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Exploitation of the heterosis of hybrid rice has shown great success in the improvement of rice yields. However, few genotypes exhibit strong restoration ability as effective restorers of cytoplasmic male sterility (CMS) in the development of hybrid rice. In this study, we developed a platform for the breeding by design of CMS restorer lines based on a library of chromosomal single segment substitution lines (SSSLs) in the Huajingxian74 (HJX74) genetic background. The target genes for breeding by design, $Rf3^4$ and $Rf4^4$, which are associated with a strong restoration ability, and *gs3*, *gw8*, Wx^{g1} and *Alk*, which are associated with good grain quality, were selected from the HJX74 SSSL library. Through pyramiding of the target genes, a restorer line, H121R, was developed. The H121R line was then improved regarding blast resistance by pyramiding of the *qBLAST11* gene. Hence, a new restorer line with blast resistance, H131R, was developed. The platform involving the $Rf3^4$ and $Rf4^4$ restorer genes would be used for the continuous improvement of restorer lines through breeding by design in rice.

Key Words: cytoplasmic male sterility, restorer, single segment substitution line, breeding by design, hybrid rice.

Introduction

Cytoplasmic male sterility (CMS) is the foundation for the exploitation of the heterosis of hybrid rice (Yuan and Tang 1999). The male sterility of CMS lines can be restored by nuclear-encoded restorer of fertility (*Rf*) genes in rice (Chen and Liu 2013). For gametophytic CMS, Chinsurah Boro II (*indica*) cytoplasm with Taichung 65 (*japonica*) nucleus (BT-CMS) is restored by the *Rf1a* or *Rf1b* gene on chromosome 10, and red-awned wild rice (*Oryza rufipogon*) cytoplasm with Liantangzao (*indica*) necleus (HL-CMS) is restored by *Rf5*, which is another *Rf1a* allele (Akagi *et al.* 2004, Hu *et al.* 2012, Kazama and Toriyama 2003, Komori *et al.* 2004, Liu *et al.* 2004, Wang *et al.* 2006). Sporophytic CMS, including wild abortive rice (*Oryza rufipogon*) cytoplasm with Eejiunan 1 (*indica*) nucleus (WA-CMS), dwarf abortive rice (*Oryza rufipogon*) cytoplasm with Xieqingzao (*indica*) nucleus (DA-CMS) and Yegong (*indica* landrace) cytoplasm with BII44-5 (*indica*) nucleus (YA-CMS) types, is restored by the *Rf3* and *Rf4* genes, located on chromosomes 1 and 10, respectively (Dai *et al.* 2015, Suresh *et al.* 2012, Yao *et al.* 1997, Zhang *et al.* 1997, 2002). Recently, the *WA352* gene, which controls WA-CMS, and the *Rf4* gene, which encodes a PPR protein, were cloned (Kazama and Toriyama 2014, Luo *et al.* 2013, Tang *et al.* 2014). CMS-based hybrid seed technology uses a three-line system that consists of a CMS line (A line), a maintainer (B line), and a restorer (R line) to produce F_1 seeds. Since China pioneered hybrid rice production in the 1970s, the yield of hybrid rice has been increased by more than 20% compared with conventional varieties and now accounts for more than half of the annual rice planting area in China (Cheng *et al.* 2007, Huang *et al.* 2014). However, few genotypes exhibit a strong restoration ability as effective restorers for CMS in the development of hybrid rice (Sharma *et al.* 2012, Singh *et al.* 2012). Therefore, the development of elite restorers is an important aim of breeding programs for hybrid rice production.

Chromosomal single segment substitution lines (SSSLs), which carry a particular chromosomal segment from a donor in the genetic background of a recipient, eliminate background noise to a large extent and are powerful tools for the genetic analysis of quantitative trait loci (QTLs) (Ebitani

Communicated by Qian Qian
Received March 15, 2016. Accepted September 22, 2016.
First Published Online in J-STAGE on November 18, 2016.
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et al. 2005, Kubo *et al.* 2002, Xi *et al.* 2006, Zhang *et al.* 2004). We have constructed a library of 1,123 SSSLs in rice using Huajingxian74 (HJX74), an elite *indica* variety from south China, as the recipient and 26 genetically diverse accessions collected worldwide as donors. Each SSSL in the library has only one chromosomal segment from a donor in the HJX74 genetic background (Zhang *et al.* 2004). These SSSLs have been employed to detect and clone QTLs for complex traits (He *et al.* 2005, Liu *et al.* 2010, Naeem *et al.* 2013, Wang *et al.* 2012, Zhang *et al.* 2012, Zhao *et al.* 2007), to assess allelic variations at loci of interest (Cai *et al.* 2013, Teng *et al.* 2012, Zeng *et al.* 2006), and to analyze gene by gene and gene by environment interactions (Chen *et al.* 2014, Liu *et al.* 2008, 2012, Zhu *et al.* 2015). To assess allelic variations at the *Rf3* and *Rf4* loci, 57 SSSLs carrying one of the loci in the substituted segments were selected from the library. Four alleles were identified at both loci in the set of SSSLs, which were designated $Rf3^1$, $Rf3^2$, $Rf3^3$ and $Rf3^4$ and $Rf4^1$, $Rf4^2$, $Rf4^3$ and $Rf4^4$, respectively, ranging from weak to strong in terms of their restoration ability. The HJX74 recipient harbors the $Rf3^4$ allele, with the strongest restoration ability, at the *Rf3* locus, but a weaker allele at the *Rf4* locus. One SSSL, W23-19-06-06-11, was found to carry the $Rf4^4$ allele, with the strongest restoration ability, in the substituted segment from the Lemont donor (Cai *et al.* 2013).

