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Fine-Mapping and Candidate Gene Analysis of *qSERg-1b* from *O. glumaepatula* to Improve Stigma Exsertion Rate in Rice

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Abstract: The stigma exsertion rate (SER) is one of the important factors affecting the seed production of hybrid rice. In the genus *Oryza*, wild rice species usually show higher SERs than cultivars. We previously identified one novel SER-QTL, *qSERg-1b*, from the single-segment substitution line (SSSL) SG22 of *O. glumaepatula*. In this study, *qSERg-1b* was fine-mapped by developing the secondary SSSLs (s-SSSLs) of SG22 and substitution mapping among the s-SSSLs. A total of 11 s-SSSLs were developed and used for *qSERg-1b* fine-mapping. As a result, *qSERg-1b* was narrowed down to the 122.59 kb physical region between InDel markers M01 and M49. There were 19 genes annotated in both *O. glumaepatula* and the HJX74 genomes at the *qSERg-1b* interval. Based on the genome re-sequencing of SG22 and HJX74, and the expression levels of the annotated functional genes, multiple nucleotide variations were found in *LOC_Os01g53630*, *LOC_Os01g53680*, and *LOC_Os01g53710*, including base substitutions and insertions/deletions of the genes, and they also showed significant differences in expression levels between SG22 and HJX74. Therefore, *LOC_Os01g53630*, *LOC_Os01g53680*, and *LOC_Os01g53710* were selected as the most possible candidate genes of *qSERg-1b* for further validation. The above results laid an important foundation for *qSERg-1b* cloning and provided valuable resources for molecular breeding of SER in rice.

Keywords: stigma exsertion rate; single-segment substitution line (SSSL); fine-mapping; substitution mapping

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Citation: Cao, L.; Dan, J.; Li, X.; Tan, Q.; Zhang, S.; Song, R.; Fu, X. Fine-Mapping and Candidate Gene Analysis of *qSERg-1b* from *O. glumaepatula* to Improve Stigma Exsertion Rate in Rice. *Agronomy* **2024**, *14*, 323. https://doi.org/10.3390/agronomy14020323

Academic Editor: Yulong Ren

Received: 31 December 2023
Revised: 28 January 2024
Accepted: 30 January 2024
Published: 1 February 2024

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1. Introduction

Hybrid rice played a very important role in boosting rice production over the past few decades. Since the success of hybrid rice being developed and planted widely in China in 1970s, the rice yield per unit area has been improved greatly by at least 20–30% compared with conventional inbred rice [1–3]. Nowadays, hybrid rice has extended to the other main rice-producing countries in Asia and has vital roles in ensuring food security [4], such as in Bangladesh, the fourth highest rice-producing country worldwide [2,4–6]. However, the low F_1 seed yield is known as the bottleneck for hybrid rice extension and commercialization. The hybrid F_1 seed is produced through the outcrossing process between the male sterile lines and the restorer lines [7], and the outcrossing rate is significantly correlated with the stigma exsertion rate (SER) of the male sterile lines [3,8]. In other words, the more spikelets with exserted stigma in a panicle of a male sterile plant, the more hybrid seeds would be produced, because the spikelets with exserted stigma receive pollen grains from the restorer lines more easily. The cultivated rice (*O. sativa*) varieties generally show low SERs to satisfy the requirement of self-pollination [9]. In order to improve the SERs of the male sterile lines, it is very essential to mine more rice germplasm resources with high SERs, and to analyze their genetic basis, as well as to explore the effective utilization methods in hybrid rice breeding.

SER in rice is a quantitative trait, and more than dozens of QTLs controlling SER have been identified and mapped on all 12 chromosomes from cultivars using different mapping populations. However, most of the QTLs showed minor effects, and several QTLs showed large genetic effects in increasing SER. For instance, *qES3* was mapped in an F_2 population of cultivar Koshihikari/IR24. The additive effect was 20.1%, which explained 31.6% of the total phenotypic variance [10], while *qES3* was also detected in a recombinant inbred line (RIL) population involving IR24 [11]. Until now, only a few SER-QTLs in rice have been fine-mapped. A chromosome segment substitution line (CSSL) population between XQZB, an indica maintainer line with a high SER, and ZH9308, an indica restorer line with a low SER, was developed, and a major QTL, *qSE11*, was identified and fine-mapped to a 350.7 kb region in the long arm of chromosome 11. The near-isogenic lines (NILs) of *qSE11* showed SER increased by 32% compared with the recipient parent ZH9308 [12]. By using the BC_4F_2 population derived from Huhan 1B and II-32B, *qSER-7* was fine-mapped to a 28.4-kb region on chromosome 7, and three candidate genes were predicted for *qSER-7* [13]. Based on a BC_4F_2 population between DaS (a japonica two-line male sterile line with a high SER) and D50 (a tropical japonica variety with a low SER), *qSE4* was fine-mapped to a 410.4 kb region on chromosome 4, and *ARF10*, an auxin-responsive factor, was selected as the most possible candidate gene for *qSE4* [14]. Tan et al. identified seven SER-QTLs in a set of single-segment substitution lines (SSSLs) of *O. glaberrima* (African cultivated rice) in the background of HJX74 (an indica cultivar), and through secondary SSSLs (s-SSSLs) substitution mapping, three QTLs, *qSER-1b*, *qSER-3*, and *qSER-8b* were further fine-mapped to the 333.0 kb, 92.5 kb, and 107.5 kb regions, respectively. Moreover, *qSER-1b* and *qSER-3* showed the respective larger additive effects of 11.5% and 11.9% [15].

