Activated Expression of Rice DMR6-Like Gene OsS3H Partially Explores the Susceptibility to Bacterial Leaf Streak Mediated by Knock-Out OsF3H04g

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Abstract: Downy Mildew Resistance 6-like (DMR6-like) genes are identified as salicylic acid (SA) hydroxylases and negative regulators of plant immunity. Previously, we identified two rice DMR6-like genes, OsF3H03g, and OsF3H04g, that act as susceptible targets of transcription activator-like effectors (TALEs) from Xanthomonas oryzae pv. oryzae (Xoc), which causes bacterial leaf streak (BLS) in rice. Furthermore, all four homologs of rice DMR6-like proteins were identified to predominantly carry the enzyme activity of SA 5-hydroxylase (S5H), negatively regulate rice broad-spectrum resistance, and cause the loss of function of these OsDMR6s, leading to increased resistance to rice blast and bacterial blight (BB). Here, we curiously found that an OsF3H04g knock-out mutant created by T-DNA insertion, osf3h04g, was remarkably susceptible to BLS and BB and showed an extreme reduction in SA content. OsF3H04g knock-out rice lines produced by gene-editing were mildly susceptible to BLS and reduced content of SA. To explore the susceptibility mechanism in OsF3H04g loss-of-function rice lines, transcriptome sequencing revealed that another homolog, OsS3H, had induced expression in the loss-of-function OsF3H04g rice lines. Furthermore, we confirmed that a great induction of OsS3H downstream and genomically adjacent to OsF3H04g in osf3h04g was primarily related to the inserted T-DNA carrying quadruple enhancer elements of 35S, while a slight induction was caused by an unknown mechanism in gene-editing lines. Then, we found that the overexpression of OsS3H increased rice susceptibility to BLS, while gene-editing mediated the loss-of-function OsS3H enhanced rice resistance to BLS. However, the knock-out of both OsF3H04g and OsS3H by gene-editing only neutralized rice resistance to BLS. Thus, we concluded that the knock-out of OsF3H04g activated the expression of the OsS3H, partially participating in the susceptibility to BLS in rice.

Keywords: rice; bacterial leaf streak; DMR6-like; susceptibility; salicylic acid
1. Introduction

One of the most important crops, rice, is widely cultivated in various regions, but it always suffers from attacks from various diseases with unfavorable climate and environmental conditions [1]. Salicylic acid (SA) is an important phytohormone in the regulation of plant defense against pathogens and abiotic factors [2,3]. In addition, the SA-mediated or -related defense response is one of the main components involved in resistance against diverse phytopathogens in rice. The nonexpressor of pathogenesis-related proteins 1 (NPR1) is the master regulator of the downstream SA signaling pathway and is recognized as an SA receptor to depolymerize into a monomer, which enters the nucleus to trigger defense-related gene expression. Other SA receptors, namely NPR3 and NPR4, are transcriptional repressors in SA-mediated defense [4,5]. Rice OsNH1, which functions as NPR1, is involved in the broad-spectrum defense against pathogens, and its mediated immunity requires the induction of cysteine-rich-receptor-like kinases gene CRK10 by TGA transcription factor TGA2.1 [6,7]. Moreover, WRKY transcription factor OsWRKY45 plays an important role in SA-mediated defense against fungal and bacterial diseases, which are independent of OsNH1 [8–10]. In addition, the mutant of rice negative defense regulators usually results in an immune burst with an elevation of SA. Loss functions of several immune repressors, including mitogen-activated protein kinase OsMPK6 [11] and OsMPK15 [12], cytochrome P450 monoxygenase SL [13], C2H2-type transcription factor Bsr-d1 [14], and C2 domain Ca2+ sensor ROD1 [15], increase broad-spectrum resistance to various phytopathogens and the accumulation of SA content in rice.

