Fine mapping and target gene identification of qSE4, a QTL for stigma exsertion rate in rice (Oryza sativa L.)

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The stigma exsertion rate (SER) is a complex agronomy phenotype controlled by multiple genes and climate and a key trait affecting the efficiency of hybrid rice seed production. Using a japonica two-line male sterile line (DaS) with a high SER as the donor and a tropical japonica rice (D50) with a low SER as the acceptor to construct a near-isogenic line [NIL (qSE4 DaS)]. Populations were segregated into 2,143 individuals of BC3F2 and BC4F2, and the stigma exsertion quantitative trait locus (QTL) qSE4 was determined to be located within 410.4 Kb between markers RM17157 and RM17227 on chromosome 4. Bioinformatic analysis revealed 13 candidate genes in this region. Sequencing and haplotype analysis indicated that the promoter region of LOC_Os04g43910 (ARF10) had a one-base substitution between the two parents. Further Reverse Transcription-Polymerase Chain Reaction (RT-PCR) analysis showed that the expression level of ARF10 in DaS was significantly higher than in D50. After knocking out ARF10 in the DaS background, it was found that the SER of arf10 (the total SER of the arf10-1 and the arf10-2 were 62.54 and 66.68%, respectively) was significantly lower than that of the wild type (the total SER was 80.97%). Transcriptome and hormone assay analysis showed that arf10 had significantly higher auxin synthesis genes and contents than the wild type and the expression of auxin signaling-related genes was significantly different. Similar results were observed for abscisic acid and jasmonic acid. These results indicate that LOC_Os04g43910 is mostly likely the target gene of qSE4, and the study of its gene function is of great significance for understanding the molecular mechanisms of SER and improving the efficiency of hybrid seed production.

KEYWORDS
stigma exsertion rate, QTL, near-isogenic line, hormone, rice
Introduction

Rice (*Oryza sativa* L.) is a staple food for billions of people worldwide (Khush, 2005). High yield has always been the primary goal of rice breeding in China for the past few decades. The successful development of hybrid rice was another milestone in enhancing rice yield following dwarf breeding. Rice is a typical self-pollinating crop (Virmani and Athwal, 1973), but the male sterile line with pollen abortion cannot self-fertilize, so the male sterile line is the key line for hybrid rice breeding. Stigma exsertion is an important agronomic trait for male sterile lines to successfully receive restorers’ pollen. Studies have shown that flowering does not mean fertilization (Kato and Namai, 1987), and the exposed stigma can maintain vigor for 4 to 6 days, which greatly improves the outcrossing rate of rice; thereby improving the seed production efficiency of hybrid rice (Yan and Li, 1987; Bi and Tan, 1988; Tian et al., 2004). Therefore, the stigma exsertion rate (SER) of male sterile lines is a key factor of hybrid rice. Increasing the SER is beneficial to increase the yield of hybrids and promote their commercialization (Virmani, 1994).

In the past two decades, dozens of QTLs related to the SER, distributed on 12 chromosomes of rice, have been detected by various germplasm resources and methods (Miyata et al., 2007; Yan et al., 2009; Liu et al., 2019; Tan et al., 2020). For this research, male sterile lines, maintainer lines, and wild rice with high stigma exsertion are commonly used. From the two-line male sterile line DaS, *qPES3*, *qPDES4*, and *qPES12* were mapped on chromosomes 3, 4, and 12, respectively (Li et al., 2017). Using the single-segment substitution line created by the maintainer line IR66897B, *qSER-2a*, *qSER2b*, and *qSER3a*, *qSER3b* were mapped on chromosomes 2 and 3, respectively (Tan et al., 2021). From the maintainer Xieqingzaob, *qSE7* (Zhang et al., 2018) and *qSE11* (Rahman et al., 2017) were mapped on chromosomes 7 and 11, respectively. Many QTLs for SER, such as *qPES8*, *qPES9*, *qRES-10* (Uga et al., 2003), *qRES-10* (Uga et al., 2003), *qSER-2* (Bakti and Tanaka, 2019), *qSER-3* (Bakti and Tanaka, 2019), *qPES-9*, and *qSER-1a* (Tan et al., 2020) have been found in wild rice, which is an important germplasm resource (Marathi et al., 2015; Marathi and Jena, 2015). *qES3* is a QTL of SER that has been mapped multiple times (Miyata et al., 2007) and later confirmed as the grain size gene *GS3* (Takano-Kai et al., 2011), which affects the SER by controlling the length of the stigma. Another gene, *qSTL3*, which also controls stigma length, was mapped from the indica rice Kasalath (Liu et al., 2015). However, neither of these two genes were cloned with SER as the mapping feature trait; only LOC_Os07g15370 has been cloned using SER as the mapping feature trait (Liu et al., 2019). Unfortunately, this gene has not been confirmed to affect SER in sterile lines. Despite substantial research, most genes only stay in the mapping stage because the stigma exsertion is not only affected by genes, but also by environmental factors (e.g., temperature, moisture, wind; Yan et al., 2009). In addition, most populations used in these studies were either *F2* populations, recombinant inbred lines, backcross inbred lines, or double haploid lines. It is difficult to exclude epistatic effects of different chromosome segments in these populations, further challenging QTL cloning of SER (Liu et al., 2015). Therefore, we presently have a poor understanding of the molecular mechanisms of SER in rice, and it is urgent to clone SER-related genes.

