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* and *Rf4*, which are associated with a strong restoration ability, and *gs3*, *gw8*, *Wxg1* 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* and *Rf4* 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*) nucleus (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 Eejunan 1 (*indica*) nucleus (WA-CMS), dwarf abortive rice (*Oryza rufipogon*) cytoplasm with Xieqingzao (*indica*) nucleus (DA-CMS) and Yeongg (*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 F₁ 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...
Breeding by design for rice restorers under SSSL library platform

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 (HXJ74), 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 HXJ74 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 allelic variations at the Rf3 and Rf4 loci, 57 SSSLs carrying allelic variations at the Rf3 and Rf4 loci, 57 SSSLs carrying allelic variations, were selected from the library. Four alleles were identified at both loci in the set of SSSLs, which were designated Rf31, Rf32, Rf33 and Rf34 and Rf41, Rf42, Rf43, and Rf44, respectively, ranging from weak to strong in terms of their restoration ability. The HXJ74 recipient harbors the Rf31 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 Rf42 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 HXJ74 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 Huaxiaohai1, which was released in 2005. Furthermore, a pyramiding line with three substituted segments in an HXJ74 genetic background was designated Huabiao1 and released to farmers in 2009 (Dai et al. 2015). Because HXJ74 is neither a CMS maintainer nor a CMS restorer, the HXJ74 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 HXJ74 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 HXJ74 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 HXJ74 SSSL library. By using the Rf31 and Rf42 restorer genes, a restorer line, H121R with a strong restoration ability was developed in the HXJ74 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 HXJ74 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 Rf31 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 HXJ74 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 HXJ4 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 Wx1 gene, W03-14-10-04-02 with the gw8 gene, and W23-19-06-06-11 with the Rf4 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 Wx1 gene were selected from an F3 population of 100 plants by marker-assisted selection (MAS). The homozygotes with gs3 and Wx1 gene were then crossed with the SSSL W03-14-10-04-02 with the gw8 gene, and the line H121 with gs3, Wx1 and gw8 genes were obtained from the F3 population. To improve the fertility restoration, the H121 line was crossed with W23-19-06-06-11 with the Rf4 gene. Good quality of restorer line, H121R, was obtained, which carried the gs3, Wx1 and gw8 genes from the H121 line and the Rf4 gene from W23-19-06-06-11 in the F3 population by MAS.
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 F1 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 Wxθ1, conferring a medium apparent amylose content (Teng et al. 2012), and Alk, conferring a high gelatinization temperature (Gao et al. 2012), 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^ 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).

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(Wxθ1)-RM204-RM402-Alk-RM539-RM541 | 1.4–68.5 | Wxθ1 | MAAC |
| 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-Alk-RM6100-RM25685 | 30.2–58.5 | Rf4^ | SRF |
| W23-07-06-05-02-02 | Lemont | 11   | RM224-qBLAST11-RM144 | 110.1–116.5 | qBLAST11 | 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.

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 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 x 20 cm x 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, Wxe1 and Alk, in three SSSLs were pyramided under the HJX74 genetic background. The pyramid line with the four genes (gs3, gw8, Wxe1 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 gene was then crossed with the H121 line. The pyramid line, carrying the gs3, gw8, Wxe1 and Alk genes from H121, the Rf4 gene from W23-19-06-06-11, and the Rf3 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...
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, Wxg1, Alk, and Rf4 genes from H121R, the qBLAST11 gene from W23-07-06-05-02-02, and the Rf34 gene from the HJX74 genetic background, was designated H131R (Fig. 2).

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 length, and filled grain number per panicle (Table 2).

### 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 $p < 0.05$.

### Table 3. Resistance to blast in HJX74, H121R, H131R and F1 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 (%) | Highly susceptible | Highly susceptible | Highly resistant | Highly resistant |

R or S is resistant or susceptible, respectively.

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**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.
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 F₁ 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 F₁ 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 F₁ 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 and Rf4 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.

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Breeding by design for rice restorers under SSSL library platform

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