In plants, SA biosynthesis is mainly related to the isochorismate and phenylalanine ammonia-lyase pathways [16]. The SA metabolism is composed of glycosylation, hydroxylation, methylation, sulfonation, and amino acid conjugations. The hydroxylation of SA into 2,5-dihydroxybenzoic acid (2,5-DHBA) and 2,3-dihydroxybenzoic acid (2,3-DHBA) by SA 5-hydroxylase (SSH) and SA 3-hydroxylase (S3H), respectively, is an inactive way to passivate plant immunity [17]. Previously, the loss function of DMR6/SSH, which encodes 2-oxoglutarate and Fe (II)-dependent dioxygenase (2OGDs), was found to confer resistance to downy mildew in Arabidopsis by the hydroxylation of SA into 2,5-DHBA [18–20]. In addition, S3H/DLO1, which is homologous to DMR6/SSH, was shown to possess the hydroxylation of SA into 2,3-DHBA in vivo of Arabidopsis [21]. Currently, several DMR6-like genes have been identified to confer resistance to phytopathogens after the loss of function in different plant species. The RNAi-mediated silencing of SdDMR6 and the gene-editing mediated knock-out of SdDMR6-1 conferred resistance to Botrytis cinerea and Phytophthora infestans in potatoes, respectively [22,23]. Similarly, the gene-editing of SdDMR6-1 enhanced tomato broad-spectrum resistance to bacterial, oomycete, and fungal pathogens [24]. Moreover, the gene-editing of OsDMR6 and MusaDMR6 reduced susceptibility to Peronospora belbahrii and Xanthomonas campestris pv. musacearum in sweet basil and banana, respectively [25,26]. Recently, rice DMR6-like genes OsF3H03g and OsF3H04g were identified as the targets of transcription activator-like effectors (TALEs) Tal2b and Tal2c, respectively, which favor Xanthomonas oryzae pv. oryzicola (Xoc) infection in rice [27,28]. Subsequently, OsF3H03g (LOC_Os03g03034), OsF3H04g (LOC_Os04g49194), and the homolog of LOC_Os10g39140 were identified as SSH, while another homolog of LOC_Os04g49210 was identified as both of S5H and SSH. Notably, these genes negatively regulate SA-mediated broad-spectrum resistance in rice [29–31].

Xoc and Xanthomonas oryzae pv. oryzae (Xoo) causes the main bacterial diseases, bacterial leaf streak (BLS) and bacterial blight (BB), respectively, in rice. They are also the model pathogens for plant–microbe interactions [32,33]. During the past few decades, over 42 BB resistance genes have been identified; however, few BLS resistance genes have been identified [34]. The first identified major BLS resistance gene, Rxox1, is a nucleotide-binding leucine-rich repeat (NLR) gene from non-host maize which confers resistance to Xoc through the recognition of non-TALE AvrRxo1 [35]. After that, Rxo1, which contains the zinc finger BED and NLR domain from Carolina Gold Select rice, confers resistance to
African Xoc strains, which is triggered by TALEs but does not rely on their ability of transcriptional activation [36–38]. A recessive resistance gene, bls1, encoding mitogen-activated protein kinase 6 (OsMAPK6), confers specific resistance to the Xoc strain JZ-8, while the overexpression of BLS1 enhances rice broad-spectrum resistance [39,40]. Recently, Xo2 characterized from Bangladeshi rice cultivar X455 was shown to confer resistance to most Chinese Xoc isolates, which involves fine mapping on chromosome 2 [41]. Mainly, the quantitative trait loci (QTLs) are responsible for rice BLS resistance [42–45]. Moreover, the constitutive expression of OsMPK6 [46], OsGH3-2 [47], OsPGIP4 [48], OsHSP18.0-Cl [49], OsMAPK10.2 [50], OsS3H1 [51], OsPGIP1 [52], OsHSFB4d [53], OsPDX1.2 [54], and Os-CIPK15 [55] enhances rice resistance to BLS. The loss or depression of the function of OsNRRB [56], OsImpa1a and OsImpa1b [57], OsALDH2b1 [58], OsEDR1 [59], OsAPS1 [60], OsF3H03g [28], and OsNRAMP1 [61] increases BLS resistance in rice. On the other hand, gene-editing of the effector-binding element (EBE) in the promoter of OsSULTR3:6, which is a target of the major virulence factor Tal2g, confers rice resistance to multiple Xoc strains [62,63]. Interestingly, editing of EBE in the promoters of OsF3H03g or OsF3H04g confers rice-specific resistance to RS105/Tal2b and RS105/Tal2c, respectively [27].

