Breeding by design of CMS lines on the platform of SSSL library in rice

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Abstract Greatly increased yield by utilizing of heterosis has been a great success in rice over the past 40 years. The cytoplasmic male sterility (CMS) line is a key line of hybrid rice. However, the CMS line was very difficult to develop following the traditional breeding methods. Here, we report an example to develop and improve CMS lines sustainably by using the strategy of breeding by design under the platform of single segment substitution line (SSSL) library in the Huajingxian74 (HJX74) genetic background. The CMS lines HZA and HBA with the wild abortive (WA) cytoplasm from Zhenshan97A and BobaiA, respectively, were firstly developed in the HJX74 background through marker-assisted selection. Then, four isonuclear alloplasmic CMS lines with the WA, dwarf wild abortive (DA), and Yegong abortive (YA) types of CMS cytoplasm, respectively, were developed by using the HZB maintainer line. According to the goal of design, E5-HXA and E5-HBA were developed by pyramiding five target genes, *Wx^{g1}*, *ALK*, *fgr*, *gs3*, and *OsMADS50*, located on the chromosomal substituted segments in four SSSLs. The two CMS lines show the phenotypes of earlier in heading date, longer and narrower in grain shape, lower

in amylose content, and fragrant smell. Thus, the goal of design was achieved on the platform of SSSL library. Compared with that in the E5-HXA line carrying the *rf3* and *rf4* genes of XieqingzaoA, the CMS in the E5-HBA line with the *rf3* and *rf4* genes from BobaiA was susceptible to environmental impact and easy to be restored. Therefore, the balance between sterility and its restored ability in CMS lines should be concerned in breeding by design.

Keywords CMS · SSSL · Molecularbreeding · Breeding by design · Rice

Introduction

Rice (*Oryza sativa*) is the most important staple crop of the world and about half of the population in the world lives on it. Over the past decades, two tremendous leaps in enhancing rice yield have been achieved by the utilization of semi-dwarf gene in rice breeding (Spielmeyer et al. 2002), and by the utilization of heterosis in hybrid rice (Yuan 2017). The varieties of hybrid rice increased the grain yield by over 20% and had accounted for approximately 55% of the total rice planting area in China since the late 1980s (Cheng et al. 2007; Li et al. 2007).

Three-line system, which is composed by three different breeding lines: the cytoplasmic male sterility (CMS) line (A line), the maintainer line (B line), and the restorer line (R line), is a main system in hybrid rice production. In the sporophytic CMS, the CMS lines

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with wild abortive (WA) cytoplasm, dwarf wild abortive (DA) cytoplasm, and Yegong abortive (YA) cytoplasm are commonly used in hybrid rice production (Li et al. 2007; Dai et al. 2015). The CMS is restored by the fertility-restoring genes, *Rf3* and *Rf4*. The *Rf3* gene was mapped on chromosome 1 (Yao et al. 1997; Zhang et al. 1997), and the *Rf4* gene was mapped on chromosome 10 (Yao et al. 1997; Zhang et al. 2002). Recently, the *WA352* gene, which controls WA-CMS, and the *Rf4* gene, which encodes a PPR protein, were cloned (Luo et al. 2013; Kazama and Toriyama 2014; Tang et al. 2014).

