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Genetic Analysis and Fine Mapping of QTLs for Stigma Exsertion Rate in Rice

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Abstract

Stigma exsertion rate (SER) is a crucial trait that influences the seed production of hybrid rice by determining the outcrossing ability of male sterile lines (MSLs). However, the molecular genetic mechanisms underlying SER are still poorly understood. In this study, we identified 14 quantitative trait loci (QTLs) using a recombinant inbred line (RIL) population derived from B805D-MR-16-8-3 (B805D) and Hua6S. Two major QTLs, *qSE1* and *qSE9*, were validated for their effects in the residual heterozygous line (RHL) background. The RHL carrying homozygous *qSE1* region from Hua6S increased dual stigma exsertion rate (DSE) by 14.67% and 15.04%, and increased total stigma exsertion rate (TSE) by 11.73% and 13.04%, in F_{10} and F_{11} progeny, respectively. Conversely, the RHL carrying homozygous *qSE9* region from B805D showed a substantial increase of 22.72% and 14.45% in single stigma exsertion rate (SSE), an increase of 13.46% and 8.30% in TSE, and an increase in percentage of spikelets with exserted stigma (PSE) by 24.82% and 15.57%, respectively, in F_{10} and F_{11} progeny. Furthermore, examination of floral organ traits revealed that both the Hua6S allele of *qSE1* and the B805D allele of *qSE9* increased pistil size to improve SER, but they had contrasting effects on spikelet shape. Subsequently, *qSE1* and *qSE9* were fine-mapped to intervals of 246.5 kb and 341.4 kb, respectively. A combination of sequencing, expression and haplotype analysis revealed that a single nucleotide variation (T to C) in the 5′UTR region of *LOC_Os01g72020* (*OsBOP1*) was likely to be the functional variation for *qSE1*. Collectively, our work has laid a foundation for cloning the genes responsible for SER, and demonstrated that the Hua6S allele of *qSE1* and the B805D allele of *qSE9* can effectively increase SER, which could make important contributions to the genetic improvement of MSLs aimed at improving hybrid seed production.

Keywords Stigma exsertion rate, QTL, Recombinant inbred line, Residual heterozygous line, Fine mapping, Rice

Introduction

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world. A landmark achievement in the history of rice breeding occurred with the adoption of hybrid rice, which greatly enhanced rice yield and made significant contributions to global food security (Qian et al. 2016; Yuan. 2014). Nevertheless, a key issue in the practice of hybrid rice is the poor production and high cost of hybrid seeds, which depend on the outcrossing rate of MSLs (Virmani et al. 1982; Tian et al. 2004; Xie and Peng. 2016; Malik and Baba. 2018). SER is one of the key factors that influence the outcrossing ability of MSLs (Marathi and Jena. 2015; El-Namaky. 2018), and enhancing SER has been proved to be a practical approach for improving hybrid seed production by increasing the outcrossing rate of MSLs (Hasan et al. 2014; Dang et al. 2020, 2022; Prahalada et al. 2021; Tan et al. 2022a, b; Zhu et al. 2023). Therefore, SER plays a crucial role in hybrid rice seed production by influencing the outcrossing ability of MSLs.

SER is a complex quantitative trait that is controlled by polygenes and influenced by environmental conditions.

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Extensive identification of QTLs responsible for SER has been achieved through the utilization of diverse segregating populations and continuous advancements in molecular marker technologies over recent decades. To investigate the genetic basis of SER, wild rice and maintainer lines with high SER were frequently utilized for detecting QTLs associated with SER. Some SER QTLs were detected from wild rice germplasm resources (Xiong et al. 1999; Li et al. 2001; Uga et al. 2003; Miyata et al. 2007; Huang et al. 2012; Khumto et al. 2017; Bakti and Tanaka. 2019; Tan et al. 2020; Zou et al. 2020; Tan et al. 2022a, b). From the common wild rice (*O. rufipogon*, P16), *es1* on chromosome 6 for exserted stigma was identified (Xiong et al. 1999). Three major loci and two minor loci responsible for exserted stigma were identified from *O. rufipogon,* W1943, and overlapped with the loci related to rice domestication (Huang et al. 2012). Using single-segment substitution lines (SSSLs) derived from the wild rice species *O. glumaepatula,* seven QTLs were detected and distributed on chromosomes 1, 3, 5, 9, and 10 (Tan et al. 2020). The *qSER-5*, *qSER-1b* and *qSER-8b* loci were fine-mapped to regions of 92.5 kb, 333.0 kb and 107.5 kb, respectively, using SSSLs derived from the wild rice species *O. glaberrima* (Tan et al. 2022a, b). Likewise, multiple QTLs for SER were identified from rice maintainer lines (Li et al. 2014a, b; Rahman et al. 2017a, 2017b; Zhang et al. 2018; Liu et al. 2019; Tan et al. 2021). From the maintainer line XieqingzaoB, *qSE7* was detected and further fine-mapped to a 322.9 kb region on chromosome 7 (Zhang et al. 2018), and *qSE11* was fine-mapped to a 350.7 kb region on chromosome 11 (Rahman et al. 2017b). Additionally, from the maintainer line II-32B, *qSER-7* was identified and narrowed down to a 28.4 kb region on chromosome 7, and *LOC_Os07g15370* (*OsNRAMP5*) was confirmed as a candidate gene (Liu et al. 2019). Furthermore, utilizing the MSL DaS, a total of 21 QTLs were detected for SER (Wang et al. 2017). Among those, *qSE4* was subsequently fine-mapped to a 410.4 kb region where *LOC_Os04g43910* (*OsARF10*) was identified as a potential candidate gene (Guo et al. 2022). In summary, although numerous QTLs for SER have been reported in the rice genome, only a limited number of them have been fine-mapped within 350 kb, and even fewer have been successfully cloned.

SER is closely associated with both spikelet and pistil traits in rice (Miyata et al. 2007; Yan et al. 2009; Uga et al. 2010; Liu et al. 2015; Marathi and Jena. 2015; Zhou et al. 2017; Khumto et al. 2018; Jiang et al. 2021; Prahalada et al. 2021; Wei et al. 2021; Tan et al. 2023). Spikelet (grain) shape is one of the important factors that determine the yield and quality of rice (Ren et al. 2023). A series of genes related to grain shape has been cloned and functionally characterized (Fan and Li. 2019). Three major grain shape genes, *GS3*, *GW5* and *GW2* had pleiotropic effects on SER (Zhou et al. 2017). *GS3* regulated stigma length by controlling the number of cells, which in turn affected the SER (Takano-Kai et al. 2011). By knocking out the grain shape genes *GS3*, *GW8*, and *GS9*, it was possible to significantly improve SER and increase the outcrossing rate, thereby facilitating the hybrid seed production of MSLs (Zhu et al. 2023). Similarly, it was found that alleles *gs3*, *gw5*, *GW7* and *gw8* in constructed SSSLs had pleiotropic effects on grain shape and SER (Tan et al. 2023). In addition, the SER exhibited a close correlation with pistil morphology (Li et al. 2001; Yan et al. 2009; Marathi and Jena. 2015; Dang et al. 2022), particularly demonstrating a high degree of correlation with stigma length (Takano-Kai et al. 2011; Liu et al. 2015; Zhou et al. 2017; Khumto et al. 2018; Zhu et al. 2023). Dozens of QTLs associated with pistil morphology have been identified (Yan et al. 2009; Uga et al. 2010; Liu et al. 2015; Marathi and Jena. 2015; Dang et al. 2016, 2020; Khumto et al. 2018; Tan et al. 2022a, b). Among those, *qSTL3*/*SYL3* (*LOC_Os03g14850*), encoding a MADS box family gene, was proved to be responsible for the length of stigma and style (Liu et al. 2015; Dang et al. 2022). Furthermore, a genome-wide association study on pistil characteristics identified a new gene locus, *OsSYL2*, which controlled style length (Dang et al. 2020). In conclusion, spikelet and pistil are flower organs in rice that share typical developmental mechanisms, which collectively determine SER.