The floral traits suitable for self-pollination in *O. sativa* were domesticated from wild rice species, which possess outcrossing characteristics with exserted stigmas [16]. By contrast, wild rice species showed much higher SERs (always reaching 50–80%) than *O. sativa*. Herein, *O. longistaminata* showed almost 100% SER [17]. Recently, several QTLs for high SER in *O. rufipogon* have been identified, whose contribution rate to the phenotypic variations were more than 10% [18–21]. Based on an F_2 population of G46B, an indica maintainer line, and T821B, an introgression line from *O. sativa* and *O. longistaminata*, the *qPES-9* for a percentage of the total exserted stigma (PES) was detected on chromosome 9, showing a 76.60% contribution rate for phenotypic variation, and the positive allele was from T821B [22]. However, there are still fewer QTLs controlling the high SER in wild rice species that have been fine-mapped, and no causal gene has been cloned.

SSSLs showed great advantages in the fine-mapping, cloning, and design breeding of the QTLs related to complex traits in crops, including rice [23,24]. In our previous study, by using the SSSLs populations from three AA-genome wild rice species, *O. rufipogon*, *O. meridionalis,* and *O. barthii*, we identified 36 stable SER-QTLs with the mean genetic effects of 14.50–41.25%. Among them, 12 QTLs were identified as novel ones [21]. In addition, we identified 10 SER-QTLs in the SSSLs population of *O. glumaepatula*. Among the QTLs, *qSER-1b* (here we named it as *qSERg-1b*) on chromosome 1 was identified in three SSSLs, SG22, SG23, and SG25, and through the substitution mapping among these three SSSLs, *qSERg-1b* was primarily delimited to the overlapping region of RM403-RM6648 on chromosome 1 with a length of 2611.3 kb. Furthermore, *qSERg-1b* was considered a novel SER locus by comparing the marker locations with the reported SER-QTLs [25]. In the present study, based on the backcross of SG22 with HJX74 and marker-assisted selection (MAS), the s-SSSLs of SG22 were developed, and *qSERg-1b* was further fine-mapped through substitution mapping among the s-SSSLs; the candidate genes were predicted and analyzed at the *qSERg-1b* locus. Through analyzing the DNA variations of the predicted genes based on the genome re-sequencing in HJX74 and SG22, combined with the gene expression analysis, the possible candidate gene of *qSERg-1b* was selected for cloning and function validation in the future.

2. Materials and Methods

2.1. Plant Materials

SG22, an SSSL of *O. glumaepatula*, carried a SER-QTL for high SER, *qSERg-1b*, with the original substituted segment of markers RM403-RM297-RM486 on chromosome 1. HJX74, the recipient of the SSSLs showed low SER. An F_2 population and 11 s-SSSLs were developed through the backcross of SG22 with HJX74, combined with MAS. The materials were planted in the paddy fields of South China Agricultural University in Guangzhou (23°07′ N, 113°15′ E) with two cropping seasons each year, that is, the spring cropping season (March–July) and the fall cropping season (August–November) from 2019 to 2020. Plant cultivation and field management were the conventional methods in paddy fields in South China.

2.2. Phenotyping

For the investigation of SER, the main panicle of an individual plant with half to two-thirds of the spikelets flowered within 2 days were collected after 2:00 pm on the day. The panicles were softly wrapped with a fresh-keeping film and then kept in the minus 20-degree refrigerator. SER was investigated at room temperature for each panicle sample, and SER data were recorded from 10 plants in the middle of each plot in the materials of SSSLs, HJX74, and s-SSSLs. The F_2 population with 240 individuals between SG22 and HJX74 was sampled for SER investigation and genotyping. In each plant, SER was calculated as the ratio of the number of the flowered spikelets with the single- and dual-exserted stigmas to the total number of the flowered spikelets. The details of SER survey and analysis were according to the previous report [21].