Peleman and van der Voort (2003) proposed the concept of 'breeding by design'. This goal can be reached by following a three-step approach: mapping loci involved in all agronomically relevant traits, assessment of the allelic variation at those loci, and breeding by design. We proposed a strategy to practice breeding by design using the HJX74 SSSL library as a platform for rice breeding (Xi *et al.* 2006). The SSSL W14-18-6-10-1, which carries the purple pericarp gene *Pb* in the substituted segment on chromosome 4 from Lianjian33, was selected from the SSSL library. After comprehensive evaluation, the SSSL became a new variety designated Huaxiaohei1, which was released in 2005. Furthermore, a pyramiding line with three substituted segments in an HJX74 genetic background was designated Huabiao1 and released to farmers in 2009 (Dai *et al.* 2015). Because HJX74 is neither a CMS maintainer nor a CMS restorer, the HJX74 SSSL library cannot be directly used as a platform for the development of CMS lines and restorer lines. Recently, the platform was successfully improved to allow the development of CMS lines through breeding by design. Three isonuclear alloplasmic CMS lines with an HJX74 genetic background, designated W-H121A (WA type), D-H121A (DA type) and Y-H121A (YA type), and their maintainer lines were the first to be developed. The CMS lines were then improved through breeding by design to develop three new isonuclear alloplasmic CMS lines, designated D-H131A, W-H131A and Y-H131A, by pyramiding target genes from the HJX74 SSSL library (Dai *et al.* 2015). In this study, we developed a platform for the breeding by design of CMS restorer lines based on the HJX74 SSSL library. By using the $Rf3^4$ and $Rf4^4$ restorer genes, a restorer line, H121R with a strong restoration ability was developed in the HJX74 genetic background. The H121R line was then improved to develop an elite restorer line, H131R, with blast resistance. This work provides an example of conducting breeding by design of restorer lines based on the HJX74 SSSL library.

Materials and Methods

Plant materials

HXJ74, an elite variety that is planted widely in south China, is the recipient of the SSSLs and contains the $Rf3^4$ gene (Cai *et al.* 2013, Xi *et al.* 2006, Zhang *et al.* 2004). Three isonuclear alloplasmic CMS lines, W-H121A (with wild abortive cytoplasm), D-H121A (with dwarf wild abortive cytoplasm), and Y-H121A (with Yegong abortive cytoplasm), exhibit an HJX74 nuclear background, with the exception of non-functional *rf3* and *rf4* genes from a CMS line Xieqingzao A (Dai *et al.* 2015). Five SSSLs with an HXJ74 genetic background, W03-14-10-04-02, W08-15-08-28, W23-07-06-10-06, W23-07-06-05-02-02, and W23-19-06-06-11, were employed as the donors of target genes (**Table 1**).

All of the materials used in this study were grown at an experimental station of South China Agricultural University, Guangzhou (23°07′N, 113°15′E), China, in two cropping seasons from 2008 to 2014. The first cropping season was from late February to mid-July, and the second cropping season was from late July to mid-November. The seeds were sown in seed beds, and the seedlings were transplanted to fields. Seedlings were transplanted at a density of 16.7 cm × 32.4 cm, with one seedling per hill. The adopted field management procedures, including irrigation, fertilizer application and pest control, essentially followed normal agricultural practice.

Development of the restorer lines H121R and H131R

For development of the restorer line H121R, four SSSLs, W08-15-08-28 with the *gs3* gene, W23-07-06-10-06 with the Wx^{g1} gene, W03-14-10-04-02 with the *gw8* gene, and W23-19-06-06-11 with the $Rf4^4$ gene in their chromosomal substituted segments, were selected from the HXJ74 SSSL library (**Table 1**). W08-15-08-28 and W23-07-06-10-06 were first crossed, and 6 homozygous plants with *gs3* and Wx^{g1} gene were selected from an F_2 population of 100 plants by marker-assisted selection (MAS). The homozygotes with *gs3* and Wx^{g1} gene were then crossed with the SSSL W03-14-10-04-02 with the *gw8* gene, and the line H121 with *gs3*, Wx^{g1} and *gw8* genes were obtained from the F_3 population. To improve the fertility restoration, the H121 line was crossed with W23-19-06-06-11 with the $Rf4^4$ gene. Good quality of restorer line, H121R, was obtained, which carried the *gs3*, Wx^{g1} and *gw8* genes from the H121 line and the $Rf4^4$ gene from W23-19-06-06-11 in the F_3 population by MAS.

Table 1. Chromosomal substituted segments with target genes in the SSSLs

SSSL	Donor	Chr.	Substitution segment	Interval of substituted segments (cM)	Target gene	Target trait
W08-15-08-28	IR64	3	RM2453-*gs3*-RM6146-RM3646-RM16	83.0–94.9	*gs3*	LG
W23-07-06-10-06	Lemont	6	RM508-RM589-RM190(Wx^{g1})-RM204-RM402-*Alk*-RM539-RM541	1.4–68.5	Wx^{g1} *Alk*	MAAC HGT
W03-14-10-04-02	Zhong4188	8	RM256-RM5493-*gw8*-RM447	96.6–111.2	*gw8*	NG
W23-19-06-06-11	Lemont	10	RM258-RM5373-$Rf4^4$-RM6100-RM25685	30.2–58.5	$Rf4^4$	SRF
W23-07-06-05-02-02	Lemont	11	RM224-*qBLAST-11*-RM144	110.1–116.5	*qBLAST-11*	HRB

LG, long grain; MAAC, medium apparent amylose content; HGT, high gelatinization temperature; NG, narrow grain; SRF, strong restoration of fertility; HRB, high resistance to blast.

For improvement of blast resistance in the H121R restorer line, an SSSL, W23-07-06-05-02-02, was selected from the SSSL library. W23-07-06-05-02-02 carries the *qBLAST-11* gene in their chromosomal substituted segments in the HXJ74 genetic background (**Table 1**). The H121R line was crossed with W23-07-06-05-02-02. The restorer line with blast resistance, H131R with the desirable homozygous alleles in all target genes out of about 1000 lines was obtained from the F_3 population through MAS using the linked markers.