To address the challenge posed by the limited understanding of molecular basis of SER, we investigated the genetic basis of SER using the RIL population developed from B805D-MR-16-8-3 (B805D) and Hua6S, and constructed RHL populations to validate genetic effects of two QTLs, *qSE1* and *qSE9*. Furthermore, *qSE1* was narrowed down to an interval of 246.5 kb. A combination of expression analysis, sequencing and haplotype analysis revealed that a single nucleotide variation (T to C) in the 5'UTR of the candidate gene *LOC_Os01g72020* (*OsBOP1*) was likely to be the functional variation of *qSE1*. Additionally, *qSE9* was fine-mapped to a 341.4 kb region. These findings present new QTLs and potential genes that could be useful in developing MSLs with high out-crossing rate, contributing to increasing seed production and decreasing seed cost for hybrid rice.

Materials and Methods

Population and Cultivation

The detailed process of population development was illustrated in Fig. S1a. A population consisting of 135 RILs was derived from a cross between B805D-MR-16-8-3 (B805D) and Hua6S (Zhou et al. 2021). B805D-MR-16-8-3 is an *indica* variety sourced from

the rice core germplasm collection (Zhao et al. 2014), and exhibits high SER values (Fig. S2a–d; Table S2). Hua6S is a photoperiod-thermo-sensitive genic male sterile line developed by Huazhong Agricultural University. The RHLs carrying heterozygous *qSE1* and *qSE9* regions were developed through a series of self-crosses via maker-assisted selection (MAS) respectively (Fig. S1a).

The RIL populations were planted in year 2017, while RHL populations of *qSE1* and *qSE9*, and recombinants were planted from year 2019 to 2021, during the normal rice growing seasons at the Experimental Farm of Huazhong Agricultural University in Wuhan, China (N 30.49°, E 114.36°).

Trait Evaluation

To determine the degree of SER, ten main stem panicles were collected from each line after the end of flowering for RILs, while three panicles were collected from each plant for RHLs and recombinants. By counting the number of spikelets with single exserted stigma (SES), dual exserted stigma (DES), and no exserted stigma (NES), the SER was calculated (Fig. S1c). The SER was further divided into four traits: single stigma exsertion rate (SSE), dual stigma exsertion rate (DSE), total stigma exsertion rate (TSE) and percentage of spikelets with exserted stigma (PSE). The four SER traits were calculated using the following formulas:

$$\mathrm{SSE}(\%) = \left(\frac{\mathrm{SES}}{\mathrm{SES} + \mathrm{DES} + \mathrm{NES}}\right) \times 100\%$$

$$\mathrm{DSE}(\%) = \left(\frac{\mathrm{DES}}{\mathrm{SES} + \mathrm{DES} + \mathrm{NES}}\right) \times 100\%$$

$$\mathrm{TSE}(\%) = \frac{2 \times \mathrm{DES} + \mathrm{SES}}{[2 \times (\mathrm{SES} + \mathrm{DES} + \mathrm{NES})]} \times 100\%$$

$$\mathrm{PSE}(\%) = \mathrm{SSE} + \mathrm{DSE}$$

For the observation of pistil morphology, ten panicles were collected from each line during the flourishing florescence for RHLs. Flowering spikelets were randomly selected to carefully separate pistils from glumes, and then photos were taken under a microscopes system (Eclipse Ni-E, Nikon, Tokyo, Japan). Pistil morphology was illustrated in Fig. S1b, and it was divided into stigma length, style length, stigma width, stigma angle, and style angle. All traits were measured using the ImageJ software (Schneider et al. 2012).

QTL Analysis and Marker Development

The genetic linkage map used for QTL mapping was constructed in a previous study (Zhou et al. 2021). QTL mapping was performed by the composite interval mapping (CIM) method using WinQTLCart2.5 software (Wang et al. 2012) with logarithm of odds (LOD) values over 2.5 as the threshold. The graphic of QTL distribution on the rice genome was drawn by R package *RIdeogram* (Hao et al. 2020). To validate the QTLs detected from RILs, SNP makers flanking the QTLs were replaced with closely linked InDel markers designed using the RiceVarMap (Zhao et al. 2014) and Primer Premier 6 software (Premier Biosoft Interpairs, Palo Alto, CA). Molecular markers for fine mapping of *qSE1* and *qSE9* were also designed using the same approach. Relevant primer sequences were shown in Table S1.

Total RNA Extraction and qRT-PCR

Total RNA was extracted from pistil separated from young panicles at the pre-heading stage of rice using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed to generate cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme biotech co, Itd, Nanjing, China). Gene expression was measured by qRT-PCR using the *OsActin (LOC_Os03g50885)* as an internal control. PCR was performed via the Applied Biosystems Quant Studio 6 Real-Time PCR System (Thermo Fisher Scientific, USA). All primers used for qRT-PCR were shown in Table S1.

Haplotype and Statistical Analysis

SNP and InDel variation information for *LOC_Os01g72020* (*OsBOP1*) were obtained from RiceVarMap (Zhao et al. 2014). The LD heatmap and gene haplotypes were constructed using SNPs and InDels (frequencies > 0.03) from the 2 kb promoter and coding regions using Haploview software (Barrett et al. 2005). Phenotypic data used for haplotype analysis was derived from a previous study (Zhou et al. 2017). The data analysis and figure making were finished with a combination of software Microsoft Excel (Microsoft Corporation, Redmond, Washington, United States), GraphPad Prism 8.0 (GraphPad Software Inc., La Jolla, CA, USA), SPSS 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp) and R (Ihaka and Gentleman. 1996).

Results

Phenotypic Description of SER in the Parents and the RIL Population

The SER performance of parents and the RIL population was assessed, which was subdivided into four traits,

namely DSE, SSE, TSE and PSE. B805D displayed significantly higher values than Hua6S for all four traits. The DSE, SSE, TSE and PSE values of B805D were 6.89%, 40.36%, 27.07% and 47.25% respectively, while that of Hua6S were 3.92%, 25.84%, 16.83% and 29.75% respectively (Table S2). The phenotypic in the RIL population ranged from 50.02 to 0.44% for DSE (Fig. S2a), from 52.81 to 9.55% for SSE (Fig. S2b), from 79.46 to 5.42% for TSE (Fig. S2c) and from 94.58 to 10.40% for PSE (Fig. S2d). The SSE, TSE and PSE followed a normal distribution, while the DSE displayed deviation from the normal distribution, based on Q–Q plots (quantile–quantile plot) and Kolmogorov-Smirnov (K-S) tests (Fig. S3; Table S2) (Ghasemi and Zahediasl. 2012).

The correlation coefficients among SSE, DSE, TSE and PSE were shown in Fig. S2e. The four traits were significantly positively correlated with each other. The highest correlation coefficient was 0.98 between TSE and PSE, while the lowest was 0.39 between DSE and SSE.