2.3. Genotyping

Marker genotyping was conducted for the experimental materials, including parental lines, F_1 hybrids, F_2 population, s-SSSLs, and intermediate materials. Both SSR and Indel markers were used in this study and they are listed in Table S1. Primers were synthesized at Tsingke Biotechnology Co., Ltd. (Beijing, China). DNA extraction from fresh leaves of young seedlings was conducted using the rapid method based on the Tris-EDTA buffer solution [26], and PCR amplificationwas performed using ETC811-384 PCR instruments (Eastwin Scientific Equipments Inc., Suzhou, China). Each PCR was carried out in a 15 μL reaction final volume, including 2.0 μL (100~150 ng /μL) template DNA, 5.3 μL 2 × Taq PCR StarMix with loading dye, 1.2 μL (10 μmol/μL) forward primer, 1.2 μL (10 μmol/μL) reverse primer, and 5.3 μL deionized and distilled water (ddH_2O). PCR products were separated by 6% denatured polyacrylamide gel and the silver staining method.

2.4. Fine-Mapping of qSERg-1b

An F_2 population with 240 individuals between SG22 and HJX74 was used for the SER investigation and genotyping using polymorphic markers and linkage analysis of *qSERg-1b* with the substituted segment of SG22 was performed using the inclusive composite interval mapping method with QTL IciMapping software V4.2 (http://www.isbreeding.net, accessed on 2 December 2022), where the logarithm of the odds (LOD) threshold was set at 2.5. To fine-map *qSERg-1b*, a large F_2 population with about 4000 individuals was used to screen the recombinants by adding new polymorphic markers in the linkage interval. After recombinant plant self-crossing and marker detection, a set of homozygous s-SSSLs covering the target region of the substituted segment in SG22 were selected for phenotyping. Then *qSERg-1b* was narrowed down to a small physical interval with substitution mapping among the s-SSSLs.

2.5. Candidate Gene Prediction

The candidate genes were predicted in the physical region of *qSERg-1b* in the genomes of Nipponbare, HJX74, and *O. glumaepatula* by searching against the websites http://rapdb.dna.affrc.go.jp/ (accessed on 23 March 2023) (Rice Annotation Project Database,

RAP-DB), http://rice.plantbiology.msu.edu/annotation_pseudo_current.Shtml (accessed on 23 March 2023) (Rice genome annotation project database, MSU-RGAP), http://192.168.87.153/ (accessed on 23 March 2023) for HJX74, and http://plants.ensembl.org/index.html (accessed on 23 March 2023) for *O. glumaepatula*. The gene expression data were downloaded from the RGAP database and RiceXPro dataset (http://ricexpro.dna.affrc.go.jp) (accessed on 23 March 2023). Through the whole genome re-sequencing of HJX74 and SG22, nucleotide variations in the upstream region and exons of the predicted candidate genes were analyzed. The possible candidate genes were selected by referencing both the relative expression levels in young panicles and the nucleotide variations in the upstream and exon regions of the predicted genes in HJX74 and SG22.

2.6. Whole Genome Re-Sequencing

The young leaves of HJX74 and SG22 were used as samples for genome re-sequencing. The genomic DNA of each sample was extracted using the CTAB method, the Paired-end (PE) library was constructed according to the Illumina standard protocol. Full sequencing was processed in Wuhan Genoseq Technology Co. Ltd, Wuhan City, China. Briefly, the genomic DNA was randomly interrupted to the 300–500 bp fragments, and then the sequencing adaptor was ligated to the end of each fragment. DNA clusters were prepared on a sequencing chip and finally processed using a HiSeq2000 sequencer. To obtain high-quality, clean data, data quality control was firstly conducted for the original data using the software Cutadpat v1.18 and Trimmomatic v.0.36. Then 85,089,602 clean reads in HJX74 and 84,156,912 clean reads in SG22 were compared with the MSU7.0 (download from http://rice.plantbiology.msu.edu (accessed on 23 March 2023)) genome sequence. As a result, the genomic coverage of HJX74 and SG22 was 87.65% and 88.03%, respectively, and the coverage depth was accordingly 40.60× and 40.13×. By using a Burrows–Wheeler Aligner (BWA, v0.7.12), 84,894 single-nucleotide polymorphisms (SNPs) and 15,938 insertion-deletions (InDels) were identified between HJX74 and SG22 for subsequent sequence variation analysis in the annotated genes.

2.7. RNA Extraction and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted from young spikelets at the pre-heading stage using the MolPure® TRIeasy Plus Total RNA Kit (Yeasen Biotechnology, Shanghai, China). Each sample was a young spikelet pool collected from three individual plants. A total of 1 μg total RNA of each sample was reverse transcribed into cDNA using the Hifair® II 1st Strand cDNA Synthesis SuperMix (Yeasen Biotechnology, Shanghai, China), and then subjected to quantitative PCR using 2× RealStar Fast SYBR qPCR Mix (GenStar, Beijing, China) on the BIO-RAD CFX 96 system. By using the $2^{-\Delta\Delta CT}$ method [27], the expression levels of the genes were normalized to that of an endogenous control, *OsActin* (*LOC_Os03g50885*). Four independent biological samples of each material were examined. The primers for qRT-PCR are listed in Table S1.