DNA extraction and marker assay

DNA was extracted from fresh young leaves using the CTAB method (Murray and Thompson 1980). Miniscale DNA extraction was carried out according to the procedure described by Zheng *et al.* (1995). The PCR profile used for amplification basically followed a previously described protocol (Cai *et al.* 2013). The PCR amplified products were analyzed via electrophoresis in 6% polyacrylamide denaturing gels and subjected to the silver staining procedure, as described by Li *et al.* (2006).

The target genes in the SSSLs were identified using SSR markers that are linkage with the genes (**Table 1**). The *gs3* gene, conferring a long-grain trait (Fan *et al.* 2006), is located in the substituted segment of chromosome 3 in SSSL W08-15-08-28. The genes Wx^{g1}, conferring a medium apparent amylose content (Teng *et al.* 2012), and *Alk*, conferring a high gelatinization temperature (Gao *et al.* 2003), are both located in the same substituted segment of chromosome 6 in W23-07-06-10-06. The *gw8* gene, conferring a narrow-grain trait (Wang *et al.* 2012), is located in the substituted segment of chromosome 8 in W03-14-10-04-02. W23-19-06-06-11 carries the $Rf4^4$ gene, conferring a strong restoration ability, in the substituted segment of chromosome 10 (Cai *et al.* 2013). The *qBLAST11* QTL, for resistance to blast, was mapped between the RM224 and RM144 markers in the substituted segment of chromosome 11 in W23-07-06-05-02-02 (Zhang *et al.* 2012) (**Table 1**).

Observation of pollen and spikelet fertility

The pollen and spikelet fertility of F_1 hybrids in testcrosses were used to evaluate the fertility restoration ability of restorer lines. Mature anthers in spikelets were collected to determine pollen fertility. The pollen was stained with a 1% (m/v) I_2-KI solution. The numbers of stainable and unstainable pollen grains in each individual were counted under an optical microscope. Twenty plants were collected for pollen and spikelet fertility traits. All statistical analyses were performed using SPSS version 18 in IBM and GraphPad Prism 5 in GraphPad Software.

Assessment of agronomic traits and grain quality

Each of the traits was tested in 40 plants per line during the second cropping season in 2012. The grain traits of grain length, grain width and grain weight were evaluated as described previously (Fan *et al.* 2006). Tests of apparent amylose content (AAC) and gelatinization temperature (GT) which was indirectly estimated via alkali spreading value (ASV), were conducted following procedures described elsewhere (Tan *et al.* 1999, Teng *et al.* 2012). All statistical analyses were performed using IBM SPSS version 18.

Evaluation of resistance to blast

Blast inoculation and the evaluation of resistance were performed followed the methods described by Zhu *et al.* (2012). Three rows of seedlings, containing 20 plants each, were planted in a greenhouse in 30 cm × 20 cm × 5 cm trays. Inoculation of the lines was performed at the 3.5–4 leaf stage using a set of 10 blast isolates selected to show a diverse spectrum of virulence. A 20 ml spore suspension (105 spores/ml) was applied to each tray using an airbrush connected to a source of compressed air. Each isolate-host combination was assessed in three replications. After inoculation, the trays were maintained in the dark for 24 h at a relative humidity of 95–100% and a temperature of 25°C, after which they were transferred to a greenhouse where the ambient temperature was maintained at 25–28°C. Six days later, disease symptoms were evaluated on a standard scale of 0–9 based on the type and size of lesions, as described by the International Rice Research Institute (IRRI 1996). Rice plants exhibiting reactions with a score of 0–3 were considered resistant, and those showing reactions with a score of 4–9 were categorized as susceptible.

Results

Development of the H121R restorer line, with a strong restoration ability and good grain quality, in the HJX74 genetic background

To improve the grain quality of HJX74 plants, four genes, *gs3*, *gw8*, *Wx*^{gl} and *Alk*, in three SSSLs were pyramided under the HJX74 genetic background. The pyramid line with the four genes (*gs3*, *gw8*, *Wx*^{gl} and *Alk*) was designated H121 (**Fig. 1a**). To improve the restoration ability of the H121 line, the SSSL W23-19-06-06-11 carrying *Rf4*^{4} gene was then crossed with the H121 line. The pyramid line, carrying the *gs3*, *gw8*, *Wx*^{gl} and *Alk* genes from H121, the *Rf4*^{4} gene from W23-19-06-06-11, and the *Rf3*^{4} gene from the HJX74 genetic background, was designated H121R (**Fig. 1a**).

To evaluate the effect of breeding by design, the target traits controlled by the target genes in the H121R line were tested. As expected, the grain shape of H121R was long-slender, with a grain length of 9.4 mm and a grain width of 2.5 mm, whereas HJX74 presented a grain length of 8.6 mm and a grain width of 2.8 mm (**Fig. 1b**, **1c**). The 1000-grain weight of H121R was 23.6 g, which was greater than the value of 20.8 g recorded in HJX74 (**Fig. 1d**). The AAC of H121R was 21.4%, which corresponded to the intermediate class and was significantly lower than the value