Peleman and van der Voort (2003) proposed the concept of “breeding by design.” To conduct the strategy of breeding by design in rice, we have developed a library consisting of chromosomal single segment substitution lines (SSSLs) in rice by using Huajingxian74 (HJX74), an elite *indica* variety from South China, as the recipient, and 40 accessions from 7 species of AA genome in the *Oryza* genus as donors. To date, more than 2000 SSSLs have been developed (Zhang et al. 2004; He et al. 2005; Xi et al. 2006; He et al. 2017; Zhao et al. 2019). The genes for agronomic importance on the substituted segments in the SSSLs were analyzed. The gene information was collected including gene location, allelic variation, interaction of gene by gene and gene by environment, molecular function, and so on (Zeng et al. 2006; Zhao et al. 2007; Liu et al. 2008; Liu et al. 2010; Liu et al. 2012; Teng et al. 2012; Wang et al. 2012; Zhang et al. 2012; Chen et al. 2014; Qin et al. 2015; Wang et al. 2015; Yang et al. 2016; Zhao et al. 2016; Zhou et al. 2017; Yang et al. 2018; Zhu et al. 2018). To assess allelic variations at the *Rf3* and *Rf4* loci, the SSSLs carrying one of the loci in the substituted segments were selected from the library. Four alleles with a weak to strong restoration ability were identified at each locus (Cai et al. 2013). Based on the SSSL library, the platform of breeding by design for CMS lines was developed. Three isonuclear alloplasmic CMS lines with the HJX74 genetic background were developed (Dai et al. 2015). Then, we developed a platform for breeding by design of the CMS restorer lines based on the SSSL library. By using the strongest restorer alleles, *Rf3*⁴ and *Rf4*⁴, a restorer line with strong restoration ability was developed in the HJX74 genetic background (Dai et al. 2016).

In this study, we used the SSSL platform to develop new CMS lines to meet the needs of hybrid rice production. A set of new CMS lines with the WA, YA, and DA

cytoplasm were developed. And then, two CMS lines with early heading and good quality were bred by design. The results indicate that the SSSL library is a powerful platform of breeding by design for CMS lines in rice.

Materials and methods

Plant materials

HJX74, an elite *indica* variety in South China, was used as the recipient of the SSSLs (He et al. 2005; Xi et al. 2006; He et al. 2017; Zhao et al. 2019). Four CMS lines used popularly in China, Zhenshan97A and BobaiA with WA cytoplasm, XieqingzaoA with DA cytoplasm, and HuanongA with YA cytoplasm, were used as the donors of abortive or sterile cytoplasm and recessive nuclear genes *rf3* and *rf4*. The maintainer line HXB (also named H121B), which was previously developed from XieqingzaoB in the HJX74 genetic background (Dai et al. 2015), was used as the donor of *rf3* and *rf4* genes to develop new maintainer lines. Four SSSLs, W23-07-06-10-06 containing *Wx^{gl}* and *ALK* genes on the substituted segment of chromosome 6 from the Lemont, W15-05-09-06-08 with a substituted segment containing the *fgr* fragrance gene on chromosome 8 from American Jasmine, W08-15-08-28 with the *gs3* gene on the substituted segment of chromosome 3 from IR64, and W23-03-08-09-27-82 with *OsMADS50* gene on the substituted segment of chromosome 3 from Lemont, were used as donors of the target genes (Table 1).

All of the plant materials used in this study were planted in two cropping seasons each year, in the experimental station at South China Agricultural University, Guangzhou (23°07'N, 113°15'E), China, from 2012 to 2017. The spring cropping season was from late February to middle July, and the fall cropping season was from late July to middle November. Field management, including irrigation, fertilizer application, and pest control, was kept according to the recommendation of the area.

Development of breeding lines

To develop maintainer lines in the HJX74 genetic background, HJX74 was crossed with BobaiB and Zhenshan97B to produce F₁ hybrids, then F₁ hybrids

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

Donor	Chr.	Substitution segment	Interval of substituted segments (cM)	Target gene	Target trait
Lemont	6	RM508-RM190 (<i>Wx^{g1}</i>) -RM225-RM253-RM402- <i>ALK</i> - RM539-PSM388-RM136-RM3-RM541	1.4–68.5	<i>Wx^{g1}</i>	MAAC
American Jasmine	8	RM515-PSM522- <i>fgr</i> -PSM525-RM223	72.1–75.7	<i>fgr</i>	HGT
IR64	3	PSM485- <i>gs3</i> -RM6146-RM3646-RM16	83–87.9	<i>gs3</i>	LG
Lemont	3	RM306-RM3202-RM3894-RM523	1.1–5.5	<i>OsMADS50</i>	EH

MAAC, medium apparent amylose content; HGT, high gelatinization temperature; LG, long grain; EH, early heading

were backcrossed with HJX74 to produce BC₁F₁, respectively. A total of 228 polymorphic SSR markers, including RM1/RM10340 linked to *rf3* and RM5373/RM6100 linked to *rf4*, were selected on the rice micro-satellite maps (Chen et al. 1997; McCouch et al. 2002; Zhang et al. 2007), to detect target genes *rf3* and *rf4* and genetic background in every generation by marker-assisted selection (MAS). After successive backcrossing, two new maintainer lines, HBB developed from BobaiB and HZB from Zhenshan97B, were developed in BC₅F₂ generations.