2.8. Data Analysis and Illustration

Trait values were primarily processed using Microsoft Excel 2016. And the One-way ANOVA and least significant difference test (LSD test) were carried out using SPSS 22.0. Values of SERs were transformed to the arcsine square root values before the test. The different significances of SERs among the materials were determined at multiple seasons at $p < 0.01$. Additive effect (a) of the SER-QTL in each SSSL was estimated by that of

$$a_i = (S_i - S_0)/2. \quad (1)$$

Here, S_0 represented the SER of HJX74; S_i represented the SER of SSSL. Charts were drawn using GraphPad Prism 9.0, and the QTL linkage map was constructed using MapChart 2.2 and Microsoft PowerPoint 2016.

3. Results

3.1. Validation of the Chromosomal Region and Effects of qSERg-1b in SG22

In SG22, the substituted segment was between markers RM403 and RM486 on chromosome 1 (Figure 1a). SG22 showed a similar plant architecture with HJX74 (Figure 1b). Here, a small F_2 population with 240 randomly selected individuals between SG22 and HJX74 was used for genotyping and phenotyping to confirm the linkage interval of *qSERg-1b* in SG22. Based on the substituted segment of SG22 on chromosome 1, five new polymorphic markers (RM1152, RM11630, RM1183, A39, and A23) in the interval of RM403 and RM486 were added to the genotype F_2 population. As a result, except for the 5 recombinants among the 240 individuals, 45, 135, and 55 individuals showed the homozygous genotype (aa) of HJX74, heterozygous genotype (Aa), and homozygous genotype (AA) of SG22, respectively. Accordingly, the mean SERs of the individuals in these three kinds of genotypes were 27.24%, 30.46%, and 35.11%, which were significantly different from each other (Figure 1c). The SER distribution in the F_2 population was in a continuous trend, ranging from 4.05–62.00% (Figure 1d), while the SERs of HJX74 (26.56%) and SG22 (39.26%) were significantly different. Through linkage analysis using QTL IciMapping, *qSERg-1b* was mapped to the 1594.75 kb interval between markers RM403 and RM1183 in the substituted segment of SG22 with an LOD score of 3.73 (Figure 1e). The additive effect and the dominant effect of *qSERg-1b* were estimated as 4.07% and −0.59%, respectively, and *qSERg-1b* explained 6.77% phenotypic variations in SG22. Thus, *qSERg-1b* was considered a minor-effect QTL whose allele from *O. glumaepatula* contributed a positive effect on SER in SG22.