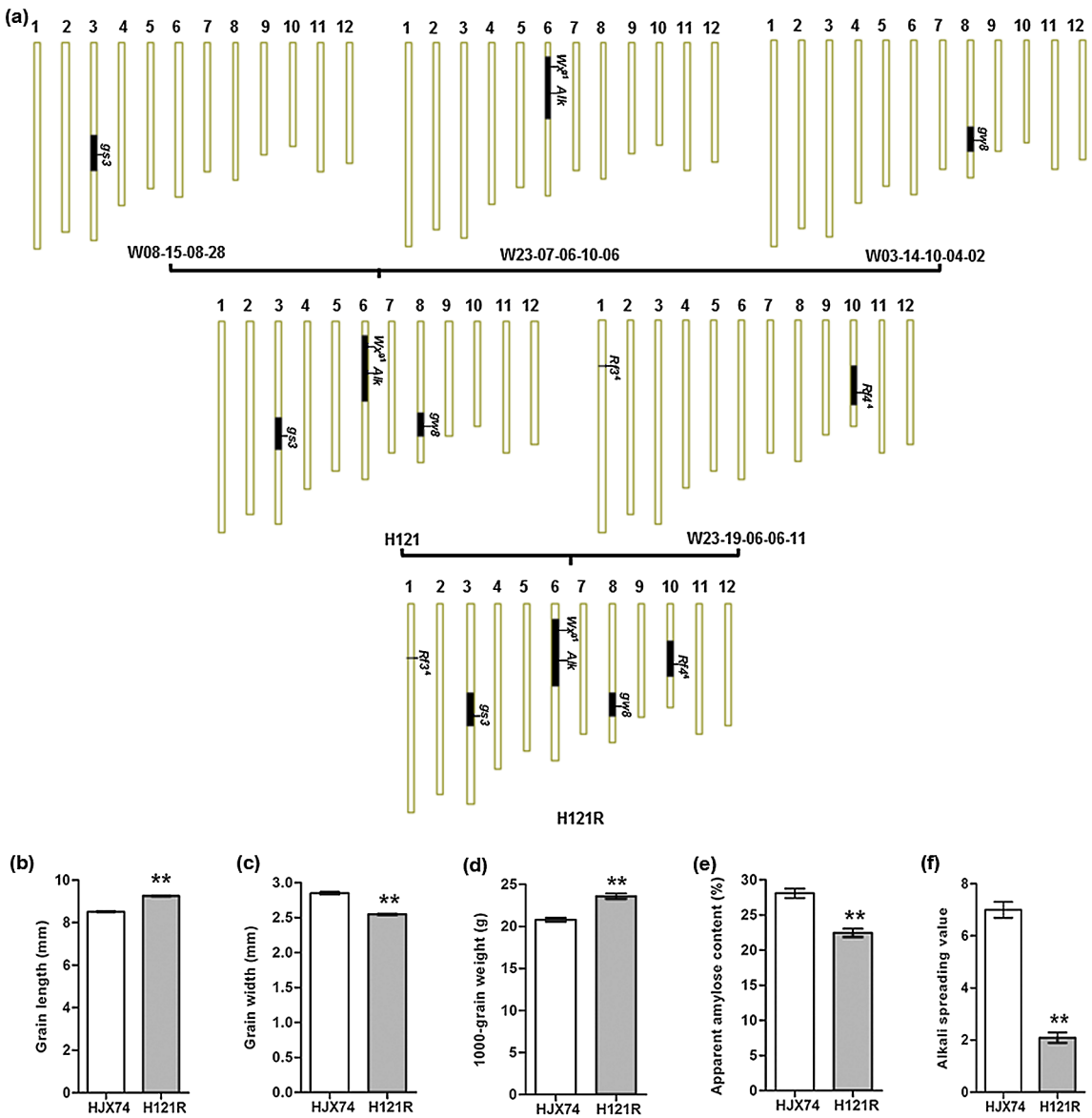

Fig. 1. Development of the H121R restorer line. (a) Development of the H121R restorer line based on the HJX74 SSSL library. H121 was developed from the cross of W08-15-08-28/W23-07-06-10-06//W03-14-10-04-02. H121R was then developed from the cross of H121/W23-19-06-06-11. The *vertical bars* are a graphical representation of the chromosomes. *Black* regions represent substitute segments with target genes, and *white* regions represent the HJX74 genetic background. (b) Grain length. (c) Grain width. (d) 1000-grain weight. (e) Apparent amylase content. (f) Alkali spreading value. *Error bars* represent SD. **, significant at the 0.01 probability level by *t* test.

Table 2. Comparison of some agronomic traits in HJX74, H121R and H131R

Trait	HJX74	H121R	H131R
Plant height (cm)	89.7±1.5	90.3±1.3 ns	89.2±0.8 ns
Heading date (d)	77.5±0.6	79.0±1.5 ns	78.2±0.7 ns
No. of panicles	7.7±0.6	8.1±1.6 ns	7.9±1.3 ns
Panicle length (cm)	22.7±1.7	23.3±1.4 ns	23.1±1.6 ns
Filled grain number per panicle	151.9±4.7	143.7±6.6 ns	147.5±5.3 ns

"ns" indicates no significant difference from the control HJX74 at $p < 0.05$.

of 28.5% obtained in HJX74 (**Fig. 1e**). Another trait related to eating and cooking, GT, was also greatly increased in H121R compared with HJX74 (**Fig. 1f**). However, there were no significant differences detected in the other main agronomic traits between H121R and HJX74, including the days to heading, plant height, number of panicles, panicle length, and filled grain number per panicle (**Table 2**).

Improvement of the blast resistance of the H121R restorer line

To improve the blast resistance of H121R, the SSSL W23-07-06-05-02-02 carrying blast resistance gene *qBLAST11* was crossed with the H121R line. The obtained pyramid line, containing the *gs3*, *gw8*, Wx^{g1}, *Alk*, and $Rf4^4$ genes from H121R, the *qBLAST11* gene from W23-07-06-05-02-02, and the $Rf3^4$ gene from the HJX74 genetic background, was designated H131R (**Fig. 2**).

Table 3. Resistance to blast in HJX74, H121R, H131R and F_1 hybrid of W-H121A/H131R

Isolate	Disease score			
	HJX74	H121R	H131R	W-H121A/H131R
04-94	1(R)	1(R)	1(R)	1(R)
Y98-66	9(S)	9(S)	1(R)	1(R)
97-322	9(S)	7(S)	1(R)	1(R)
W06-2a	8(S)	9(S)	1(R)	1(R)
93-286a	9(S)	8(S)	1(R)	1(R)
07-4a	7(S)	7(S)	1(R)	2(R)
06-141a	9(S)	8(S)	1(R)	1(R)
93-203a	5(S)	5(S)	1(R)	1(R)
00-193	9(S)	8(S)	1(R)	1(R)
00-173a	7(S)	8(S)	1(R)	1(R)
No. of infected isolates	9	9	0	0
Resistance frequency (%)	10	10	100	100
Resistance evaluation	Highly susceptible	Highly susceptible	Highly resistant	Highly resistant

R or S is resistant or susceptible, respectively.