QTL Mapping of SER in the RIL Population

A total of 14 QTLs associated with SER were identified, distributed on chromosomes 1, 2, 4, 5, 6, 8 and 9 respectively (Table 1; Fig. S4). The phenotypic variance explained by each QTL ranged from 20.15 to 6.63%, with LOD scores ranging from 7.47 to 2.52 (Table 1).

Four QTLs were detected for DSE on chromosomes 1, 4, 8 and 9 (Table 1; Fig. S4). Among them, the QTL *qDSE9* explained the highest phenotypic variance of 15.47% with a LOD value of 5.65. *qDSE8* explained 6.63% of the phenotypic variance with a LOD value of 2.52. Alleles from B805D increased DSE for *qDSE9* and *qDSE8*, with additive effects of 5.50% and 3.59%, respectively. In contrast, alleles from Hua6S increased DSE for *qDSE1* and *qDSE4*, exhibiting additive effects of 4.63% and 4.04%, and explaining 9.64% and 8.35% of the phenotypic variation, respectively.

Three QTLs were identified for SSE on chromosomes 2, 5, and 9 (Table 1; Fig. S4). The alleles from B805D increased SSE for *qSSE2* and *qSSE9*. The QTL *qSSE9* explained 20.15% of the phenotypic variation with a LOD value of 7.47. The QTL *qSSE2* exhibited a phenotypic variance of 10.30% with a LOD value of 4.07. Additionally, *qSSE5* explained 9.89% of the phenotypic variation with a LOD value of 3.86, with the allele from Hua6S increasing SSE.

Three QTLs for TSE and four QTLs for PSE were identified on chromosomes 1, 2, 4, 6 and 9, respectively (Table 1; Fig. S4). *qTSE9* and *qPSE9* explained 15.69% and 13.87% of the TSE and PSE variation respectively, and coincided with the genomic region of *qDSE9* and *qSSE9* (Table 1). This locus had the highest impact on SER and was renamed as *qSE9*. The *qPSE2* region accounted for 10.02% of the variation in PSE, and overlapped with the *qSSE2* region, which was renamed as *qSE2* (Table 1). *qTSE4* and *qPSE4* accounted for 9.17% and 9.97% of the variation in TSE and PSE, respectively, and were co-located with the *qDSE4* region, which was renamed as *qSE4* (Table 1). The *qTSE1* locus accounted for 8.05% of the variation in TSE and co-localized with *qDSE1* within a QTL cluster, which was renamed as *qSE1* (Table 1).

Table 1 Putative QTLs for SER detected in the RIL population

Trait	QTL	Chr	Physical interval (bp)	LOD	Add	PVE (%)
DSE	*qDSE1/qSE1*	1	41,923,017–42,574,450	3.6	−4.63	9.64
	qDSE4/qSE4	4	25,401,580–29,142,744	3.3	−4.04	8.35
	qDSE8	8	3,316,744–3,602,540	2.52	3.59	6.63
	qDSE9/qSE9	9	19,239,288–21,383,102	5.65	5.5	15.47
SSE	*qSSE2/qSE2*	2	10,659,278–18,494,768	4.07	3.22	10.3
	qSSE5	5	2,297,474–5,361,872	3.86	−3.16	9.89
	qSSE9/qSE9	9	19,248,045–21,615,415	7.47	4.37	20.15
TSE	*qTSE1/qSE1*	1	42,328,822–42,574,450	3.22	−4.73	8.05
	qTSE4/qSE4	4	28,358,979–30,075,347	4	−5.13	9.97
	qTSE9/qSE9	9	19,2392,88–21,615,415	6.02	6.41	15.69
PSE	*qPSE2/qSE2*	2	10,618,982–17,246,109	4.15	7.09	10.02
	qPSE4/qSE4	4	25,401,580–30,011,860	3.86	−5.92	9.17
	qPSE6	6	12,795,997–19,609,502	3.83	5.97	9.2
	qPSE9/qSE9	9	19,239,288–21,383,102	5.6	7.43	13.87

In the column of QTL, the co-located QTLs for SER were renamed as *qSE*, and different renamed QTLs were shown in different color. *Chr*, chromosome; *LOD*, logarithm of odds; *Add*, additive effect; *PVE*, the phenotypic variance explained by each QTL

Validation of *qSE1* and *qSE9* Using RHL Populations

To confirm the genetic effects of identified QTLs, we developed residual heterozygous line (RHL) populations for each QTL through a series of self-crosses and maker-assisted selection (MAS), and then reconducted QTL analysis (Fig. S1a). Two QTLs, *qSE1* and *qSE9*, significantly influenced SER in RHL populations (Fig. 1; Table S3, S4).

qSE1 explained 31.77% of the variation for DSE, 12.49% for SSE, 33.45% for TSE, and 29.94% for PSE in the RHL population (Table S3). Significant difference was observed among alleles for all traits, except for the SSE between lines carrying the B805D genotype and the heterozygous genotype (Fig. 1c–j; Table S4). RHL-*qSE1*^{B805D} (homozygous for the B805D *qSE1* allele in the RHL background) and RHL-*qSE1*^{Hua6S} (homozygous for the Hua6S *qSE1* allele in the RHL background) exhibited differences of 14.67% for DSE, 11.74% for TSE, 8.81% for PSE. However, the SSE of RHL-*qSE1*^{B805D} was only 5.86% higher than that of RHL-*qSE1*^{Hua6S}, which could be attributed to our calculation method based on counting spikelets with exerted stigma on rice panicle. RHL-*qSE-1*^{Hua6S} showed a relatively high number of spikelets with dual-exerted stigma, resulting in a decreased number of spikelets with single-exerted stigma per panicle. These findings suggested that *qSE1* is a genetic locus primarily impacting DSE, thereby influencing TSE and PSE.

qSE9 explains 65.39% of the variation for DSE, 10.66% for SSE, 11.07% for TSE, and 11.37% for PSE in the RHL population (Table S3). Significant difference was observed among alleles for all traits (Fig. 1m–t; Table S4). RHL-*qSE9*^{Hua6S} (homozygous for the Hua6S *qSE9* allele in the RHL background) and RHL-*qSE9*^{B805D} (homozygous for the B805D *qSE9* allele in the RHL background) displayed differences of 2.09% for DSE, 22.73% for SSE, 13.45% for TSE, and 24.82% for PSE.