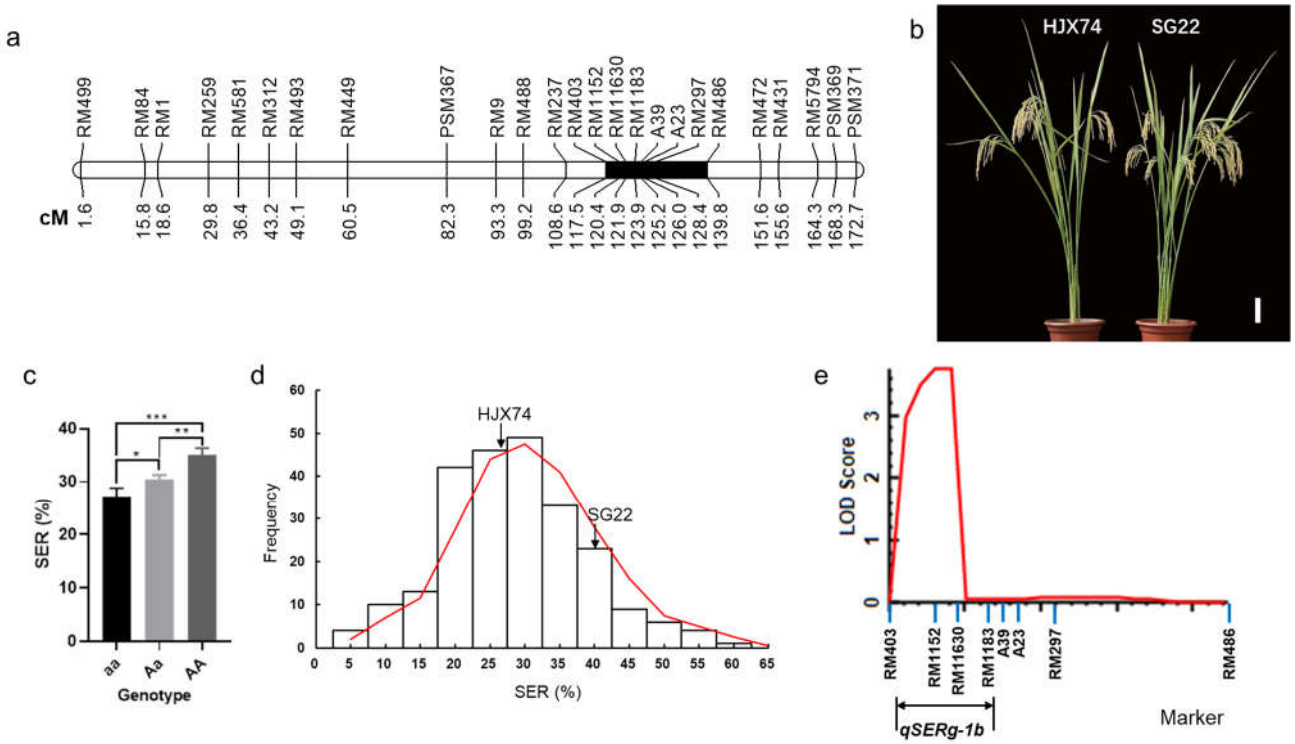

Figure 1. Distribution of SER and QTL intervals in the F_2 population of HJX74/SG22. (**a**) The substituted segment interval of SG22 on chromosome 1, which was the black square between markers RM403 and RM486; (**b**) plant architectures of two parents: left is HJX74, right is SG22, and the scale bar is 10 cm; (**c**) SER in different genotypes in the F_2 population; (**d**) the distribution frequency of the SER in the F_2 population; (**e**) the linkage interval of *qSERg-1b*. "*", "**", and "***" represent the significant differences at 0.05, 0.01, and 0.001 levels, respectively.

3.2. Fine-Mapping of *qSERg-1b*

To fine-map *qSERg-1b*, the recombinant plants were selected from about 4000 individuals in the F_2 population using 15 polymorphic InDel markers and 7 SSR markers (Table S1). The informative recombinant lines were self-crossed, and combined with MAS to obtain homozygous s-SSSLs. As a result, 11 genotypes of homozygous s-SSSLs family lines were developed and used to investigate the SER and fine-map *qSERg-1b* across three cropping seasons in 2019–2020 (Figure S1). The mean SERs of HJX74 and SG22 in three cropping seasons were 24.56% and 38.69%, respectively. Among the s-SSSLs (designated from R1 to R11), six s-SSSLs (R3, R4, R5, R6, R10, and R11) showed significantly higher SERs (33.87–40.18%) than HJX74 at $p < 0.01$, and they shared the common substituted segment interval between InDel markers M01 and M49. Meanwhile, the other five s-SSSLs (R1, R2, R7, R8, and R9) showed lower SERs (24.07–27.25%) which were non-significantly different from HJX74. Thus, through substitution mapping, *qSERg-1b* was narrowed down to a 122.59 kb interval between markers M01 and M49 (Figure 2).

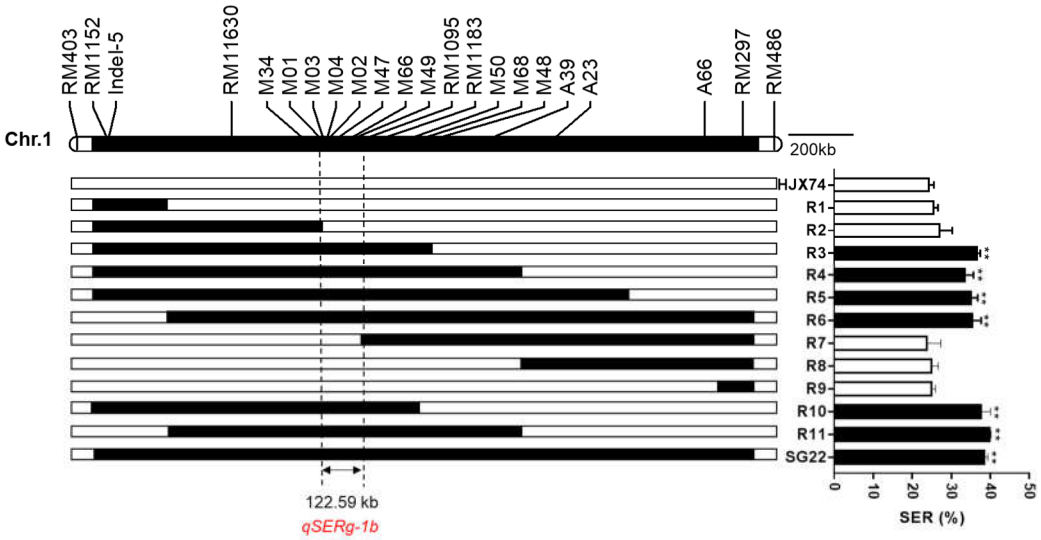

Figure 2. Fine-mapping of *qSERg-1b* through the substitution mapping among the s-SSSLs. R1~R11 are the s-SSSLs family lines derived from SG22. "**" represents the significant differences at 0.01 level.