To develop a four-segment pyramiding line, W23-07-06-10-06 with the *Wx^{g1}* and *ALK* genes, W15-05-09-06-08 with the *fgr* gene, W08-15-08-28 with the *gs3* gene, and W23-03-08-09-27-82 with the *OsMADS50* gene in their substituted chromosomal segments were selected from the HJX74-SSSL library (Table 1). Two double segment substitution lines (DSSLs) were developed from the crosses of W23-07-06-10-06/W15-05-09-06-08 and W08-15-08-28/W23-03-08-09-27-82, respectively. Then, the two DSSLs were crossed to develop a four-segment pyramiding line by MAS.

To develop early heading and good quality CMS lines, HXB and HBB were respectively crossed with the four-segment pyramiding line. Then, the F₁ hybrids were backcrossed with the four-segment pyramiding line. Finally, two new maintainer lines, E5-HXB developed from HXB and E5-HBB from HBB, were respectively obtained in BC₁F₂ generations by MAS.

Examination of pollen and spikelet fertility

To examine pollen fertility, 6–9 mature flowers were collected from the upper one-third of the panicles of plants during the flowering time. The pollens were stained with 1% I₂-KI solution containing 0.1% (w/v)

iodine and 1% (w/v) potassium iodide. More than 300 pollen grains were randomly counted per plant under an optical microscope. The numbers of stainable pollens and un-stainable pollens in each individual were recorded. To observe spikelet fertility, three panicles per plant were harvested during the maturation time. Ten to twenty plants were recorded for each variation.

Investigation of traits and statistical analysis

Each of the traits was tested in 10–20 plants per line during the fall cropping season in 2012–2017. The apparent amylose content (AAC) and gelatinization temperature (GT), which was indirectly estimated via alkali spreading value (ASV), were tested following procedures described in previous studies (Teng et al. 2012; Teng et al. 2013). Leaves and dehulled rice were used to evaluate the fragrance. To test fragrance using leaves, one or two leaves per line were cut into small pieces and put into 15-mL tubes with 10 mL of 1.7% KOH. After 30 min, the boxes were opened and immediately smelled (Sood and Siddiq 1978). To test fragrance using dehulled rice grains, 10 seeds from each line were chewed individually. Rice materials were then scored as fragrant or non-fragrant. Statistical analyses of the tested traits were processed by using Microsoft Excel 2010 and SPSS version 18.0.

Results

Development of new maintainer lines, HZB and HBB, in the HJX74 genetic background

To develop two new maintainer lines in the HJX74 genetic background, HJX74 was crossed with

Fig. 1 Genotypes and phenotypes of HJB and HBB. **a** Plant morphology of HJB74, HJB, and HBB. Scale bar, 15 cm. **b** Position of the target substituted segments in the genome of HJB and HBB. Vertical bars are a graphical representation of chromosomes. Black parts are substituted segments from Zhenshan97B/BobaiB with target genes, and white parts are

genetic background from HJB74. **c–e** Grain shapes of HJB74, HJB, and HBB. Scale bar, 2 cm. **f** The number of panicles per plant of HJB74, HJB, and HBB. **g** Plant height of HJB74, HJB, and HBB. **h** Heading date of HJB74, HJB, and HBB. Error bars represent SD