To evaluate the reaction of the plants to blast in uniform blast nursery, HJX74, H121R and H131R were tested against 10 representative blast isolates collected in Guangdong province. The results indicated that the frequency of resistance in H131R was 100%, which was much higher than the frequency of 10.0% recorded in HJX74 and H121R (**Table 3**). However, there were no significant differences detected in the other main agronomic traits, including the days to heading, plant height, number of panicles, panicle

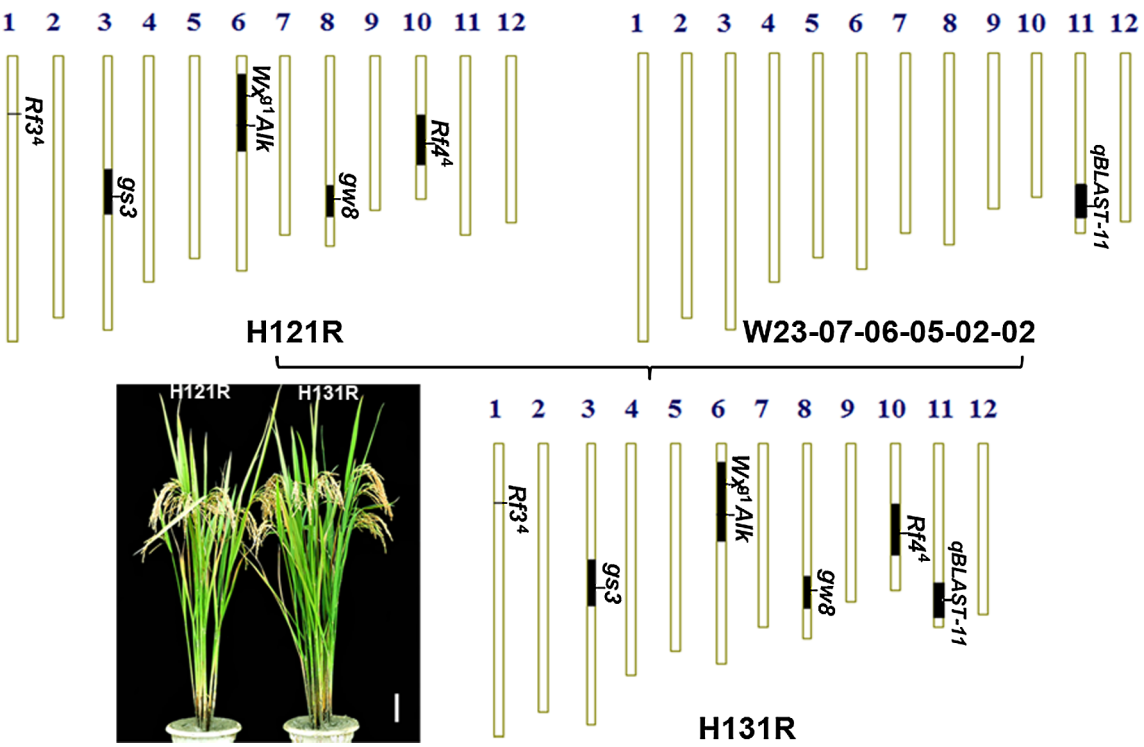

Fig. 2. Improvement of the H121R restorer line regarding blast resistance. H131R was developed from the cross of H121R/W23-07-06-05-02-02. The *vertical bars* are a graphical representation of the chromosomes. *Black* regions represent substitute segments with target genes, and *white* regions represent the HJX74 genetic background.

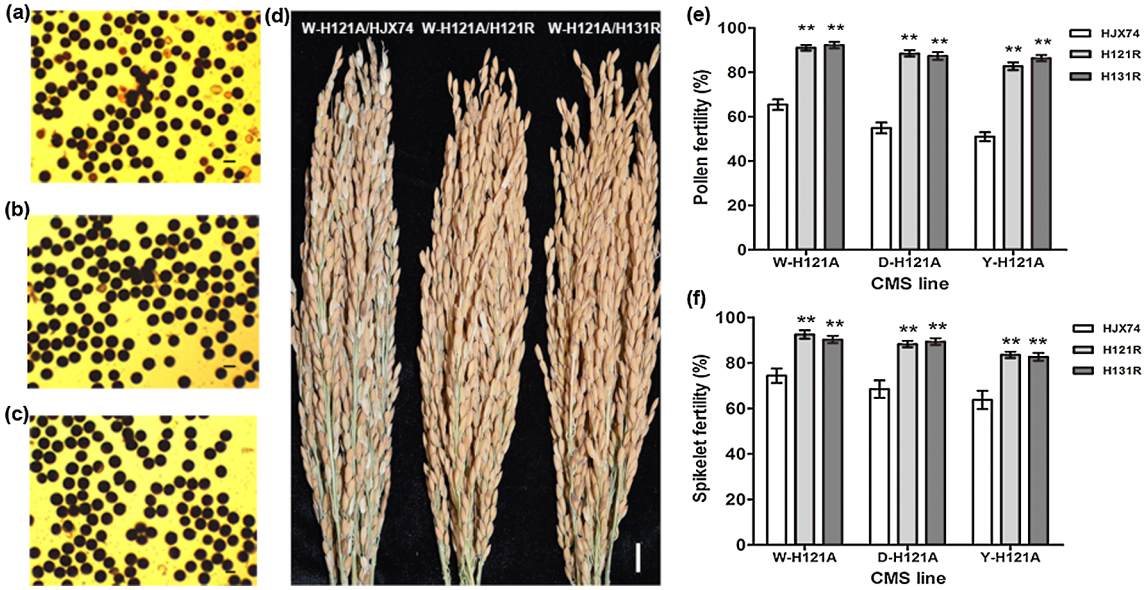

Fig. 3. Restoration ability of the restorer lines H121R and H131R. (a) Pollen fertility in the F_1 hybrid of W-H121A/HJX74. *Scale bar*, 50 μm. (b) Pollen fertility in the F_1 hybrid of W-H121A/H121R. *Scale bar*, 50 μm. (c) Pollen fertility in the F_1 hybrid of W-H121A/H131R. *Scale bar*, 50 μm. (d) Spikelet fertility in the F_1 hybrids of W-H121A/HJX74, W-H121A/H121R, and W-H121A/H131R. *Scale bar*, 1 cm. (e) Pollen fertility of F_1 hybrids from the crosses between the restorer lines HJX74, H121R and H131R and the CMS lines W-H121A, D-H121A, and Y-H121A, respectively. (f) Spikelet fertility of F_1 hybrids from the crosses between the restorer lines HJX74, H121R and H131R and the CMS lines W-H121A, D-H121A, and Y-H121A, respectively. *Error bars* represent SD. **, significant at the 0.01 probability level by *t* test.

length and filled grain number per panicle among H131R, H121R and HJX74 (**Table 2**).