3.3. Gene Annotation and Candidate Gene Prediction of *qSERg-1b*

Gene annotation at the *qSERg-1b* interval was carried out in genomes of Nipponbare, HJX74, and *O. glumaepatula.* In the reference genome of Nipponbare, the physical region of *qSERg-1b* (30,763,812–30,886,398 bp on chromosome 1 harbored 22 annotated genes according to RAP-DB, including 2 hypothetical genes, 3 genes for hypothetical proteins, 14 genes for specific functional proteins, and 3 genes with no functional information, while according to MSU-RGAP, there were 17 annotated genes in Nipponbare (4 genes for expressed proteins, 1 for transposon proteins, 1 for retrotransposon proteins, and 11 for specific functional proteins) (Table S2). In the genomes of HJX74 and *O. glumaepatula*, 19 genes were annotated and, herein, 17 showed as the same genes as that of Nipponbare (Table S3). From searching the databases of both FPKM (Fragments Per Kilobase per Million mapped reads) (FPKM values of candidate genes in pistils > 8) and RiceXPro spatio-temporal gene expression data of the reference genome (raw signal intensity of candidate genes in pistils > 350), six functional genes with higher expression levels in rice pistils were selected for the expression analysis (Table S4).

The qRT-PCR results in young spikelets at the pre-heading stage indicated that the relative expression levels of the three predicted genes were significantly different between SG22 and HJX74 (Figure 3). Among them, the relative expression level of *LOC_Os01g53630* in SG22 was significantly higher than that in HJX74 at $p < 0.01$, while the expression levels of *LOC_Os01g53680* and *LOC_Os01g53710* in SG22 were significantly lower than

those in HJX74. The additional three genes, *LOC_Os01g53600*, *LOC_Os01g53610*, and *LOC_Os01g53700* showed non-significant expression differences between SG22 and HJX74. In addition, based on the genome re-sequencing analysis of SG22 and HJX74, nucleotide variations in *LOC_Os01g53630*, *LOC_Os01g53680*, and *LOC_Os01g53710* were found between HJX74 and SG22. In *LOC_Os01g53630*, a total of 13 variations were in the upstream region of the gene, including eight base substitutions and five indels, while 14 variations were found at the exons, including five nonsynonymous and eight synonymous base substitutions, and one non-frameshift deletion. And there was one base substitution at the upstream and two synonymous base substitutions in the exon in *LOC_Os01g53680*. As for *LOC_Os01g53710*, 10 base substitutions were found at the upstream, while 1 non-synonymous base substitution in exon resulted in the amino acid variation (Table 1 and Table S5). Taken together, due to the above three functional genes with both DNA structure variation and expression differences between HJX74 and SG22, they would be selected as the possible candidate genes of *qSERg-1b* for further validation.

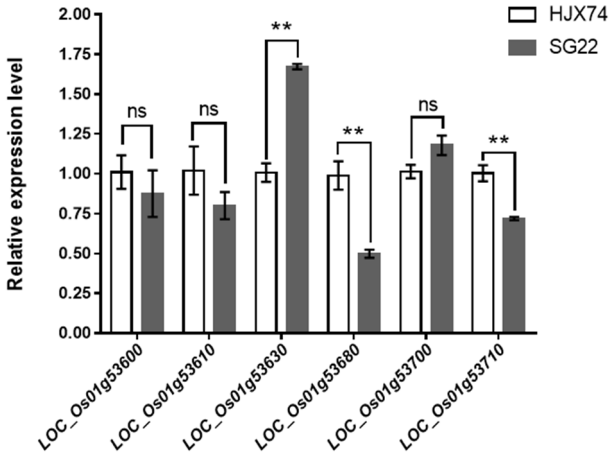

Figure 3. Relative expression levels of the candidate genes in young spikelets at the pre-heading stage. The mean ± se values were obtained from four biological replicates. Data analysis adopted an independent-sample *t*-test based on the relative expression values. "**" represents the significant differences at 0.01 level. "ns" means non-significant differences.

Table 1. The possible candidate genes selected for *qSERg-1b*.