maintainer lines Zhenshan97B and BobaiB to produce F_1 hybrids, respectively. Then, five times successive marker-facilitated backcrossing with HJB74 was performed to develop BC_5F_1 populations. Two new HJB74 maintainer lines, HJB and HBB, with the *rf3* and *rf4* genes derived from Zhenshan97B and BobaiB, were bred in the BC_5F_2 generation, respectively (Fig. 1a). In the HJB line, only two chromosomal substituted segments from Zhenshan97B were detected in the HJB74 genetic background, a 39.1-cM-length segment containing the *rf3* locus on chromosome 1 and a 3.6-cM-length segment containing the *rf4* locus on chromosome 10. Similarly, the HBB line carries a 27.6-cM-length segment with *rf3* locus on chromosome 1 and a 39.9-cM-length segment with *rf4* locus on chromosome 10 from BobaiB in the HJB74 genetic background (Fig. 1b).

Because only two chromosomal segments were substituted in HJB and HBB lines compared with HJB74, no significant differences of agronomic traits, such as grain shape, panicle number per plant, plant height, and heading date, were detected

between the maintainer lines (HJB and HBB) and HJB74 (Fig. 1c–h).

Development of isonuclear alloplasmic CMS lines of HJB

Using the maintainer line HJB as the nuclear donor, four isonuclear alloplasmic CMS lines, WA-HZA1, WA-HZA2, DA-HZA, and YA-HZA, were developed (Fig. 2a). To develop the isonuclear alloplasmic CMS lines, four CMS lines, Zhenshan97A and BobaiA with WA cytoplasm, XieqingzaoA with DA cytoplasm, and HuanongA with YA cytoplasm, were used as cytoplasm donors to cross with HJB. After successive backcrossing for six times, the pollens of all BC_6F_1 plants were completely sterile in the four populations. The anthers were white and slender, and the pollen grains were shrinking and un-stained with 1% I_2 -KI solution (Fig. 2d–g). As control, the anthers and pollen grains in maintainer line HJB were normal (Fig. 2h). In the following successive backcrossing, the sterility was complete and steady with 100% of abortive pollens from

Fig. 2 Phenotypes of four isonuclear alloplasmic CMS lines. **a** Plant morphology of WA-HZA1, DA-HZA, HZB, WA-HZA2, and YA-HZA. The cytoplasm of WA-HZA1, DA-HZA, WA-HZA2, and YA-HZA was derived from BobaiA, XieqingzaoA, Zhenshan97A, and HuanongA, respectively. Scale bar, 15 cm. **b, c**

Heading date and plant height of four isonuclear alloplasmic CMS lines. Error bars represent SD. **d–h** Morphology of anthers and pollen grains of WA-HZA1, DA-HZA, WA-HZA2, YA-HZA, and HZB, respectively. Pollen grains were stained with 1% I₂-KI; Scale bars, 1 mm (anthers); 50 μ m (pollen)

generation to generation in the four populations. In consequence, four isonuclear alloplasmic CMS lines, WA-HZA1, WA-HZA2, DA-HZA, and YA-HZA, in the HJX74 genetic background were developed.

The traits of isonuclear alloplasmic CMS lines were tested and compared with each other. The plant heights were in 72.2–73.9 cm and the heading dates were 81.3–83.1 days with no significant difference among four CMS lines (Fig. 2b, c). These results indicated that the cytoplasm from WA, DA, and YA did not cause difference in the traits among the isonuclear alloplasmic CMS lines.

Improvement of the CMS maintainer lines of HBB and HXB

To conduct breeding by design of CMS lines with early heading and good quality, four SSSLs with the target

genes *Wx^{g1}* and *ALK* from Lemont, *fgr* from American Jasmine, *gs3* from IR64, and *OsMADS50* gene from Lemont were selected from the SSSL library to develop pyramiding line with those loci (Fig. 3; Table 1). At first, two DSSLs were respectively developed from the crosses of W23-07-06-10-06/W15-05-09-06-08 and W08-15-08-28/W23-03-08-09-27-82. And then, a line with the four substituted segments, named E5, was developed from the cross of the two DSSLs by MAS (Fig. 3). Using the maintainer line HBB developed in this study, and the maintainer line HXB (H121B) developed previously in our lab (Dai et al. 2015) as the donors of *rf3* and *rf4*, two new maintainer lines, E5-HBB and E5-HXB, were respectively developed from the crosses between the maintainer lines (HBB and HXB) and E5 by MAS (Fig. 4a).