In our previous study, the *F2* population was constructed using the japonica two-line male sterile line, DaS, and tropical japonica, D50, to map QTL of SER, *qPES3* on Chr3, *qPDES4* on Chr4, and *qPES12* on Chr12, and the contribution rates were 25.6, 17.86, and 16.98%, respectively (Li et al., 2017). In this study, we further constructed a near-isogenic line NIL (*qSE4-ins*), whose SER was significantly higher than that of D50. The *qSE4* gene was narrowed within 410.4 kb on chromosome 4 using the backcross inbred populations of BC1;F2 and BC1;F2. Within this region, a possible stigma exsertion-related gene was identified, and the function of the gene was preliminarily studied. These results provide a significant breakthrough for molecular level research on the SER of rice and have important value for the hybrid rice with high and stable yield in seed production.

Materials and methods

Population and field experiments

The process of constructing the mapping population is shown in Figure 1. In our previous study, QTL *qSE4* was detected for a dual SER using an *F2* population derived from a cross between D50 and DaS (Li et al., 2017). D50 is a tropical japonica with a low SER. DaS is a japonica two-line male sterile line with a high SER selected from the offspring of indica-japonica hybrids.

We fine-mapped *qSE4* by constructing a NIL with respect to *qSE4*. To this end, an *F2* line with the DaS genotype in the *qSE4* region was selected to successively backcross with D50 for four generations. The SSR markers RM17157 and RM17303 were used for marker-assisted selection (MAS) of each generation among the segregating progenies. Finally, we used the BC1;F2 and BC1;F2 populations to map *qSE4* and selected two BC1;F2 individuals as NILs. To test recovery rate of the NILs, we used 134 pairs of polymorphic SSR markers between DaS and D50, which evenly distributed on 12 rice chromosomes. It was found that the genotypes of all the markers of the two NILs were the same as those of D50 except for RM17207 and RM17227.

The *qSE4* knockout vector was constructed using the BGK003 vector of the Biogle kit and sent to Wuhan Biorun Company for the transformation of the parental DaS.

All materials were grown at the experimental field of China National Rice Research Institute or the test field in Lingshui, Hainan Province, China. Standard crop management practices were followed.
Phenotypic evaluation

After full flowering of the rice plants, 6 main panicles were randomly selected from each line, and the single (SSE), dual (DSE), and total (TSE) SER were investigated. SSE (%) = single stigma exposed spikelets / total spikelets × 100%; DSE (%) = dual stigma exposed spikelets / total spikelets × 100%; TSE (%) = SSE + DSE.

DNA extraction and molecular marker screening

The genomic DNA was extracted from fresh leaves using the Sodium Dodecyl Sulfate (SDS) method (Orjuela et al., 2010). Polymerase Chain Reaction (PCR) was performed in 12 μl reaction volumes containing 2 template DNA, 5 μl of 2 × T5 Super PCR Mix (PAGE; TSINGKE, Beijing, China), 1 μl of 10 μmol/μL primer pairs, and 4 μl of double distilled H₂O. Target DNA segments were amplified with the following program, 95°C for 5 min, followed by 35 cycles at 95°C for 10 s, 60°C for 34 s, followed at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from young panicles at the pre-heading stage (Liu et al., 2019) using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was reverse transcribed to generate cDNA using the Rever Tra Ace qPCR RT Kit (Toyobo, Osaka, Japan). 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 3 Real-Time PCR System (Thermo Fisher Scientific, USA). The PCR program was 95°C for 5 min, followed by 40 cycles at 95°C for 5 s, 60°C for 34 s, followed at 95°C for 15 s, 60°C for 1 min, 95°C for 15 s.