Previously, we demonstrated that the overexpression of the DMR6-like gene OsF3H03g and OsF3H04g reduced rice resistance to BLS and resulted in a decrease in SA content. Moreover, the gene-editing or suppressing of OsF3H03g conferred rice resistance to BLS [27,28]. Recently, studies have revealed that the gene-editing of OsF3H04g improves broad-spectrum resistance to rice blast and BB [29,31]. Unexpectedly, in this study, we identified an OsF3H04g T-DNA insertion mutant, osf3h04g, which was more susceptible to BLS and BB, as well as a tremendous reduction in SA content. However, the gene-editing mediated knock-out of OsF3H04g showed a slight increase in the susceptibility to BLS and a mild decrease in SA content. To explain the turnover functions of osf3h04g, transcriptome profiling was performed to reveal the activation of OsS3H by inserting T-DNA carrying quadruple enhancer elements that complemented the loss function of OsF3H04g and promoted the hydroxylation of SA to negatively regulate rice immunity. We revealed that the T-DNA mutant osf3h04g was composed of the knock-out of OsF3H04g and the constitutive activation of OsS3H mediated by the T-DNA carried enhancer elements, while the OsF3H04g loss function mutant induced a slight expression of OsS3H by an unknown mechanism. Furthermore, the knock-out of both OsF3H04g and OsS3H restored part of the rice resistance to BLS. Thus, we conclude that the loss function of OsF3H04g activates OsS3H to partially suppress the susceptibility to BLS in rice.

2. Results

2.1. T-DNA Insertion Line osf3h04g Is More Susceptible to BLS and BB

Previously, we found that the overexpression of the DMR6-like gene OsF3H03g, which was targeted by Tal2b, could reduce rice resistance to Xoc and Xoo, while the loss-of-function could enhance the defense response of rice to the pathogens [28]. Subsequently, the overexpression of another DMR6-like gene, OsF3H04g, which is a target of Tal2c, resulted in increased susceptibility to Xoc [27]. However, the enhanced resistance to Xoc for the loss function of OsF3H04g is still unclear. To better understand the defense response involved with OsF3H04g, we obtained a T-DNA activation tagging line (PF_3A-05627.L) from the rice T-DNA mutant library [64]. The homozygous mutant osf3h04g was identified as activation tagging vector pGA2715 inserted in the second exon of OsF3H04g (Figure 1A,B). Then, we performed qRT-PCR to reveal that OsF3H04g had decreased expression by a large margin in osf3h04g (Figure 1C). After that, we performed inoculation with the Xoc strains RS105 and HGA4 (hypervirulence strain containing Tal2b-Tal2e) on osf3h04g. As shown in Figure 1D, both RS105 and HGA4 caused more severe lesions in osf3h04g than in wild-type Dongjin (DJ). The lesion lengths were 2.32 ± 0.14 cm and 2.44 ± 0.17 cm in osf3h04g, which were longer than that of 1.82 ± 0.15 cm and 2.13 ± 0.10 cm in DJ caused by RS105 and HGA4, respectively (Figure 1E). Then, we also
performed inoculation with Xoo strain PXO99 on DJ and osf3h04g. More severe disease symptoms and longer lesion lengths caused by PXO99 were also observed in osf3h04g than in DJ (Figure 1F,G). Thus, we revealed that the T-DNA insertion rice line osf3h04g is a null mutant and severely susceptible to BLS and BB.

Figure 1. The identified T-DNA insertion line osf3h04g was susceptible to BLS. (A) Structure diagram for the T-DNA insertion in osf3h04g. (B) Identification of OsF3H04g T-DNA insertion rice osf3h04g by PCR amplification. The primer pairs LP1/LP2 and LB2/LP2 were used to amplify the genomic DNA fragment of OsF3H04g and the recombinant fragment of T-DNA and genome, respectively. (C) The expression level of OsF3H04g in osf3h04g. OsACTIN was used as a reference gene. Data represent means ± SD, n = 3. (D, E) Image of lesion expansions (D) and diagram of lesion lengths (E) in DJ and osf3h04g at 14 days post inoculation (dpi) with RS105 and HGA4. Data represent means ± SD, n = 10. (F, G) Phenotype of lesion expansions (F) and diagram of lesion lengths (G) in DJ and osf3h04g at 14 dpi with Xoo PXO99. Data represent means ± SD, n = 15. Asterisks indicate significant differences between DJ and osf3h04g. (* p ≤ 0.05; ** p ≤ 0.01, Student’s t-test).