The traits controlled by the target genes in the E5-HBB and E5-HXB were investigated to evaluate the

Fig. 3 Development scheme of E5 based on the HJX74 SSSL library. Two DSSL lines were developed from the crosses of W23-07-06-10-06/W15-05-09-06-08 and W08-15-08-28/W23-03-08-09-27-82, then, E5 was developed from the cross of the two DSSL

lines. 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

improvement. In heading date, a significant difference was detected between the maintainer lines (E5-HBB with 65.9 days, and E5-HXB with 65.8 days) and HJX74 (74.8 days) (Fig. 4b). Compared with HJX74 with a grain length of 8.29 mm, grain width of 2.64 mm, and 1000-grain weight of 21.5 g, longer, more slender grain and heavier grain weight were detected in E5-HBB and E5-HXB with grain lengths of 10.13 mm and 10.28 mm, grain widths of 2.60 mm and 2.60 mm, and 1000-grain weights of 26.2 g and 26.5 g, respectively (Fig. 4c–e). For the traits of cooking and eating quality, intermediate class apparent amylose content (AAC) in E5-HBB (22.5%) and E5-HXB (22.7%) was very significantly lower than the value 24.8% of the recipient HJX74 (Fig. 4f). Alkali spreading value (ASV) was greatly decreased in E5-HBB and E5-HXB compared with HJX74 (Fig. 4g). As expected, fragrance was detected in E5-HBB and E5-HXB with the effect of *fgr* gene in exception of HJX74. These results indicated

that the goal of breeding designed can be well achieved under the SSSL platform.

Difference in the CMS between E5-HBA and E5-HXA

Two new CMS lines, E5-HXA carrying the *rf3* and *rf4* genes from XieqingzaoA and E5-HBA carrying *rf3* and *rf4* from BobaiA, were developed by successive backcross using of E5-HXB with HXA and of E5-HBB with HBA, respectively. Similar to their maintainer lines, the grain shape of E5-HBA and E5-HXA was long-slender compared with HJX74 (Fig. 5a, b). The E5-HXA showed pollen sterility completely and steadily in 9 cropping seasons from 2013 to 2017 (Fig. 5c, d; Table 2). Interestingly, the pollen fertility in E5-HBA was complete and steady in fall cropping seasons (from Jul to Nov), but incomplete and unsteady in spring cropping seasons (from Feb to

Fig. 4 Development of the E5-HBB and E5-HXB maintainer lines. **a** Development schemes of the E5-HBB and E5-HXB maintainer lines based on the HJX74 SSSL library. E5-HBB was developed from the cross of HBB and E5 (a four-segment pyramiding line). E5-HXB was developed from the cross of HXB and E5 in the same way. 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** Heading date. **c** Grain length. **d** Grain width. **e** 1000-grain weight. **f** Apparent amylose content. **g** Alkali spreading value. Error bars represent SD. Single asterisk (*) and double asterisks (**) represent significance at the 0.05 and 0.01 probability level by *t* test

Jul) (Fig. 5e, f; Table 2). In spring cropping seasons, the pollen grains of E5-HBA presented spherical abortion type, stained abortion type, and even some fertility (Fig. 5e; Table 2). By contrast, typical abortion type of pollen grains was always observed in fall cropping seasons of E5-HBA (Fig. 5f). These results indicated that the CMS in the E5-HBA line was more susceptible to environmental factors compared with that in other CMS lines.