RNA-seq analysis

Young panicles of DaS and arf10 at the pre-heading stage were harvested and immediately frozen in liquid nitrogen.
RNA-seq analysis was performed by Novogene (Beijing, China). After the RNA was reversed to double-stranded cDNA, qualified libraries were constructed, then sequenced by the Illumina NovaSeq 6,000. Differential expression analysis between the two comparisons was performed using the DESeq2 R package (1.20.0). The resulting p-values were adjusted using the Benjamini and Hochberg’s approach for controlling the false discovery rate. Thresholds of padj < 0.05 and |log2foldchange| > 1 were set for significant differential expression. GO enrichment analysis and KEGG enrichment analysis of differentially expressed genes were both analyzed by the cluster Profiler R package (3.8.1).

Measurement of free IAA, ABA, and JA content

Young panicles of DaS and arf10 at the pre-heading stage were harvested and immediately frozen in liquid nitrogen. The samples (100 mg) were resuspended with liquid nitrogen and homogenized with 400 µl of acetonitrile (50%) which contained mixed internal standards and extracted at 4°C. Then centrifuged at 12,000 rpm for 10 min. The supernatant passed through the HLB sorbent (first flow-through fraction) and then was eluted subsequently with 500 µl of acetonitrile (30%; second flow-through fraction). These two fractions were combined into the same centrifuge tube and mixed well. Finally, these solutions were injected into the LC-MS/MS system for analysis.

Statistical analysis

One-way ANOVA was used to test the statistically significant differences among tested varieties, which was performed using SPSS 22.0 software (IBM Inc.).

Results

Development of the near isogenic line for qSE4

Based on previous research, qSE4 was mapped between markers RM17157 and RM17303 (Li et al., 2017; Figure 2A). One line from the F2 population was selected for four rounds of backcrossing with D50, and near-isogenic lines (NILs) were used to isolate qSE4 (Figure 1). The markers RM17157 and RM17303 were used for marker-assisted selection in the segregating progenies carrying the DaS qSE4 allele during each backcross generation. After continuous backcrossing for four generations and selling, the genetic background became similar to that of the recurrent parent D50, except for the substituted target segments. Two individuals with the highest recovery rate to the recurrent parent were selected among the segregating progeny, i.e., NIL (qSE4D50)-1 and NIL (qSE4D50)-2, which carry the homozygous allele of DaS in the region of the qSE4.

The NIL (qSE4D50)-1 had increased exsertion rates of 22.14, 32.35, and 54.49% for single stigma exsertion rate (SSE), dual stigma exsertion rate (DSE), and total stigma exsertion rate (TSE), respectively, when compared to the recurrent parent D50. The NIL (qSE4D50)-2 had increased exsertion rates of 17.25, 36.33, and 53.58% for SSE, DSE, and TSE, respectively (Table 1; Figure 3). This indicated that qSE4 is responsible for the high stigma exsertion rate in NIL (qSE4D50).

Among the random selection of 100 individuals in the BC2F3 population, the marker RM17157 was used to validate the effect of qSE4. The Chi-squared test revealed the phenotypic separation ratio was fitted to 1:2:1 (X^2 = 3.74 < X^2_0.05,2 = 5.99), suggesting that the effect of qSE4 is likely controlled by one genetic locus (Table 2).

Fine mapping of qSE4

Selfing of some BC2F2 individual plants to produce BC2F3, and one heterozygote recombinant from the BC2F2 population that carried the target QTL region from DaS was backcrossed with D50 to produce a larger BC2F3 population. A total of 2,143 individuals were used for fine mapping the qSE4 using three additional polymorphic SSR markers between DaS and D50. Five homozygous recombinant lines and one heterozygote line in the QTL region were analyzed for fine mapping (Figure 2B). The phenotypic performance of the SERs varied from 24.07 to 79.72% in the recombinant lines. The total SERs of A2, A3, A4, and A5 were similar to that of D50 and significantly lower than that of A1 which was similar to DaS. The total SER of heterozygote A6 was between that of the two parents (Figure 2B). Based on genetic and phenotypic analysis, the location of qSE4 was narrowed to a 410.4-kb region between the RM17207 and RM17227 (Figure 2C).