MSU_Locus ID	Annotation	Nonsynonymous Mutations in Exon Regions
LOC_Os01g53630	ulp1 protease family protein, putative, expressed	exon10: A1231G; I411V exon8: C1068A; D356E exon4: 724_741del; 242_247del exon2: A376C; K126Q exon1: T341C; L114P exon1: T287G; L96W
LOC_Os01g53680	6-phosphofructokinase, putative, expressed	None
LOC_Os01g53710	Dual-specificity protein phosphatase, putative, expressed	exon6: A977G; N326S

4. Discussion

Many important agronomical traits in rice are controlled by both major-effect QTLs and minor-effect QTLs [28]. Minor QTLs play roles in the regulation of complex agronomic traits and are widely utilized in commercial rice varieties [29]. However, due to small genetic effects and environmental sensitivity, only a few minor QTLs have been fine-mapped. For example, minor QTLs *qTGW1.2b* [30] and *qTGW7b* [31] for rice grain weight were fine-mapped to a 44.0 kb region and 86.2 kb region, respectively. Moreover, Wu et al. [32] cloned and characterized a minor-effect QTL, *DTH2*, for days to heading, which is a target of human selection for adaptation to long-day conditions during rice domestication and/or

improvement. It demonstrated that the minor QTLs play key roles in crop adaptation and breeding. Since the genetic effect of the minor QTLs is affected by the environment, and their mapping and cloning are difficult, more repeatable results and genetic materials with simple backgrounds are needed.

Stigma exsertion is an important trait in both rice domestication and breeding. A lot of SER-QTLs have been detected in rice. However, fewer QTLs have been fine-mapped, because most of them showed minor additive effects. Previously, by using the SSSLs populations from four AA-genome wild rice species, *O. glumaepatula*, *O. barthii*, *O. meridionalis*, and *O. rufipogon*, we identified dozens of stable SER-QTLs on 12 chromosomes, most of which showed minor additive effects [21,25]. In the present study, we fine-mapped a novel minor-QTL, *qSERg-1b*, for SER in an SSSL-SG22 from *O. glumaepatula*. *qSERg-1b* was delimited to the 122.59 kb interval between InDel markers M01 and M49 on chromosome 1, and the allele from *O. glumaepatula* showed a positive effect in increasing SER in SG22 with an 8.02% additive effect. Stigma exsertion is related to the plant growth and developmental process, and it may also connect with plant hormone signaling pathways. Through the comprehensive analysis of gene annotation, relative expression levels, combined with the genome re-sequencing of the two parents, SG22 and HJX74, some helpful information and clues for screening the candidate genes of *qSERg-1b* were provided for the future study. The results proved that SSSL is an excellent material type for identifying minor QTLs. Furthermore, the derived s-SSSLs are very reliable and important materials for the fine-mapping and cloning of minor QTLs. However, time is needed to develop the s-SSSLs covering the whole substituted segment of the original SSSL.

Three possible candidate genes were predicted for *qSERg-1b*, that is, *LOC_Os01g53630* for the putative ulp1 (ubiquitin-like protease 1) protease family protein, *LOC_Os01g53680* for the putative 6-phosphofructokinase, and *LOC_Os01g53710* for the putative dual-specificity protein phosphatase. However, there is no report about the cloning and functional analysis of these three genes in rice. Ulp1 protease is related to sumoylation, which is a major post-translational process. Sumoylation could regulate plant signaling, development, and responses to hormonal and environmental cues through transcriptional regulation of determinant gene expression [33]. For example, the *AtSIZ1* gene for E3 SUMO ligase in *Arabidopsis thaliana* negatively regulates abscisic acid signaling [34], but positively regulates SLY1-mediated GA signaling and plant development [35]. The relationship of *LOC_Os01g53630* in the present study with high SER is worth exploring, combined with GA signaling pathway in the pistil development of rice. 6-phosphofructokinase is known to catalyze the phosphorylation of fructose-6-phosphate to fructose-1, 6-bisphosphate, which is a key regulatory step in the glycolytic pathway. In rice seedlings, it was found that 6-phosphofructokinase (PFK) has isozymes that play an adaptive role after *Rhizoctonia solani* infection, and those of PFK are expressed at higher levels within a short time after infection [36]. In the seed germination process, energy production plays an important role. The glycolytic pathway and citrate cycle in the respiration pathway supply energy during *Phyllostachys edulis* seed germination; moreover, the respiration intensity increased significantly with the 6-phosphofructokinase (PFK) enzyme peaking in stage II. [37]. At present, *LOC_Os01g53680*, one of the candidate genes for *qSERg-1b*, is annotated for the putative 6-phosphofructokinase in rice; the energy supply is needed in stigma exsertion and the role of 6-phosphofructokinase in the stigma exsertion process could be probed in the future, which would be helpful in verifying the causal gene of *qSERg-1b*. Additionally, plants possess many protein kinases with broad substrate specificity, and dual-specificity phosphatase is one of these family members [38]. Mitogen-activated protein kinase (MAPK) belongs to a subgroup of dual-specificity phosphatase, which was found to function in plant growth and development and abiotic and biotic stress tolerances, such as in *Arabidopsis thaliana* [39] and the common bean [40]. In rice, the dual-specificity phosphatase gene *ERECTA1* (*OsER1*) was found to act upstream of the OsMKKK10-OsMKK4-OsMPK6 cascade to negatively regulate the spikelet number per panicle, and the OsER1-OsMKKK10-OsMKK4-OsMPK6 pathway is required to modulate cytokinin metabolism [41]. However, there are a lot of