To evaluate the restored ability of CMS lines, two restorer lines developed in our lab, P46 and Rnn, were test-crossed with the E5-HBA and E5-HXA in the fall cropping season of 2017, respectively. The higher degree of restoration was detected in E5-HBA in terms of pollen fertility than that in E5-HXA when test-crossed with P46 and Rnn (Fig. 5g, i). The pollen fertility in the F₁ hybrids

from CMS line E5-HBA (95.6% in P46 and 95.7% in Rnn) was significantly higher than that from CMS line E5-HXA (90.6% in P46 and 92.7% in Rnn) (Fig. 5g, i) although the difference was not found in the spikelet fertility (Fig. 5h, j). These results indicated that the CMS in the E5-HBA line was easily restored than that in the E5-HXA line.

The pollen sterility of E5-HBA was influenced by environment

The pollen sterility of E5-HBA was incomplete and unsteady in the spring cropping season in 2013–2017 (Table 2). To test the period from incomplete sterility to complete sterility, the E5-HBA CMS line was sowed in 6 times, from Mar 2 to Jul 13 in 2015. The pollen fertility at the 6 sowing times was 5.03–5.07%, 1.11–1.42%, 0.65%, 0–0.12%, 0, and 0 in E5-HBA CMS

Fig. 5 Phenotypes of E5-HXA and E5-HBA. **a, b** Grain shapes of E5-HXA and E5-HBA. Scale bar, 1 cm (**a**); 0.5 cm (**b**). **c, d** Morphology of pollen grains of E5-HXA in spring cropping season (**c**) and fall cropping season (**d**). **e, f** Morphology of pollen grains of E5-HBA in spring cropping season (**e**) and fall cropping season (**f**). Pollen grains were stained with 1% I₂-KI; Scale bar, 50 μ m. **g** Pollen fertility of the F₁ hybrids from the crosses between E5-HBA, E5-HXA, and restorer line P46. **h** Spikelet

fertility of the F₁ hybrids from the crosses between E5-HBA, E5-HXA, and restorer line P46. **i** Pollen fertility of the F₁ hybrids from the crosses between E5-HBA, E5-HXA, and restorer line Rnn. **j** Spikelet fertility of the F₁ hybrids from the crosses between E5-HBA, E5-HXA, and restorer line Rnn. (H1), E5-HXA/P46; (H2), E5-HBA/P46; (H3), E5-HXA/Rnn; (H4), E5-HBA/Rnn. Error bars represent the SD, $n = 12$

Table 2 Pollen fertility of E5-HBA and E5-HXA in 9 crop seasons

CMS	Pollen fertility (%)								
	2013 fall	2014 spring	2014 fall	2015 spring	2015 fall	2016 spring	2016 fall	2017 spring	2017 fall
E5-HXA1	0	0	0	0	0	0	0	0	0
E5-HXA2	0	0	0	0	0	0	0	0	0
E5-HBA1	0	24.09 $\pm$ 5.91	0	8.6 $\pm$ 4.68	0	0.92 $\pm$ 0.42	0	3.14 $\pm$ 1.51	0
E5-HBA2	0	16.89 $\pm$ 6.36	0	11.3 $\pm$ 2.65	0	1.25 $\pm$ 1.01	0	–	0

Data shown are mean $\pm$ SE, $n = 10$

line, and was, as control, 0 in all E5-HXA CMS lines (Table 3). The result indicated that the pollen sterility in E5-HBA was incomplete and unsteady when seed is sown before June 1, while complete and steady when seed is sown after June 1.

Discussion

Breeding by design, which can be achieved by three key procedures: mapping loci involved in all agronomic relevant traits; assessment of the allelic variation at those loci; and breeding by design of superior genotypes comprising a combination of favorable alleles at all loci, was proposed by Peleman and van der Voort (2003). We have been working on breeding by design in rice following a strategy of the three steps more than 20 years. At the first step, we developed a library of single segment substituted lines (SSSLs) in rice by the using HJX74 as the recipient and 40 accessions from 7 species of AA genome in *Oryza* as donors. To date, more than 2000 SSSLs were developed, in which a rich of genes from AA genome of the *Oryza* genus was collected (He et al. 2005; Xi et al. 2006; He et al. 2017; Zhao et al. 2019). At the second step, we detected the genotypes on the chromosomal substituted segments in the SSSLs by mapping of genes or QTLs of important traits (He et al. 2005; Zhao et al. 2007; Liu et al. 2010; Zhang et al. 2012; Naeem et al. 2013; Yang et al. 2016; Zhou et al. 2017; Zhu et al. 2018), by assessing allelic variations at the loci of interest (Zeng et al. 2006; Teng et al. 2012; Cai et al. 2013), by analyzing interaction of gene by gene and gene by environment (Liu et al. 2008; Liu et al. 2012; Chen et al. 2014; Qin et al. 2015; Zhao et al. 2016; Yang et al. 2018), and by cloning genes of agronomic important (Wang et al. 2012; Wang et al. 2015). A large gene information has been collected from the SSSL