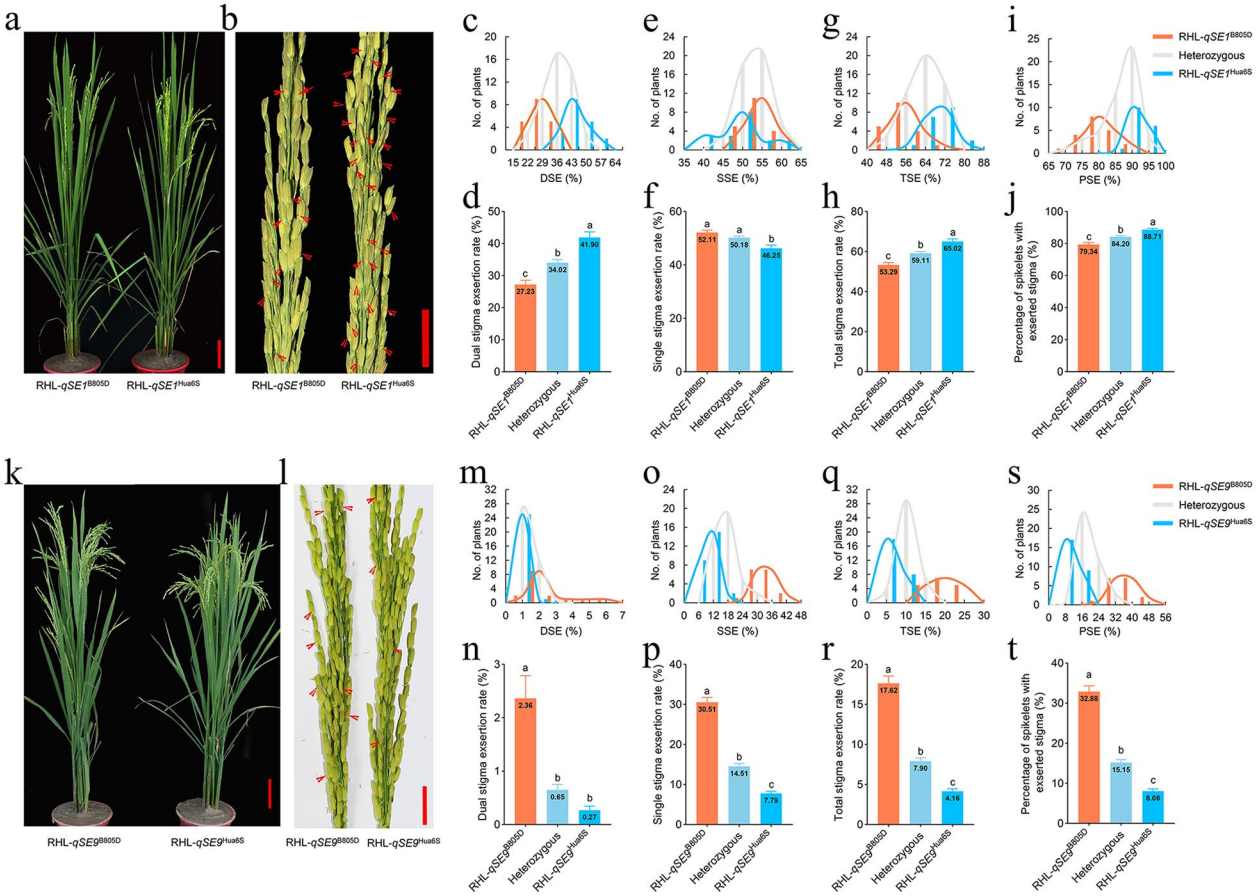


Fig. 1 The phenotype of RHLs for *qSE1* and *qSE9*. Plant type of RHL-*qSE1*^{B805D} and RHL-*qSE1*^{Hua6S} (**a**), RHL-*qSE9*^{B805D} and RHL-*qSE9*^{Hua6S} (**k**) at heading stage. Scale bar, 10 cm. Panicle of RHL-*qSE1*^{B805D} and RHL-*qSE1*^{Hua6S} (**b**), RHL-*qSE9*^{B805D} and RHL-*qSE9*^{Hua6S} (**l**). The red arrows pointed to exserted stigmas. Scale bar, 2 cm Frequency distribution of dual stigma exsertion rate (DSE) (**c, m**), single stigma exsertion rate (SSE) (**e, o**), total stigma exsertion rate (TSE) (**g, q**), percentage of spikelets with exserted stigma (PSE) (**i, s**) in the RHL population. Comparison of DSE (**d, n**), SSE (**f, p**), TSE (**h, r**), and PSE (**j, t**) in the RHL population. Different letters indicated significant differences at $p < 0.05$ (one-way analysis of variance followed by Tukey's multiple-comparison test)

Floral Characteristics in the RHL Populations

To characterize the role of *qSE1* and *qSE9* in influencing SER, we examined the spikelet and pistil traits in RHLs of *qSE1* and *qSE9*.

For *qSE1*, the spikelet length and width of RHL-*qSE-1*^{Hua6S} were higher than those of RHL-*qSE1*^{B805D} (Fig. 2a, e and f; Table S5). Correspondingly, RHL-*qSE1*^{Hua6S}, which had higher SER values except for SSE (Fig. 1c–j; Table S4), displayed increased style length and stigma length as well as width, along with an increase in stigma angle and the length of stigma and style (Fig. 2b and g–l; Table S5). Correlation analysis showed significant positive correlation between SER traits (except for SSE) and floral organ traits (Fig. S5a). These findings clearly demonstrate that *qSE1* synchronously regulates spikelet size, pistil growth and the SER in rice.