Candidate gene analysis of qSE4

After removing unknown retrotransposons, transposons, and putative genes (Li et al., 2021) within the 410.4-kb region, 13 genes remained (Figure 4A) according to the Rice Genome Annotation Database (rice.plantbiology.msu.edu, MSU-version_7.0). Sequencing analysis found that the two parents had one base difference in the promoter region of LOC_Os04g43910 (A for DaS and G for D50), but no difference in the coding region (Figure 4B). Gene expression analysis showed that the expression level of LOC_Os04g43910 in parental DaS was significantly higher than in D50. The expression level of lines carrying the DaS allele in progenies was also significantly higher than that carrying the D50 allele (Figure 4C). The differences of LOC_Os04g43910 among different cultivars were further
analyzed, and it was found that the cultivars of haplotype G were mostly wide-grained cultivars with lower SERs. The haplotype A cultivars were mostly slender grains with higher SERs (Figure 4D). Therefore, LOC_Os04g43910 is predicted as the candidate gene. Further, LOC_Os04g43910 (ARF10) was knocked out in the DaS background using CRISPR/Cas9 technology, two mutants which named arf10-1 and arf10-2 were obtained. The arf10-1 and arf10-2 mutant plants contained 1-bp deletion and 1-bp insertion, respectively, in the exon of ARF10 (Figure 5A). These two mutations caused the premature appearance of stop codons in the ARF10 gene, resulting in a significant decrease in the expression of ARF10 (Figure 5B). The SERs were significantly reduced in both knockout lines compared to the wild type (Figures 5C–H; Table 3). These results further confirmed that ARF10 was most likely to be the causal gene for the qSE4 influencing the SER in rice.

Transcriptome analysis of ARF10 knockout plants

RNA-sequencing (RNA-seq) was conducted to show the transcriptomic variation in the young panicle between the wild-type DaS and the mutant arf10 (Figure 6). A total of 10,414
differentially expressed genes (DEGs) were detected (padj < 0.05), of which 53.7% (5,597 genes) were up-regulated and 46.3% (4,817 genes) were down-regulated in the arf10 plant compared with the expression levels in DaS plant (Figure 6A; Supplementary Figure 1). We randomly selected 8 genes to analyze their expression levels via qRT-PCR. The results were consistent with the results of the transcriptome (Supplementary Figure 2). GO analysis reveals DEGs enrichment in biological processes, cellular components, and molecular functions. Metabolic process, biochemical reaction, and biosynthetic process represent 41.32, 16.51, and 11.85% of total DEGs, respectively, and were the largest subcategories in the biological process. Ribosome, cell periphery, and external encapsulating structure represent 30.17, 21.55, and 14.08% of total DEGs, respectively, and were the largest subcategories in the cellular component. Enzyme activity, binding, and transmembrane transporter activity represent 43.67, 28.85, and 3.90% of total DEGs, respectively, and were the largest subcategories in the molecular function (Supplementary Figure 3). KEGG pathway analysis showed that ribosome, phenylpropanoid biosynthesis, and plant hormone signal transduction were the most significantly enriched pathways (padj < 0.05) of DEGs (Figure 6D).

Since ARF10 is an auxin-responsive factor, we detected the hormone content of DaS and arf10 and analyzed the expression of hormone synthesis and signaling-related genes. Compared with wild-type DaS, the content of IAA, ABA, and JA in the knockout line arf10 was significantly increased (Figure 6B). It was further found that the genes OsTAR1, OsYUC11, OsYUCCA7, OsYUC9, and OsYUCCA4 for the IAA synthesis pathway; OsNCED1 and OsNCD4 for the ABA synthesis pathway; OsAOS3 and OsAOS4 for the JA synthesis pathway were significantly higher in arf10 than in DaS (Figure 6C). In addition, the genes of IAA, ABA, and JA signaling pathways were also...
expressed significantly differently in arf10 and DaS (Supplementary Figure 4). These results suggested that hormones are key factors affecting the SER.

Discussion

The application of hybrid rice has greatly improved rice yields, but the low efficiency of hybrid seed production limits its promotion in Southeast Asia (Xie, 2008). The increase of stigma exsertion can increase hybrid seed production and promote the commercialization of hybrid rice (Zhou et al., 2017). In the past two decades, dozens of QTLs of the SER have been mapped and validated its function by knocking out ARF10 in the two knockout lines (Chen et al., 2007). Recently, there was a major breakthrough in the study of stigma exsertion in legumes. It was found that qSE4 is a QTL that controls stigma exsertion, which is not related to its expression level, so the loss of qSE4 function promotes tomato stigma retraction, while Style2.1 and SE3.1 are key genes that regulate the two-step process of stigma from exsertion to insertion during tomato domestication (Shang et al., 2021).

Because it is beneficial to the application of heterosis, stigma exsertion is a research hotspot for many crops. So far, only a few genes related to stigma exsertion have been cloned from limited germplasm resources. To detect reproducibly across different germplasm resources.