library. At the third step, we conducted breeding by design using favorable genes selected in the library. For example, we have developed two varieties Huaxiaohei 1 and Huabiao 1, two CMS lines H121A and H131A, and two restorer lines H121R and H131R (Dai et al. 2015; Dai et al. 2016). In this study, we developed two new CMS lines HBA and HZA, four isonuclear alloplasmic CMS lines WA-HZA1, WA-HZA2, DA-HZA, and YA-HZA, and two CMS lines with early heading and good quality, E5-HBA and E5-HXA, on the SSSL platform. These results indicate that the SSSL library is a powerful platform for breeding by design in rice. Based on the gene information collected from the SSSL library, a series of varieties or lines could be designed. Then, the design could be achieved by pyramiding of the target genes in the SSSLs with the HJX74 genetic background.

In hybrid rice production, one of the key characteristics of CMS lines is of complete and steady sterility. In some CMS lines, the fertility can be easily influenced by the environmental factors. It was found that, for example, the individuals carrying the same genotypes in the same population from the WA-type *indica* CMS lines LongtefuA and BobaiA exhibited different fertility levels in different environmental conditions (Huang 1997; Lin and Cai 2002). In this study, we developed two CMS lines with early heading and good quality in the E5 genotype, E5-HXA and E5-HBA. The CMS in the E5-HXA line carrying the *rf3* and *rf4* genes from XieqingzaoA was complete and steady in different cropping seasons. However, the CMS in the E5-HBA line with the *rf3* and *rf4* genes of BobaiA was complete and steady in the fall cropping seasons, while incomplete and unsteady in the spring cropping seasons. It implies that the CMS in the different CMS lines carrying different *rf3* and *rf4* gene sources was in different fertility levels. We also found that the restored ability of E5-

Table 3 Pollen fertility of E5-HXA and E5-HBA on six sowing dates in 2015

CMS	Pollen fertility (%)					
	Sowing on 3/2	Sowing on 3/18	Sowing on 4/1	Sowing on 5/1	Sowing on 6/1	Sowing on 7/13
E5-HBA1	5.70 ± 1.87	1.11 ± 1.11	—	0	0	0
E5-HBA2	5.03 ± 1.04	1.42 ± 0.66	0.65 ± 0.40	0.12 ± 0.09	0	0
E5-HXA1	0	0	0	0	0	0
E5-HXA2	0	0	0	0	0	0

Data shown are mean ± SD, *n* = 10

HBA was better than that of E5-HXA in terms of the pollen fertility of the F₁ hybrids derived from test crosses. These results indicated that in CMS lines, the higher in sterility level, the higher in abortive degree of pollens, the higher in sterility steadiness, but the lower in restored ability for CMS. The sterility and its restored ability in CMS lines depend on the *rf3* and *rf4* gene sources. Therefore, when designing CMS lines using *rf3* and *rf4* gene resources in the SSSL library, the balance between sterility and its restored ability in the lines should be concerned.

Author Contributions X.L. performed most of the experiments and prepared the data and the drafting of the manuscript; Z.D., W.Y., Q.T., Q.L., and J.G. performed some of the experiments; H.Z. performed the field experiments; G.L. performed the biometry; S.W. supervised this study and revised the manuscript; and G.Z. designed the experiments, supervised this study, and wrote the manuscript. All the authors have discussed the results and contributed to the drafting of the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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