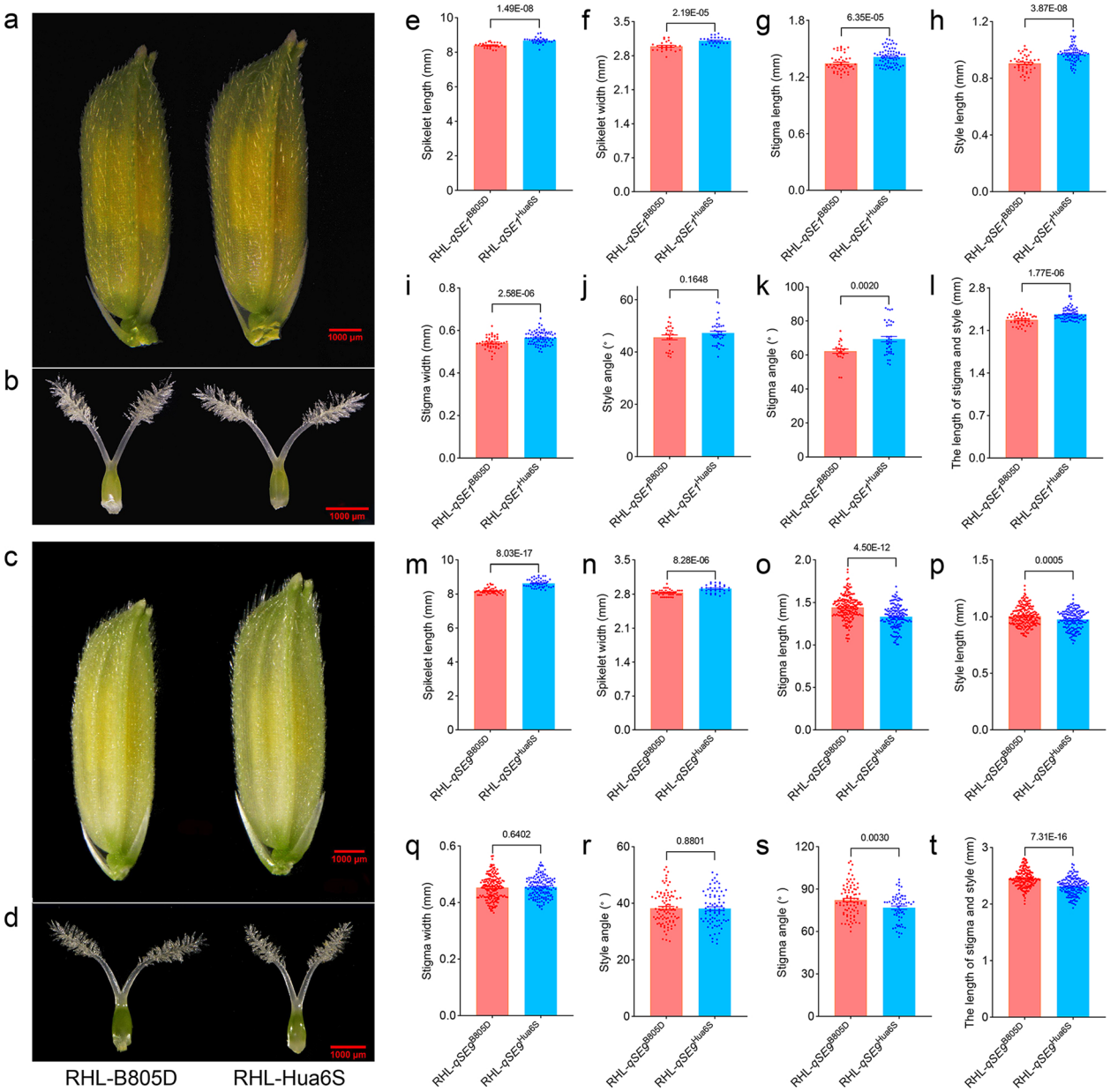


Fig. 2 Floral morphology of RHLs for *qSE1* and *qSE9*. Spikelet morphology of RHLs for *qSE1* (**a**) and *qSE9* (**c**). Pistil morphology of RHLs for *qSE1* (**b**) and *qSE9* (**d**). Scale bar, 1000 um. Spikelet length (**e, m**), spikelet width (**f, n**), stigma length (**g, o**), style length (**h, p**), stigma width (**i, q**), style angle (**j, r**), stigma angle (**k, s**), and the length of stigma and style (**i, t**) of RHLs for *qSE1* and *qSE9*, respectively. *P* values were based on two-tailed Student's *t* test

For *qSE9*, the spikelet length and width of RHL-$qSE9^{B805D}$ were lower than those of RHL-$qSE9^{Hua6S}$ (Fig. 2c, m and n; Table S5). The pistil traits of the RHL-$qSE9^{B805D}$, including stigma length, style length, stigma angle and the length of stigma and style, were significantly higher than those of RHL-$qSE9^{Hua6S}$ (Fig. 2d and o–t; Table S5). There was a significant positive correlation between SER traits and pistil length, but negative correlation with spikelet length and width (Fig. S5b). These results indicate that the *qSE9* allele from B805D has positive effects on pistil traits to increase SER, but negatively affects spikelet size simultaneously.

Fine Mapping of *qSE1*

To fine map *qSE1*, a segregating population consisting of 5880 individuals was developed from several F_{10} plants carrying heterozygous *qSE1* region, and 217 recombinant individuals were identified by DNA markers S1-1 and S1-6 (Table S1). The effect of *qSE1* was further confirmed using 360 RHLs at F_{11} generation. Significant difference was observed among alleles for DSE, TSE and PSE in RHLs (Table S4; Fig. S6). For individuals with homozygous B805D genotype, DSE ranged from 29.80 to 12.37%, with a mean value of 21.41%; TSE ranged from 55.64 to 37.63%, with a mean value of 48.35%; PSE ranged from 83.42 to 62.90%, with a mean value of 75.28%. For individuals with homozygous Hua6S genotype, DSE ranged from 49.78 to 27.04%, with a mean value of 36.45%; TSE ranged from 71.65 to 53.62%, with a mean value of 61.84%; PSE ranged from 94.35 to 80.19% with a mean value of 87.24% (Table S4; Fig. S6).

In order to accurately deduce the genotype of *qSE1*, recombinants with DSE values less than 20.00% and TSE values less than 48.00% were tentatively defined to carry homozygous *qSE1* regions from B805D, while recombinants with DSE values greater than 35.00% and TSE values greater than 60.00% were tentatively defined to carry homozygous *qSE1* regions from Hua6S. Thus, 98 out of 217 recombinants were selected for further study (Table S6). Subsequently, four novel InDel markers were developed in the *qSE1* region (Table S1), and used to conduct genotyping of 98 recombinants. 11 recombinant types were classified based on the genotype of six markers (Fig. 3b; Table S6). The mean phenotypic values of all recombinants belonging to the same recombinant type were calculated and defined as the values of each

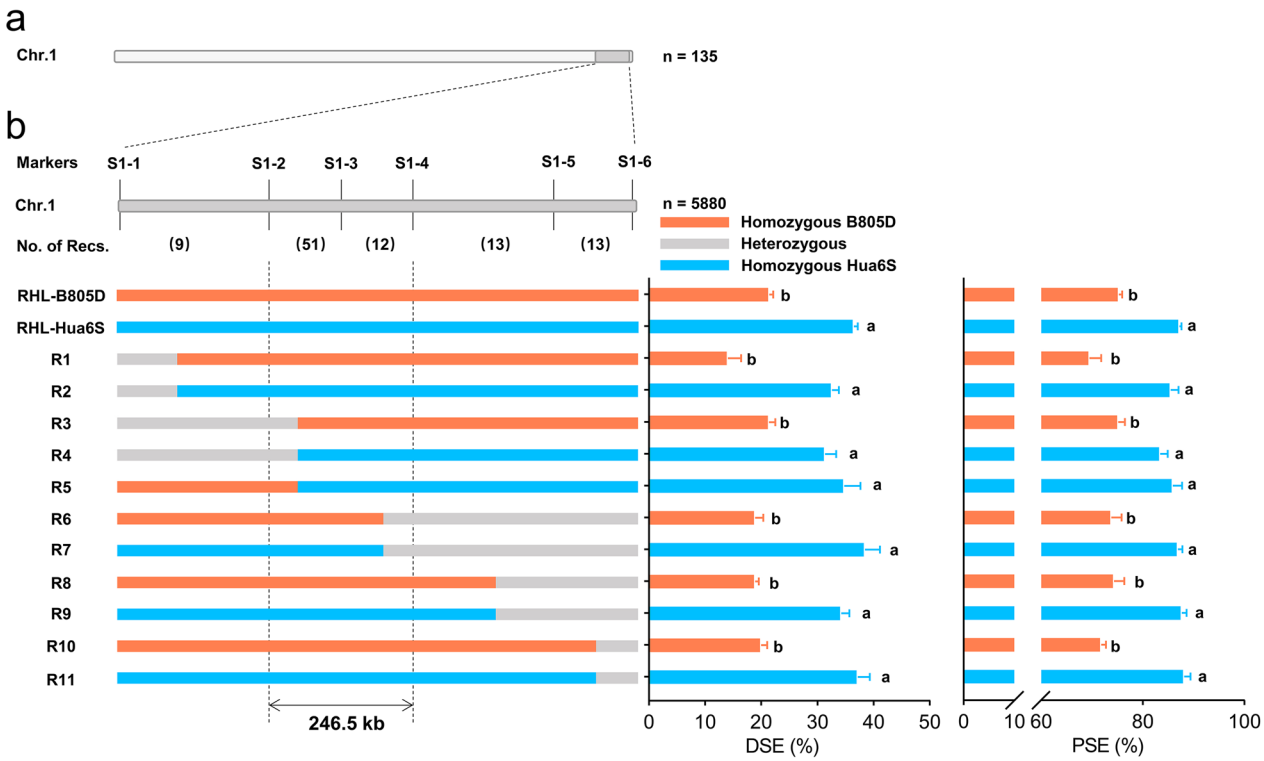


Fig. 3 Fine mapping of *qSE1*. **a** The *qSE1* locus was initially mapped on the long arm of chromosome 1 using 135 RILs, with the dark grey region indicating its approximate interval. **b***qSE1* was finely mapped to a region of 246.5 kb. Six markers were used to perform genotyping of recombinants screened from 5880 F11 plants. The numbers in brackets indicated the number of recombinant crossover events occurred between flanking markers. Graphical genotypes and phenotype statistics were shown for different recombinant types. Different letters indicated significant differences at $p < 0.05$ (one-way analysis of variance followed by Tukey's multiple-comparison test)

recombinant type, which was exploited to accurately deduce the genotype of *qSE1*. Based on the analysis of marker genotype and *qSE1* genotype of 11 recombinant types, *qSE1* was finally delimited to a region of 246.5 kb between markers S1-2 and S1-4 (Fig. 3b).