Restoration ability of the two restorer lines

To assess the restoration ability of the restorer lines, H121R, H131R and HJX74 were test crossed with the W-H121A, D-H121A and Y-H121A CMS lines, respectively. The restoration ability of the restorer lines H121R and H131R was much stronger than that of HJX74 in terms of both pollen fertility and in spikelet fertility (**Fig. 3a–3d**). The pollen fertility and spikelet fertility of the F_1 hybrids derived from H121R and the CMS lines W-H121A, D-H121A and Y-H121A were 95.4% and 93.8%; 91.9% and 91.4%; and 88.9% and 85.6%, respectively. Similarly, the pollen fertility and spikelet fertility of the F_1 hybrids derived from H131R and the CMS lines W-H121A, D-H121A and Y-H121A were 94.3% and 95.8%; 92.5% and 90.7%; and 87.8% and 86.4%, respectively. The pollen fertility and spikelet fertility in the F_1 hybrids derived from HJX74 and the CMS lines W-H121A, D-H121A and Y-H121A, which were used as controls, were only 68.5% and 72.7%; 57.3% and 66.9%; and 48.6% and 62.1%, respectively. These results indicate that the goal of breeding to achieve restoration of fertility in the restorer lines H121R and H131R was achieved through pyramiding of the target genes in the platform.

Discussion

Following the strategy of breeding by design proposed by Peleman and van der Voort (2003), we developed inbred varieties and CMS lines under the platform of HJX74 SSSL library in rice (Dai *et al.* 2015). In this study, the SSSL library was successfully improved with the $Rf3^4$ and $Rf4^4$ genes into a platform for developing restorer lines. These results indicate that the HJX74 SSSL library is a powerful platform for breeding by design in rice, not only for inbred varieties but also for CMS lines, maintainers and restorers in hybrid rice. As a platform for breeding by design, the HJX74 SSSL library has several advantages. First, HJX74, the recipient parent of the SSSL library, is an elite variety that has been widely planted in south China in the past decade. Second, the substituted segments in the SSSL library cover the entire rice genome, with more than 18 equivalents of the rice genome. Third, the substituted segments in the library come from 26 genetically diverse donors. Fourth, each of the SSSLs shares the same HJX74 genetic background, with only one substituted segment from a donor (Xi *et al.* 2006, Zhang *et al.* 2004). Therefore, the three-steps involved in breeding by design proposed by Peleman and van der Voort (2003) can be conducted using the platform of the HJX74 SSSL library (Dai *et al.* 2015).

Since China pioneered hybrid rice production in the 1970s, the sporophytic CMS system has been the most important system employed in hybrid rice development, both in China and around the world (Cheng *et al.* 2007, Yuan and Tang 1999). Sporophytic CMS, including the WA, DA and YA types, is controlled by the *WA352* gene (Luo *et al.* 2013). The restoration of fertility in sporophytic CMS lines is controlled by two restorer genes, *Rf3* on chromosome 1

and *Rf4* on chromosome 10 (Cai *et al.* 2014, Tang *et al.* 2014, Xie *et al.* 2002, Yao *et al.* 1997, Zhang *et al.* 1997, 2002). CMS-based hybrid seed technology uses a three-line system consisting of a CMS line (A line), a maintainer line (B line), and a restorer line (R line). The A line exhibits male-sterile cytoplasm and two non-functional nuclear restorer genes, *rf3* and *rf4*. The B line displays normal fertile cytoplasm but contains the same nuclear genome as the A line. The R line possesses two functional nuclear restorer genes, *Rf3* and *Rf4*. Therefore, the three-line system is basically a CMS/*Rf* system. Understanding CMS and fertility restoration in the three-line system lays a foundation for breeding by design of the "three lines" in hybrid rice. In this study, the HJX74 SSSL library was successfully developed into a platform for developing restorer lines through breeding by design. Taken together with the previous development of CMS lines and maintainer lines using the platform of the HJX74 SSSL library (Dai *et al.* 2015), the HJX74 SSSL library has become a platform for breeding by design for the "three lines" in hybrid rice. Employing this platform, a series of "three lines" can be designed according to the goals regarding improvement. The available "three lines" developed on the platform can be then improved by the use of target genes selected from the HJX74 SSSL library. Thus, various new superior versions of the "three lines" can be developed easily and effectively under the SSSL platform. It is expected that the sustainable improvement of these "three lines" will facilitate hybrid rice development.

Acknowledgements

This work was supported by grants from the major program of transgenic new variety breeding of China (2014ZX08009-037B) and from the National Natural Science Foundation of China (91435207 and U1031002).

Literature Cited

Akagi, H., A. Nakamura, Y. Yokozeki-Misono, A. Inagaki, H. Takahashi, K. Mori and T. Fujimura (2004) Positional cloning of the rice *Rf-1* gene, a restorer of BT-type cytoplasmic male sterility that encodes a mitochondria-targeting PPR protein. Theor. Appl. Genet. 108: 1449–1457.

Cai, J., Q. Liao, Z. Dai, H. Zhu, R. Zeng, Z. Zhang and G. Zhang (2013) Allelic differentiations and effects of the *Rf3* and *Rf4* genes on fertility restoration in rice with wild abortive cytoplasmic male sterility. Biol. Plant. 57: 274–280.