RM1113 as far from RM17207 and RM17227 on Chromosome 4 (Figures 2C, D). The relationship between the haplotype in the S. 364 base upstream of ATG and stigma exsertion phenotype in different rice varieties. TSE: total stigma exsertion rate; L/W: length/width. The data represent the mean values ± SD (n = 6), **p 0.01.
was significantly lower than of the wild type (Figure 5B), and the SER was also significantly lower than that of the wild type (Figure 5C). These results point to ARF10 as a target gene of qSE4.

Auxin response factors (ARFs) are a class of transcription factors that regulate the response to plant auxins (Wang et al., 2007). Twenty-five ARFs family member genes were found in rice, which plays important functions in rice disease resistance (Qin et al., 2020; Zhang et al., 2020; Zhao et al., 2020), root growth (Qi et al., 2012; Shen et al., 2013; Shao et al., 2019), and plant morphology (Attia et al., 2009; Sakamoto et al., 2013; Zhang et al., 2015; Huang et al., 2016, 2021; Chen et al., 2018; Liu et al., 2018). ARF25 may regulate the expression of the auxin synthesis gene OsYUCCAs to affect the auxin content of rice roots (Qi et al., 2012), while ARF16 may regulate auxin redistribution (Shen et al., 2015). Compared with wild DaS, the auxin content and five auxin synthesis genes in arf10 increased significantly (Figures 6B,C), suggesting that ARF10 may also regulate the expression of auxin synthesis genes to affect the auxin content in rice young panicles. Auxin has a dual effect on plant growth, low concentrations promote growth and high concentrations inhibits growth, therefore, the high concentration of auxin in arf10 may be the reason for its reduced SER. Consistent with this notion, low concentrations of IAA were found in tobacco

### TABLE 3 The stigma exsertion rates of DaS, arf10-1 and arf10-2.

| Traits            | DaS          | arf10-1      | arf10-2      |
|-------------------|--------------|--------------|--------------|
| Single stigma     | 40.15 ± 2.24 | 28.48 ± 2.48** | 35.56 ± 2.37* |
| exsertion rate (%)|              |              |              |
| Dual stigma       | 40.82 ± 2.17 | 34.05 ± 1.95** | 31.12 ± 2.45** |
| exsertion rate (%)|              |              |              |
| Total stigma      | 80.97 ± 2.50 | 62.54 ± 2.22** | 66.68 ± 1.14** |
| exsertion rate (%)|              |              |              |

arf10 is a knockout line of LOC_Os04g43910. Trait values were shown as mean values ± standard deviation values. *p≤0.05, **p≤0.01.
to promote stigma growth while high concentrations inhibited stigma growth (Chen and Zhao, 2008).

Various biochemical processes in plants are regulated by hormone crosstalk. Auxin also performs some functions in conjunction with other hormones. Auxin and abscisic acid jointly control seed dormancy in Arabidopsis thaliana (Liu et al., 2013), and together with brassinolide regulate plant height and leaf angle (Liu et al., 2018). The contents of ABA and JA in arf10 were significantly higher than those of the wild type (Figure 6B), and their synthetic genes also increased significantly (Figure 6C), suggesting that ARF10 may be involved in the synthesis of ABA and JA. KEGG analysis showed that the differential genes were enriched in the hormone signaling pathway (Figure 6D, Supplementary Figure 4).

These results all indicated that IAA, ABA, and JA may jointly regulate the degree of stigma exertion in rice.

The development of NILs is an effective strategy for studying QTLs as the interference of the noise from the background is significantly reduced (Ding et al., 2011). In our study, NIL of the qSE4 carrying DaS allele in the D50 background significantly increased the stigma exsertion rate compared with the recurrent parent, D50 (Figure 3E), suggesting that qSE4 is responsible for the increased SER in NIL. This not only provides the basis for the subsequent cloning of the qSE4 target gene but also provides molecular markers linked to the qSE4 target gene. Molecular markers can be applied to gene pyramid breeding in rice with a high SER. Cultivating rice male sterile lines with a high SER can increase the yield of hybrid rice seed
production, reduce the production cost of hybrid rice, and promote hybrid rice development with health and sustainability.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA850518.

Author contributions

HP and SZ designed experiments. GN, WY, CW, TS, AR, WX, HS, TS, SG, JG, XL and WL performed experiments. GN analyzed data and compiled figures. GN wrote the manuscript. SZ edited the final manuscript. All authors contributed to the article and approved the submitted version.

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Supplementary materials

The Supplementary materials for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2022.959859/full#supplementary-material
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