Fine Mapping of *qSE9*

To narrow down the *qSE9* interval, a segregating population consisting of 7840 individuals was developed from several F_{10} plants carrying heterozygous *qSE9* region, and 238 recombinant individuals were identified by DNA markers S9-1 and S9-8 (Table S1). The effect of *qSE9* was further confirmed using 354 RHLs at F_{11} generation (Table S4; Fig. S7). For individuals with homozygous B805D genotype, SSE ranged from 31.28 to 15.37%, with a mean value of 20.15%; TSE ranged from 17.57 to 8.08%, with a mean value of 11.24%; PSE ranged from 33.21 to 15.65%, with a mean value of 21.38%. For individuals with homozygous Hua6S genotype, SSE ranged from 9.24 to 2.02%, with a mean value of 5.70%; TSE ranged from 4.96 to 1.01%, with a mean value of 2.94%; PSE ranged from 9.58 to 2.02%, with a mean value of 5.81% (Table S4; Fig. S7).

Taking that no overlap was observed for each SER trait between individuals with B805D genotype and Hua6S genotype into consideration, 135 out of 238 recombinants with tentatively defined homozygous *qSE9* regions from B805D or Hua6S were selected for further study (Table S7). Subsequently, six novel InDel markers were developed in the *qSE9* region (Table S1), and used to conduct genotyping of 135 recombinants. 16 recombinant types were classified based on the genotype of eight markers (Fig. 4b; Table S7). Based on the analysis of marker genotype and *qSE1* genotype of 16 recombinant types, *qSE9* was finally delimited to an interval between S9-3 and S9-5 with an estimated length of 341.4 kb (Fig. 4b).

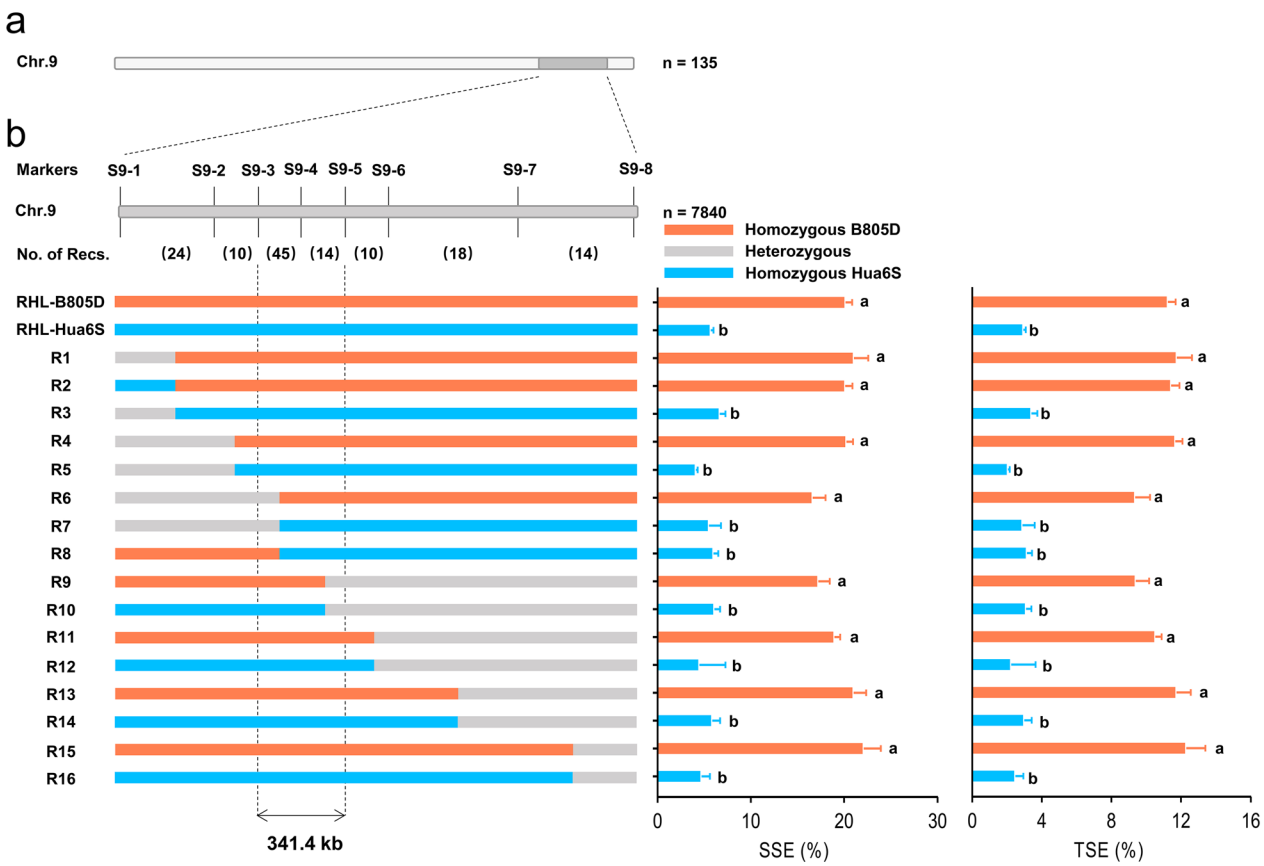


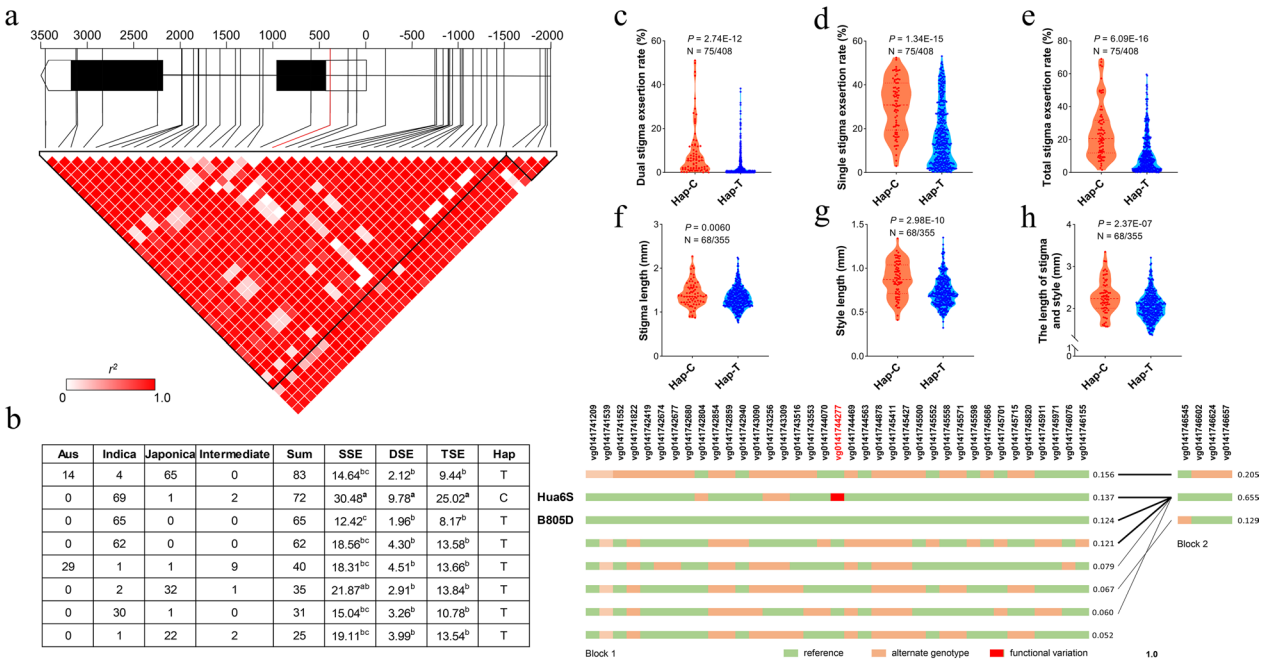
Fig. 4 Fine mapping of *qSE9*. **a** The *qSE9* locus was initially mapped on chromosome 9 using 135 RILs, with the dark grey region indicating its approximate interval. **b** Fine mapping *qSE9* to a 341.4 Kb region. Eight markers were used to perform genotyping of recombinants screened from 7840 F_{11} plants. The numbers in brackets indicated the number of recombinant crossover events occurred between flanking markers. Graphical genotypes and phenotype statistics were shown for different recombinant types. Different letters indicated significant differences at $p < 0.05$ (one-way analysis of variance followed by Tukey's multiple-comparison test)