Cai, J., Z. Dai, H. Zhu, R. Zeng, Z. Zhang, T. Ma and G. Zhang (2014) Comparative analysis of fertility restoration genes for WA, Y, and DA cytoplasmic male sterility in rice. Biol. Plant. 58: 241–246.

Chen, J., X. Li, C. Cheng, Y. Wang, M. Qin, H. Zhu, R. Zeng, X. Fu, Z. Liu and G. Zhang (2014) Characterization of epistatic interaction of QTLs *LH8* and *EH3* controlling heading date in rice. Sci. Rep. 4: 4263.

Chen, L. and Y. Liu (2013) Male sterility and fertility restoration in crops. Annu. Rev. Plant Biol. 65: 579–606.

Cheng, S., J. Zhuang, Y. Fan, J. Du and L. Cao (2007) Progress in research and development on hybrid rice: a super-domesticate in China. Ann. Bot. 100: 959–966.

Dai, Z., Q. Lu, X. Luan, J. Cai, H. Zhu, Z. Liu, R. Zeng, Z. Zhang, S. Wang, L. Zheng *et al.* (2015) Development of a platform for breeding by design of CMS lines based on an SSSL library in rice (*Oryza sativa* L.). Euphytica 205: 63–72.

Ebitani, T., Y. Takeuchi, Y. Nonoue, T. Yamamoto, K. Takeuchi and M. Yano (2005) Construction and evaluation of chromosome segment substitution lines carrying overlapping chromosome segments of indica rice cultivar 'Kasalath' in a genetic background of japonica elite cultivar 'Koshihikari'. Breed. Sci. 55: 65–73.

Fan, C., Y. Xing, H. Mao, T. Lu, B. Han, C. Xu, X. Li and Q. Zhang (2006) *GS3*, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, encodes a putative transmembrane protein. Theor. Appl. Genet. 112: 1164–1171.

Gao, Z., D. Zeng, X. Cui, Y. Zhou, M. Yan, D. Huang, J. Li and Q. Qian (2003) Map-based cloning of the *ALK* gene, which controls the gelatinization temperature of rice. Sci. China, C, Life Sci. 46: 661–668.

He, F., Z. Xi, R. Zeng, A. Talukdar and G. Zhang (2005) Identification of QTLs for plant height and its components by using single segment substitution lines in rice (*Oryza sativa*). Rice Sci. 12: 151–156.

Hu, J., K. Wang, W. Huang, G. Liu, Y. Gao, J. Wang, Q. Huang, Y. Ji, X. Qin, L. Wan *et al.* (2012) The rice pentatricopeptide repeat protein RF5 restores fertility in Hong-Lian cytoplasmic male-sterile lines via a complex with the glycine-rich protein GRP162. Plant Cell 24: 109–122.

IRRI (1996) Standard evaluation system for rice, 4th edn. International Rice Research Institute, Manila, Philippines: p. 52.

Kazama, T. and K. Toriyama (2003) A pentatricopeptide repeat-containing gene that promotes the processing of aberrant *atp6* RNA of cytoplasmic male-sterile rice. FEBS Lett. 544: 99–102.

Kazama, T. and K. Toriyama (2014) A fertility restorer gene, *Rf4*, widely used for hybrid rice breeding encodes a pentatricopeptide repeat protein. Rice 7: 28.

Komori, T., S. Ohta, N. Murai, Y. Takakura, Y. Kuraya, S. Suzuki, Y. Hiei, H. Imaseki and N. Nitta (2004) Map-based cloning of a fertility restorer gene *Rf-1* in rice (*Oryza sativa* L.). Plant J. 37: 315–325.

Kubo, T., Y. Aida, K. Nakamura, H. Tsunematsu, K. Doi and A. Yoshimura (2002) Reciprocal chromosome segment substitution series derived from japonica and indica cross of rice (*Oryza sativa* L.). Breed. Sci. 52: 319–325.

Li, W., R. Zeng, Z. Zhang, X. Ding and G. Zhang (2006) Fine mapping of locus *S-b* for F_1 pollen sterility in rice (*Oryza sativa* L.). Chin. Sci. Bull. 51: 675–680.

Liu, G., Z. Zhang, H. Zhu, F. Zhao, X. Ding, R. Zeng, W. Li and G. Zhang (2008) Detection of QTLs with additive effects and additive-by-environment interaction effects on panicle number in rice (*Oryza sativa* L.) with single-segment substitution lines. Theor. Appl. Genet. 116: 923–931.

Liu, G., H. Zhu, S. Liu, R. Zeng, Z. Zhang, W. Li, X. Ding, F. Zhao and G. Zhang (2010) Unconditional and conditional QTL mapping for the developmental behavior of tiller number in rice (*Oryza sativa* L.). Genetica 138: 885–893.

Liu, G., H. Zhu, G. Zhang, L. Li and G. Ye (2012) Dynamic analysis of QTLs on tiller number in rice (*Oryza sativa* L.) with single segment substitution lines. Theor. Appl. Genet. 125: 143–153.

Liu, X., X. Xu, Y. Tan, S. Li, J. Hu, J. Huang, D. Yang, Y. Li and Y. Zhu (2004) Inheritance and molecular mapping of two fertility-restoring loci for Honglian gametophytic cytoplasmic male sterility in rice

(*Oryza sativa* L.). Mol. Genet. Genomics 271: 586–594.

Luo, D., H. Xu, Z. Liu, J. Guo, H. Li, L. Chen, C. Fang, Q. Zhang, M. Bai, N. Yao *et al.* (2013) A detrimental mitochondrial-nuclear interaction causes cytoplasmic male sterility in rice. Nat. Genet. 45: 573–577.

Murray, M. and W.F. Thompson (1980) Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321–4325.

Naeem, M., S. Freed and G. Zhang (2013) Molecular genetic studies of heading date gene *OsMADS50* by using single segment substitution lines in rice. Int. J. Agric. Biol. 15: 631–639.