2.2. Downregulated Defense-Related Genes and Salicylic Acid Content in osf3h04g

To clarify that the regulation of gene expression in osf3h04g caused more susceptibility to BLS, we performed transcriptome sequencing for osf3h04g and wild-type DJ. The result showed that, in contrast with DJ, there were 826 differentially expressed genes (DEGs), which included 258 upregulated and 568 downregulated DEGs in osf3h04g (Table S1). After that, we performed a GO analysis of these DEGs and focused on those genes involved in the defense and stress response. The results showed that few genes were involved in defense response, and many genes were grouped into response to stress among the upregulated DEGs. However, there were several genes belonging to the GO terms of defense response, response to stress, and hormones, including the SA-related and JA-related response, among the downregulated DEGs (Figure S1). After that, we created a heatmap for these defense and stress response genes. The result showed that a considerable number of pathogenesis-related (PR) genes, ROS-related genes, and transcriptional factor genes showed repressed expression, while a few hormone-related genes, including ethylene- and gibberellin-related genes, had induced expression (Figure 2A). Among the downregulated DEGs in osf3h04g, we observed that a cluster of SA-related genes was downregulated (Figure 2A,B). OsPR1a and OsPR1b showed dramatically reduced expression in osf3h04g (Figure 2A,B). OsWRKY45 and OsNPR3, which are involved in the SA-mediated signaling pathway, had significantly decreased expression in osf3h04g (Figure
2A,B). The abovementioned results indicate that the defense response and SA-related signaling pathway are alleviated in osf3h04g, which may relate to the reduced resistance to BLS and BB.

Previously, OsF3H04g was identified as an S5H as well as OsF3H03g and other homologs [29–31]. Overexpression of either OsF3H04g or any of the other three homologs could decrease SA content and SA-related genes expression, and the knock-out of any of the three homologs could accumulate SA content and activate broad-spectrum resistance [27–31]. Here, our results were different from those expected in osf3h04g, as SA-related genes had a suppressed expression as well as increased susceptibility. Then, we measured the content of SA in osf3h04g. The result showed that the SA content in osf3h04g was 0.94 ± 0.04 µg/g, which was dramatically reduced compared with that of 11.15 ± 0.68 µg/g in wild-type DJ (Figure 2C). To clarify whether the reduction in SA in osf3h04g was related to the synthesis, we then quantified the expression of the SA synthesis-related genes OsPAL and OsICS. However, the result showed that these two SA synthesis-related genes did not have reduced expression in osf3h04g (Figure 2D). Thus, the decreased SA content and normal expression of synthesis-related genes imply that other aspects of SA metabolism may be activated in osf3h04g.
2.3. Gene-Editing of OsF3H04g Increases Moderate Susceptibility to BLS in Rice