isozymes for ulp1 protease, 6-phosphofructokinase, and dual-specificity phosphatase in plants, and each isozyme is related to a different responsible gene. Till now, there have no clear-cut results on the gene function analysis in rice about the predicted candidate genes in present study. Thus, cloning the target gene of *qSERg-1b* and probing the regulating mechanism in SER improvement will be important in future work. Different vectors, such as complementary vectors, overexpression vectors, and/or candidate genes editing using CRISPR/Cas9 technology, should be constructed to obtain genetic materials for function verification.

The minor-effect QTL is usually difficult to use in crop breeding. However, QTL pyramiding breeding has been tried to use as a practicable way to use minor QTLs in the improvement of crop quantitative traits. For example, Shao et al. [42] revealed a combination of two minor resistant QTLs, *Qphs.hwwg-5A.1* and *Qphs.hwwg-3B.1*, for pre-harvest sprouting (PHS) in wheat greatly reduced the PHS. Moreover, the combination of these two minor QTLs together with the major resistant QTL *Qphs.hwwg-3A.1* reduced PHS sprouting by 50% or more than the genotype with only *Qphs.hwwg-3A.1*. In our previous study, several pyramiding lines (PLs) of SER-QTLs in rice showed that different epistatic and pyramid effects existed among PLs with different QTLs [21]. Tan et al. [43,44] reported the negative epistatic effects and improved SER by aggregating more SER-QTLs in PLs among SSSLs from different donors. Even if the QTL had a minor additive effect, it could contribute to SER improvement in PLs. Although the epistatic interactions were complicated within pyramided QTLs, it is worth developing more aggregation combinations involving minor QTLs to screen promising PLs for SER in rice.

5. Conclusions

In this study, we have fine-mapped a novel minor SER-QTL, *qSERg-1b*, from the SSSL of *O. glumaepatula*. The results shed light on the fact that substitution mapping in the s-SSSLs is an effective and reliable method for the fine-mapping of minor QTLs and map-based gene cloning. In the future, it is worth trying to clone *qSERg-1b* and analyze the functional mechanism in controlling SER in rice. In addition, the breeding way could be tried through developing and selecting the elite pyramiding lines in *qSERg-1b* with the SER-QTLs identified in other SSSLs, then transferring the SER-QTLs in PLs to cytoplasmic male sterile lines to improve the SERs of male sterile lines in rice.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14020323/s1: Figure S1: SER distribution of the secondary SSSLs during three cropping seasons, (a), (b), and (c) the SER values in the second cropping season of 2019, the first cropping season of 2020, and the second cropping season of 2020, respectively, (d) the mean SER across the three cropping seasons, R1-R11, the secondary SSSLs; Table S1: DNA markers used for QTL mapping and candidate genes quantitative real-time PCR in this study; Table S2: Gene annotation within the interval of *qSERg-1b* from the database of Nipponbare, *O. sativa*; Table S3: Gene annotation within the interval of *qSERg-1b* from the database of *O. glumaepatula* and HJX74; Table S4: The expression values of functional genes within the interval of *qSERg-1b* from the database of *O. sativa*; Table S5: List of the candidate genes with nucleotide variations at upstream and exon regions between two parents based on the re-sequencing results.

Author Contributions: Conceptualization, X.F.; validation, L.C., J.D., X.L., and S.Z.; formal analysis, L.C., J.D. and X.L.; investigation, L.C., X.L., J.D. and S.Z.; resources, Q.T., R.S. and X.F.; writing—original draft preparation, X.F., L.C. and J.D.; writing—review and editing, X.F., J.D., R.S., L.C., Q.T. and S.Z.; visualization, L.C., J.D. and Q.T.; supervision, X.F.; project administration, X.F.; funding acquisition, X.F. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, Grant number: 32171967, the National Natural Science Foundation of China, Grant number: 31671762, the Guangzhou Science and Technology Program Key Project, Grant number: 201804020086.

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Acknowledgments: Our thanks go to Guiquan Zhang for his suggestions in this manuscript, and also go to Haitao Zhu at SCAU for his help in field management.

Conflicts of Interest: The authors declare no conflicts of interest.

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