Identification of Candidate Genes for *qSE1* and *qSE9*

In the candidate region of *qSE1*, thirty-one predicted ORFs were annotated in the reference genome of Nipponbare (http://rice.uga.edu/, MSU-version_7.0), excluding four encoding retrotransposon or transposon protein (Table S8). The levels of mRNA expression of thirty-one genes in pistil of RHL-$qSE1^{Hua6S}$ and RHL-$qSE1^{B805D}$ were examined by qRT-PCR (Fig. S8). The results showed that twelve genes displayed differential expression between the two RHLs, while ten genes did not express in pistil (Fig. S8; Table S8). Notably, among the thirty-one genes, *LOC_Os01g72020* (*OsBOP1*) was found to be involved in the development of leaves and spikelet organ in rice (Toriba et al. 2019). Comparison of genomic sequences revealed four variants between the parental alleles of *OsBOP1*, including three in the intron and a SNP (T for B805D and C for Hua6S) in the 5'UTR (Fig. 5b). Moreover, a significant differential expression of *OsBOP1* was observed between the two RHLs (Fig. S8). Consequently, *LOC_Os01g72020* (*OsBOP1*) emerged as a promising candidate gene underlying *qSE1*.

The genomic sequence of *OsBOP1* was further analyzed using the rice germplasm collection consisting of 533 accessions (Table S9) (Zhao et al. 2014). A total of forty-one polymorphisms were identified in the 2 kb promoter and coding regions of *OsBOP1*, with high linkage disequilibrium (LD) observed among these variants (Fig. 5a). Four variations were located in the coding regions, while none were identified in the promoter region between B805D and Hua6S (Fig. 5b). Eight major alleles were identified, and the Hua6S allele displayed significantly higher SER values than the other alleles (Fig. 5b), suggesting that variations vg0141742804 and vg0141744277 may serve as functional variants. Notably, variation vg0141744277 was the SNP observed in the 5'UTR between the parent alleles. Based on these findings, we classified the eight alleles into two haplotypes: Hap-T and Hap-C. These two haplotypes exhibited significant differences both in terms of SER values and pistil traits (Fig. 5c–h). Therefore, the SNP in the 5'UTR of *LOC_Os01g72020* is likely to be the functional variant underlying *qSE1*.

Within the *qSE9* region, a total of forty-three predicted ORFs were annotated in the reference genome of Nipponbare (http://rice.uga.edu/, MSU-version_7.0), excluding five encoding retrotransposon, transposon and hypothetical protein respectively (Table S10). Among



Aus	Indica	Japonica	Intermediate	Sum	SSE	DSE	TSE	Hap
14	4	65	0	83	14.64^{bc}	2.12^{b}	9.44^{b}	T
0	69	1	2	72	30.48^{a}	9.78^{a}	25.02^{a}	C
0	65	0	0	65	12.42^{c}	1.96^{b}	8.17^{b}	T
0	62	0	0	62	18.56^{bc}	4.30^{b}	13.58^{b}	T
29	1	1	9	40	18.31^{bc}	4.51^{b}	13.66^{b}	T
0	2	32	1	35	21.87^{ab}	2.91^{b}	13.84^{b}	T
0	30	1	0	31	15.04^{bc}	3.26^{b}	10.78^{b}	T
0	1	22	2	25	19.11^{bc}	3.99^{b}	13.54^{b}	T

Fig. 5 Haplotype analysis of the candidate gene *LOC_Os01g72020* (*OsBOP1*). **a** Gene structure and linkage disequilibrium (LD) display of *OsBOP1*. The predicted functional variation (marked in red) was observed within the 5'UTR of *OsBOP1*. The color scheme represented the LD relationships between DNA polymorphisms, based on r^2 values. Two triangles indicated the two haplotype blocks divided by software Haploview. **b** Haplotypes of *OsBOP1* in the rice core germplasm collection were depicted on the left panel. Different letters indicated significant differences among phenotypic values at $p < 0.05$ (one-way analysis of variance followed by Tukey's multiple-comparison test). Variation information was displayed on the right panel. Variation IDs from website RiceVarMap2 were shown on the top (Zhao et al. 2014), with the predicted functional variation highlighted in red. Block1 and 2 correspond to the two triangles in (**a**). **c–h** Phenotypic comparison between two haplotypes for dual stigma exsertion rate (DSE) (**c**), single stigma exsertion rate (SSE) (**d**), total stigma exsertion rate (TSE) (**e**), stigma length (**f**), style length (**g**), the length of stigma and style (**h**). *p* value was calculated based on two-tailed Student's *t* tests

those, nineteen genes displayed differential expression levels in the pistil of RHL-*qSE9*^{Hua6S} and RHL-*qSE9*^{B805D} (Fig. S9). Notably, the gene *LOC_Os09g32944* (*OsSPL18*) was previously reported to positively regulate grain width and thickness by influencing cell proliferation to modulate glume development (Yuan et al. 2019). *OsSPL18* exhibited significant differential expression in the pistil of the two RHLs. The gene *LOC_Os09g32540* (*LGD1*), which encodes a protein containing a von Willebrand factor type A (vWA) domain, was reported to be involved in pleiotropic regulating of rice vegetative growth and development (Thangasamy et al. 2012). *LGD1* exhibited high expression levels in the pistil and showed higher expression in RHL-*qSE9*^{B805D} than in RHL-*qSE9*^{Hua6S} (Fig. S9). Additionally, *LOC_Os09g32948* encoding a MADS-box protein was reported to play a role in regulating floral organ development (Shen et al. 2023). It showed high expression level in the pistil but no difference was observed between two RHLs (Fig. S9). *LOC_Os09g32680* encoding a cyclin protein showed high expression level in the pistil as well (Fig. S9), suggesting its potential role in modulating cell development and division for affecting pistil growth. More work was required to further narrow down the interval of *qSE9* and to ascertain the functional gene.