The T-DNA line of osf3h04g showed more susceptibility to BLS and BB; however, our previous study revealed that the overexpression of this gene also increased rice susceptibility to BLS [27]. To clarify that the loss function of OsF3H04g is associated with increased susceptibility, we also generated the gene-editing lines of OsF3H04g mediated by the CRISPR-Cas9 system in the ZH11 background. Among the OsF3H04g gene-editing rice lines, only one type of mutation, a “T” base in the U3-gRNA target region and a “C” deletion in the U6a-gRNA target region were found in a total of eight T0 generation lines (Figure S2). Then, we randomly selected two lines of CR-04g-11 and CR-04g-12 to inoculate RS105 in T1 generation, which showed that the lesions in CR-04g-11 and CR-04g-12 developed slightly more rapidly than in wild-type ZH11 (Figure 3A). Furthermore, the lesion lengths were 2.30 ± 0.17 cm and 2.35 ± 0.16 cm in CR-04g-11 and CR-04g-12, respectively, which were mildly longer than that of 2.07 ± 0.17 cm in ZH11 (Figure 3B). After that, the SA content in the OsF3H04g gene-editing lines was also measured, which showed about 20 percent reductions in CR-04g-11 and CR-04g-12 compared with ZH11 (Figure S3). To validate the increased susceptibility, the gene-editing lines were also generated in the DJ background. A “T” base and a “C” base deletion in the U3-gRNA and U6a-gRNA target regions, respectively, were identified in CR-04g-DJ-2, while only a “C” base deletion in the U6a-gRNA target region was identified in CR-04g-DJ-5 (Figure S2). Similar to CR-04g-11 and CR-04g-12 in ZH11, CR-04g-DJ-2, and CR-04g-DJ-5 showed more susceptibility to RS105 with longer lesion lengths than that of DJ (Figure 3C,D). Interestingly, CR-04g-DJ-2 and CR-04g-DJ-5 also enhanced BB susceptibility to the Xoo strain of PXO99 (Figure S4). The abovementioned results indicate that gene-editing of OsF3H04g also increases rice susceptibility to BLS and decreases the SA level. Thus, our results demonstrate that the loss-of-function OsF3H04g compromises rice resistance to BLS.
Figure 3. Gene-editing of OsF3H04g comprised rice resistance to BLS. (A,B) Image of lesion expansions (A) and diagram of lesion lengths (B) for the OsF3H04g gene-editing lines (CR-04g-11 and CR-04g-12) caused by Xoc RS105 at 14 dpi in ZH11. (C,D) Image of lesion expansions (C) and diagram of lesion lengths (D) in DJ for the OsF3H04g gene-editing lines (CR-04g-DJ-2 and CR-04g-DJ-5) inoculated with RS105 at 14 dpi. Data represent means ± SD, n = 10. Asterisks indicate significant differences between wild-type and OsF3H04g gene-editing lines. (*p ≤ 0.05, Student’s t-test).

2.4. RNA-Seq Analysis for Gene Expression Change in Loss-of-Function OsF3H04g Rice

Here, we found that the OsF3H04g T-DNA line, as well as the gene-editing lines, increased rice susceptibility to BLS. The RNA-seq analysis revealed the defense response genes were seriously suppressed in the OsF3H04g T-DNA line. To reveal the differential regulation of defense response in the OsF3H04g gene-editing lines, we also performed the transcriptome sequencing for the OsF3H04g gene-editing line CR-04g-12 at the same condition of transcriptome sequencing for OsF3H04g overexpression line described in a previous report [27]. After comparing with wild-type ZH11, we found 878 upregulated and 181 downregulated DEGs in CR-04g-12 (Figure 4A, Table S2). Then, we compared these DEGs in CR-04g-12 with those identified in osf3h04g, which showed that 79 upregulated and 21 downregulated DEGs were common (Figure 4A, Table S3). After that, we performed the gene function analysis for these 100 common DEGs. The result showed that five upregulated and three downregulated DEGs were involved in disease resistance, while thirteen upregulated and three downregulated DEGs were related to stress tolerance (Figure 4B). Among these DEGs involved in disease resistance, a benzothiadiazole-induced rice blast resistance gene OsPibH8 [65], a salicylic acid methyltransferase gene OsBISAMT1 [66], and a glutathione S-transferase gene OsGSTU24 [67] showed suppressed expression in osf3h04g and CR-04g-12 (Figure 4B). However, a DMR6-like gene, OsS3H, which is a negative regulator in rice broad-spectrum resistance [29,30], had highly induced expression in osf3h04g and mildly induced in CR-04g-12 (Figure 4B).

Figure 4. RNA-seq analysis for common differentially expressed genes in two loss-of-function OsF3H04g rice lines. (A) Venn diagram for the upregulated and downregulated DEGs in the OsF3H04g T-DNA line (osf3h04g) and gene-editing line (CR-04g-12). The number of the common DEGs between osf3h04g and CR-04g-12 is marked with bold red text. (B) Heatmap of the common
DEGs involving disease resistance and stress resistance in osf3h04g and CR-04g-12. (C) The expression level of the common DEGs OsBISAMT1, OsPibH8, and OsGSTU24 in osf3h04g and CR-04g-12. OsACTIN was used as an internal control. Data represent means ± SD, n = 3.