Peleman, J.D. and J.R. van der Voort (2003) Breeding by design. Trends Plant Sci. 8: 330–334.

Sharma, S., S. Singh, R. Nandan and M. Kumar (2012) Identification of restorers and maintainers for CMS lines of rice (*Oryza sativa* L.). Indian J. Plant Genet. Resour. 25: 186–188.

Singh, U., J. Lal and H. Kumar (2012) Isolation and evaluation of restorers and examining possibility of developing new version of CMS lines for upland rainfed rice hybrids. Environ. Ecol. 30: 872–876.

Suresh, P.B., B. Srikanth, V.H. Kishore, I.S. Rao, L.R. Vemireddy, N. Dharika, R.M. Sundaram, M.S. Ramesha, K.R.S.S. Rao, B.C. Viraktamath *et al.* (2012) Fine mapping of *Rf3* and *Rf4* fertility restorer loci of WA-CMS of rice (*Oryza sativa* L.) and validation of the developed marker system for identification of restorer lines. Euphytica 187: 421–435.

Tan, Y., J. Li, S. Yu, Y. Xing, C. Xu and Q. Zhang (1999) The three important traits for cooking and eating quality of rice grains are controlled by a single locus in an elite rice hybrid 'Shanyou 63'. Theor. Appl. Genet. 99: 642–648.

Tang, H., D. Luo, D. Zhou, Q. Zhang, D. Tian, X. Zheng, L. Chen and Y. Liu (2014) The rice restorer *Rf4* for wild-abortive cytoplasmic male sterility encodes a mitochondrial-localized PPR protein that functions in reduction of *WA352* transcripts. Mol. Plant 7: 1497–1500.

Teng, B., R. Zeng, Y. Wang, Z. Liu, Z. Zhang, H. Zhu, X. Ding, W. Li and G. Zhang (2012) Detection of allelic variation at the *Wx* locus with single-segment substitution lines in rice (*Oryza sativa* L.). Mol. Breed. 30: 583–595.

Wang, S., K. Wu, Q. Yuan, X. Liu, Z. Liu, X. Lin, R. Zeng, H. Zhu, G. Dong, Q. Qian *et al.* (2012) Control of grain size, shape and quality by *OsSPL16* in rice. Nat. Genet. 44: 950–954.

Wang, Z., Y. Zou, X. Li, Q. Zhang, L. Chen, H. Wu, D. Su, Y. Chen, J. Guo, D. Luo *et al.* (2006) Cytoplasmic male sterility of rice with Boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. Plant Cell 18: 676–687.

Xi, Z., F. He, R. Zeng, Z. Zhang, X. Ding, W. Li and G. Zhang (2006) Development of a wide population of chromosome single-segment substitution lines in the genetic background of an elite cultivar of rice (*Oryza sativa* L.). Genome 49: 476–484.

Xie, J., J. Zhuang, Y. Fan, G. Tu, Y. Xia and K. Zheng (2002) Mapping of fertility-restoring genes with main effects and epistatic effects for CMS-DA in rice. J. Genet. Genomics 29: 616–621.

Yao, F., C. Xu, S. Yu, J. Li, Y. Gao, X. Li and Q. Zhang (1997) Mapping and genetic analysis of two fertility restorer loci in the wild-abortive cytoplasmic male sterility system of rice (*Oryza sativa* L.). Euphytica 98: 183–187.

Yuan, L. and C. Tang (1999) Retrospect, current status and prospect of hybrid rice. Rice Sci. 4: 3–6.

Zeng, R., Z. Zhang, F. He, Z. Xi, A. Talukdar, J. Shi, L. Qin, C. Huang and G. Zhang (2006) Identification of multiple alleles at the *Wx* locus and development of single segment substitution lines for the alleles in rice. Rice Sci. 13: 9–14.

Zhang, G., Y. Lu, T.S. Bharaj, S.S. Virmani and N. Huang (1997) Mapping of the *Rf-3* nuclear fertility-restoring gene for WA cytoplasmic male sterility in rice using RAPD and RFLP markers. Theor. Appl. Genet. 94: 27–33.

Zhang, G., R. Zeng, Z. Zhang, X. Ding, W. Li, G. Liu, F. He, A. Tulukdar, C. Huang, Z. Xi *et al.* (2004) The construction of a library of single segment substitution lines in rice (*Oryza sativa* L.). Rice Genet. Newsl. 21: 85–87.

Zhang, Q., Y. Liu, G. Zhang and M. Mei (2002) Molecular mapping of the fertility restorer gene *Rf-4* for WA cytoplasmic male sterility in rice. J. Genet. Genomics 29: 1001–1004.

Zhang, Y., J. Yang, Z. Shan, S. Chen, W. Qiao, X. Zhu, Q. Xie, H. Zhu, Z. Zhang, R. Zeng *et al.* (2012) Substitution mapping of QTLs for blast resistance with SSSLs in rice (*Oryza sativa* L.). Euphytica 184: 141–150.

Zhao, F., H. Zhu, X. Ding, R. Zeng, Z. Zhang, W. Li and G. Zhang (2007) Detection of QTLs for important agronomic traits and analysis of their stabilities using SSSLs in rice. China Agric. Sci. 6: 769–778.

Zheng, K., P. Subudhi, J. Domingo, G. Magpantay and N. Huang (1995) Rapid DNA isolation for marker assisted selection in rice breeding. Rice Genet. Newsl. 12: 255–258.

Zhu, H., Z. Liu, X. Fu, Z. Dai, S. Wang, G. Zhang, R. Zeng and G. Liu (2015) Detection and characterization of epistasis between QTLs on plant height in rice using single segment substitution lines. Breed. Sci. 65: 192–200.

Zhu, X., S. Chen, J. Yang, S. Zhou, L. Zeng, J. Han, J. Su, L. Wang and Q. Pan (2012) The identification of *Pi50(t)*, a new member of the rice blast resistance *Pi2/Pi9* multigene family. Theor. Appl. Genet. 124: 1295–1304.