Discussion

Understanding the genetic architecture of SER in rice could facilitate the genetic improvement of SER in MSLs, further enhancing the outcrossing ability of MSLs and hybrid rice seed production. In the past two decades, numerous QTLs for SER and related traits have been identified and are distributed across all 12 chromosomes. In the present study, a total of 14 QTLs were detected from the RIL population derived from a cross betweeen parent Hua6S and B805D, including 4 QTLs for DSE, 3 QTLs for SSE, 3 QTLs for TSE and 4 QTLs for PSE (Table 1). Among those, the QTL *qSE9* was close to *qTSSL9*, a locus influencing the length of stigma and style (Dang et al. 2020). The *qSE4* region encompassed the intervals of *qSER-4.1* (Xu et al. 2019) and *qPSES4* (Wang et al. 2017), and it was suggested that *LOC_Os04g43910* (*ARF10*) may serve as a potential target gene within this genomic region (Guo et al. 2022). The *qSE2* region overlapped with the region of *qSTL2*, a locus conferring stigma length (Dang et al. 2020). *qSSE5* coincided with the regions of *qTSE-5a* (Li et al. 2014a), *qSER-5.1* (Xu et al. 2019), and *qSER-5* (Tan et al. 2022a, b). *qDSE8* was co-located with *qTSE8* (Liu et al. 2022). The *qPSE6* region overlapped with that of *qSER-6.1* (Xu et al. 2019), *qPES-6* (Lou et al. 2015), and *qTSSL6*, a locus influencing the length of stigma and style (Dang et al. 2020). In addition, *qSE1* was novel.

Despite numerous QTLs associated with SER and related traits in rice have been identified, cloning of genes conferring high SER remains a great challenge. The utilization of secondary mapping populations, such as near-isogenic line (NIL) population and RHL population, represents an effective approach for fine mapping and cloning of genes for SER(Guo et al. 2022; Liu et al. 2019; Rahman et al. 2017b; Tan et al. 2021, 2022a, b). In the present study, RHL populations of F_{10} and F_{11} generations were exploited to validate genetic effects of *qSE1* and *qSE9*, and conduct fine mapping of the two QTLs (Fig. S1). It should be noted that RHL populations were developed from RHLs selected from RILs of F_7 generation, due to the great difficulty in identifying RHLs from RILs of higher generations. Thus, RILs of low generations from F_5 to F_7 are valuable genetic materials for two reasons. On one hand, they carry homozygous regions of more than 93% genome that could facilitate accurate evaluation of target traits for QTL mining. On the other hand, they carry heterozygous regions of less than 7% genome that could facilitate the screening of RHLs of target QTLs for effect validation and fine mapping. In short, a combination of RILs of low generations and derived RHLs provided solid material foundation for fine mapping of *qSE1* and *qSE9* in this study, which could be of great use in fine mapping of other QTLs of interest.

Previous studies have revealed that SER is influenced by multiple factors, with spikelet shape and pistil size being two key factors. SER related traits are positively correlated with spikelet length, stigma length, style length and stigma angle, but negatively correlated with spikelet width, stigma width and style angle (Dang et al. 2016, 2020, 2022; Virmani et al. 1973; Virmani et al. 1974, 1987; Uga et al. 2003; Yan et al. 2009; Zhou et al. 2017; Zhu et al. 2023). In the present study, both the *qSE1* allele from Hua6S and the *qSE9* allele from B805D significantly increased stigma length, style length, the length of stigma and style, and stigma angle, and the *qSE1* allele from Hua6S increased stigma width additionally (Fig. 2). Thus, both *qSE1* and *qSE9* increase pistil size to improve SER. In contrast, the *qSE1* allele from Hua6S significantly increased spikelet length and width, while the *qSE9* allele from B805D decreased spikelet length and width. In addition, several grain shape genes, namely *gs3*, *gw8*, *gs9*, *gw5* and *gw7*, could effectively enhance SER through decreased spikelet width and increased spikelet length (Tan et al. 2023; Zhu et al. 2023). These results suggest that different genes conferring high SER have different effects on spikelet shape, all of which may be likely to increase pistil size. Therefore, we can boldly hypothesize that pistil size rather than spikelet shape is the key factor in determining SER. Cloning the functional genes underlying *qSE1* and *qSE9* and elucidating their molecular

mechanisms will contribute to a better understanding of the relationship among SER, pistil size and spikelet shape.

In the present study, *LOC_Os01g72020* (*OsBOP1*) was identified as the candidate gene for *qSE1* with the combination of sequencing, expression and haplotype analysis (Fig. 5). *OsBOP1* encodes a Broad Complex BTB domain with Ankyrin repeat region protein, with a high expression level in pistil (http://rice.uga.edu/, MSU-version_7.0). In a previous study, *BOP1* and its homologous genes was reported to influence flower development in *Arabidopsis* (Hepworth et al. 2005) and rice (Toriba et al. 2019). Significant differences in spikelet shape and pistil size were observed between the B805D allele and Hua6S allele of *qSE1* in the RHL population (Fig. 2; Table S5). The Hua6S allele exhibited higher expression level in the pistil than the B805D allele (Fig. S8), possibly due to the SNP in the 5'UTR of *OsBOP1*. As the 5'UTR is of great importance to the regulation of transcription and translation of downstream coding genes (Ryczek et al. 2023), the SNP is likely to be the functional variation underlying *qSE1*. It is exciting to note that the allele frequency of Hap-C (Hua6S allele) with higher SER is far lower than that of Hap-T (B805D allele) in diverse cultivated rice (Fig. 5b), suggesting that the Hua6S allele of *qSE1* could be a promising target in the genetic improvement of male sterile lines.

Conclusion

In this study, we detected 14 QTLs for SER using the RILs derived from B805D and Hua6S, and confirmed the effects of two major QTLs, *qSE1* and *qSE9*, using RHLs. Both *qSE1* and *qSE9* improved SER by increasing pistil size and were fine mapped to intervals of 246.5 kb and 341.4 kb, respectively. A combination of sequencing, expression and haplotype analysis revealed that *LOC_Os01g72020* (*OsBOP1*) was likely to be the candidate gene for *qSE1*. The Hua6S allele of *qSE1* and B805D allele of *qSE9* will be favorable for molecular breeding of MSLs with high outcrossing ability, contributing to the development of hybrid rice and ensuring global food security.

Abbreviations

MSL	Male sterile line
SER	Stigma exsertion rate
SSSLs	Single-segment substitution lines
RIL	Recombinant inbred line
QTL	Quantitative trait loci
RHL	Residual heterozygous line
SES	Spikelets with single exserted stigma
DES	Spikelets with dual exserted stigma
NES	Spikelets with no exserted stigma
DSE	Dual stigma exsertion rate
SSE	Single stigma exsertion rate
TSE	Total stigma exsertion rate
PSE	Percentage of spikelets with exserted stigma
MSL	Male sterile line
MAS	Maker-assisted selection
SNP	Single nucleotide polymorphism

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-024-00752-6.

Additional file 1.

Additional file 2.

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Not applicable.

Author Contributions

Professor Yuqing He designed and supervised the study; Mr Hanyuan Yang performed the experiments; Miss Yin Zhou, Miss Enyu Liu, Miss Ping Sun,Dr Rongjia Liu, Mr Haozhou Gao, Mr Zerui Xu, Miss Ping Yang, Miss Xingyue Wang participated in the experiments. Miss Yiting Ao provided the plant materials. Mr Guanjun Gao, Mr Qinglu Zhang and Dr Lizhong Xiong help to field management. Mr Hanyuan Yang and Miss Yin Zhou analyzed the date; Mr Hanyuan Yang wrote the manuscript. Mr Yin Zhou, Dr Pingbo Li and Professor Yuqing He revised the manuscript. All authors read and approved the final manuscript.

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Data Availability

No datasets were generated or analysed during the current study.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Competing Interest

The authors declare no competing interests.

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