2.5. The OsS3H Is Activated Expression by the Adjacent Enhancer Elements in osf3h04g

We found that the homologous OsS3H demonstrated a much more highly induced expression in the OsF3H04g T-DNA line than that in the gene-editing line. It was located downstream and genomic adjacent to OsF3H04g (Figure 5A). A previous report showed that the T-DNA vector, pGA2715, contained four enhancer elements of the 35S promoter, which may affect the expression of genes upstream and downstream of the T-DNA inserted position [64]. Thus, we checked the expression levels of Os04g49180, OsF3H04g (Os04g49194), OsS3H (Os04g49210), and Os04g49220, which were close to the T-DNA in osf3h04g (Figure 5A). The result showed that the expression of Os04g49180, which was upstream of the T-DNA, was not changed significantly in osf3h04g or in the OsF3H04g gene-editing lines (Figure 5B). OsF3H04g had a dramatically decreased expression in osf3h04g compared to no significant change in expression in the ZH11 gene-editing lines of CR-04g-11 and CR-04g-12 (Figure 5C). Surprisingly, OsS3H activated a high expression of about 250-fold in osf3h04g, which was downstream of the T-DNA in osf3h04g, but only increased expression by several folds in CR-04g-11 and CR-04g-12 (Figure 5D). The Os04g49220 gene, which was downstream of the T-DNA and OsS3H, showed about a three-fold induction in osf3h04g compared with DJ, and no significant change in expression was observed in the OsF3H04g gene-editing lines (Figure 5E). The abovementioned results indicate that a massive accumulation of OsS3H expression in osf3h04g is primarily caused by the quadruple 35S enhancer elements, which may also relate to the enhanced susceptibility to BLS and reduction in the SA level.

Figure 5. The OsS3H gene was activated expression by the T-DNA insertion in osf3h04g. (A) Structure diagram of the inserted T-DNA (pGA2715) and gene structure around T-DNA in the fourth chromosome of osf3h04g. Quadruple E indicates 35S enhancer elements. (B–E) Expression level of Os04g49180 (B), Os04g49194 (OsF3H04g) (C), Os04g49210 (OsS3H) (D), and Os04g49220 (E) genomic
adjacent to the T-DNA insertion position in osβh04g and OsF3H04g gene-editing lines (CR-04g-11 and CR-04g-12). OsACTIN was used as an internal control. Data represent means ± SD, n = 3.

2.6. OsS3H Plays a Negative Role in Rice Resistance to BLS and BB

The highly induced expression of OsS3H may be associated with increased susceptibility and decreased SA content in osβh04g. Therefore, to clarify whether the induction of OsS3H is related to the susceptibility, we generated the OsS3H overexpression (OE) rice lines. Two OsS3H OE lines (S3HOE-13 and S3HOE-22) of 15 individual transgenic rice lines were selected for further study. After inoculation with RS105, we found that the lesion symptoms in OsS3H OE lines were more severe than that in the wild-type ZH11 (Figure 6A). As shown in Figure 6B, the lesion lengths in S3HOE-13 (2.74 ± 0.20 cm) and S3HOE-22 (2.86 ± 0.22 cm) were significantly longer than that of 2.16 ± 0.20 cm in ZH11. Furthermore, the increased lesion lengths were consistent with the constitutively enhanced expression of OsS3H in S3HOE-13 and S3HOE-22 (Figure S5). After that, we also performed inoculation with Xoo strain PXO99 in OsS3H OE lines. The result showed that longer lesion lengths caused by PXO99 were observed in S3HOE-13 and S3HOE-22 than those in ZH11 (Figure S6A,B). In addition, we also measured the SA content in the OsS3H OE lines. Compared with ZH11, the SA content was decreased by over 50 percent in both S3HOE-13 and S3HOE-22 (Figure S7). We then quantified the gene expression of OsPR1b, OsNPR3, and OsWRKY45 in these lines, which showed that these SA-related genes had decreased expression in S3HOE-13 and S3HOE-22 compared with ZH11 (Figure S8A). The abovementioned results demonstrate that the overexpression of OsS3H results in a reduction in SA content and more susceptibility to BLS and BB in rice.

Figure 6. OsS3H negatively regulated rice resistance to BLS. (A,B) Image of lesion expansions (A) and diagram of lesion lengths (B) in ZH11 and the OsS3H OE lines (S3HOE-13 and S3HOE-22) caused by RS105 at 14 dpi. Data represent means ± SD, n = 10. Asterisks indicate significant differences between ZH11 and the OsS3H OE lines. (** p ≤ 0.01, Student’s t-test). (C,D) Image of lesion expansions (C) and diagram of lesion lengths (D) in ZH11 and the OsS3H gene-editing lines (CR-s3h-8 and CR-s3h-15) at 14 dpi with RS105. Data represent means ± SD, n = 10. Asterisks indicate significant differences between ZH11 and the OsS3H gene-editing lines. (** p ≤ 0.01, Student’s t-test).
We also performed the gene-editing of OsS3H in ZH11 rice. Among the OsS3H gene-editing lines, two of them were identified as an “A” base and as a “T” base inserted in the genome of CR-s3h-8 and CR-s3h-15, respectively (Figure S9). Then, we performed inoculation with RS105 in CR-s3h-8 and CR-s3h-15. The results showed that the symptoms in CR-s3h-8 and CR-s3h-15 developed more slowly than that in ZH11 (Figure 6C). The lesion lengths were 1.34 ± 0.15 cm and 1.41 ± 0.14 cm in CR-s3h-8 and CR-s3h-15, respectively, which were apparently shorter than that of 2.08 ± 0.17 cm in ZH11 (Figure 6D). Furthermore, we also performed inoculation with PXO99 in these gene-editing lines. Compared with ZH11, lighter disease symptoms and shorter lesion lengths in CR-s3h-8 and CR-s3h-15 were observed (Figure S6C,D). Then, we also performed the quantification of SA content in the OsS3H gene-editing lines. The results showed that a significantly increased SA level was displayed in CR-s3h-8 and CR-s3h-15 compared with ZH11 (Figure S7). After that, the SA-related genes were examined in terms of expression level in the OsS3H gene-editing lines. The expression levels of OsPR1b, OsNPR3, and OsWRKY45 were all enhanced several times in CR-s3h-8 and CR-s3h-15 compared to those in ZH11 (Figure S8B). Thus, our results indicate that OsS3H plays a negative role in the regulation of SA content and the defense response to BLS and BB in rice.

2.7. Gene-Editing Both OsF3H04g and OsS3H Neutralizes Rice Resistance to BLS

The gene-editing of OsF3H04g showed increased susceptibility to BLS and induced expression of OsS3H; however, the overexpression of OsS3H reduced rice resistance, and the gene-editing of OsS3H resulted in increased rice resistance. To verify whether the induction of OsS3H expression in the OsF3H04g gene-editing rice line was related to the increased susceptibility, we generated the double gene-editing line of OsF3H04g and OsS3H rice in the ZH11 background. Two OsF3H04g and OsS3H double gene-editing lines named CR-04g&s3h-1 and CR-04g&s3h-11 were both identified as a “T” base deletion in the U3-gRNA target region in the OsF3H04g genome, and as a “T” base insertion in the U6a-gRNA target region in the OsS3H genome (Figure S10). Then, we simultaneously performed inoculation with RS105 in CR-04g&s3h-1 and CR-04g&s3h-11, as well as in the OsF3H04g or OsS3H single gene-editing lines as a parallel. The results showed that the lesion extensions and lesion lengths in CR-04g-11 and CR-04g-12 were more rapid and longer than that in wild-type ZH11, and CR-s3h-8 and CR-s3h-15 were significantly slower and shorter than that in ZH11 as described above; however, the lesion extension and lesion lengths in CR-04g&s3h-1 and CR-04g&s3h-11 were in an intermediate level between the OsF3H04g and OsS3H single gene-editing lines but still slower and shorter than that in ZH11 (Figure 7A). Thus, the abovementioned results indicate that the double knock-out of OsF3H04g and OsS3H neutralizes the increased susceptibility to BLS for the OsF3H04g single gene-editing line.
Figure 7. Gene-editing of OsS3H neutralized the rice susceptible to BLS mediated by the knock-out of OsF3H04g. (A) Image of lesion expansions and diagram of lesion lengths in ZH11, OsF3H04g gene-editing lines (CR-04g-11 and CR-04g-12), OsS3H gene-editing lines (CR-s3h-8 and CR-s3h-15), and OsF3H04g and OsS3H double gene-editing lines (CR-04g&s3h-1 and CR-04g&s3h-11) in the ZH11 background at 14 dpi with RS105. (B) Image of lesion expansions and diagram of lesion lengths in the OsF3H04g/OsSAH2 gene-editing line (sah2), OsF3H04g/OsSAH2, and OsS3H/OsSAH3 double gene-editing line (sah2&sah3) in the ZH17 background at 14 dpi with RS105. Data represent means ± SD, n = 10. The letters on the columns mean significant differences at a value of \( p \leq 0.05 \) (LSD test).

A previous study showed that the gene-editing of OsF3H04g/OsSAH2 and OsS3H/OsSAH3 increased resistance to rice blast, sheath blight, and BB in the ZH17 background [29]. Here, we also performed inoculation with Xoc strain RS105 in the OsF3H04g/OsSAH2 single gene-editing line, as well as the OsF3H04g/OsSAH2 and OsS3H/OsSAH3 double gene-editing line in the ZH17 background, which showed that the gene-editing of OsF3H04g/OsSAH2 also slightly increased susceptibility to BLS, while the gene-editing of OsF3H04g/OsSAH2 and OsS3H/OsSAH3 markedly increased resistance to BLS (Figure 7B). Then, we performed inoculation with PXO99 in the OsF3H04g and OsS3H double gene-editing lines in the ZH11 background. The result showed that the gene-editing of OsF3H04g in the ZH11 background had no significant effect on rice resistance to BB, while the double gene-editing of OsF3H04g and OsS3H increased rice resistance to BB, and the degree of increased resistance in the double gene-editing lines was still less than that in OsS3H gene-editing lines (Figure S11). Our results indicate that the gene-editing of OsF3H04g in ZH11 and ZH17 rice varieties increases susceptibility to BLS, which could be neutralized by the knock-out of OsS3H.

3. Discussion

DMR6 and DMR6-like genes were previously found as downy mildew susceptibility genes in Arabidopsis [18,19]. They usually function as SA hydroxylase to repress SA-mediated defense [20,21]. Recently, several DMR6-like genes have been identified in potatoes [23], tomato [24], sweet basil [25], banana [26], grapevine [68], and rice [27–31], that enhance resistance to pathogens after the loss of function in corresponding plants. In this study, we identified the loss function of a rice DMR6-like gene OsF3H04g for either T-DNA insertion or gene-editing, which unexpectedly resulted in increased susceptibility to BLS. However, the OsF3H04g T-DNA activation tagging line showed increased susceptibility to BLS and extremely decreased SA content compared to that of the gene-editing
rice lines. We also revealed that another DMR6-like gene, OsS3H, located downstream of OsF3H04g, showed a highly induced expression in the T-DNA line, which was mainly caused by the insertion of the quadruple enhancer elements in the T-DNA sequence. OsS3H also has a slightly induced expression in the gene-editing lines by an unknown regulation. We then confirmed that OsS3H had a negative role in rice defense against Xoc, and gene-editing of both genes only partially recovered rice resistance to BLS. Consequently, our results revealed an abnormal case of the DMR6-like gene in rice susceptible to BLS, of which the loss of function could regulate the expression of a genomic downstream homolog to supplement the SA metabolism.

Recent studies have shown that the gene-editing of OsF3H04g/OsSAH2/OsS3H12 increased resistance to BB and blast in rice varieties ZH17 and Nipponbare [29,31]. However, in this study, we revealed that the loss function of this gene increased susceptibility to BLS in rice varieties DJ and ZH11 (Figures 1 and S4). We also found that the gene-editing of OsSAH2 in the ZH17 background slightly increased susceptibility to BLS (Figure 7). In addition, the OsF3H04g T-DNA line in the DJ background was more susceptible to BB, which may predominantly relate to a great induction of OsS3H. The OsF3H04g gene-editing lines of DJ also showed increased susceptibility to BB, while the OsF3H04g gene-editing lines of ZH11 showed no significant effect on susceptibility to BB (Figures 1, S4, and S11). Thus, we propose that the gene-editing of OsF3H04g has a distinct role in rice defense against BLS and BB, as well as a diversity of susceptibility to BB in different rice backgrounds. We observed that the backgrounds of rice varieties used for the generation of OsF3H04g knock-out lines are different. Comparing analyses from two recent publications, the rice variety Nipponbare is more resistant to Xoo PXO99, causing a shorter lesion length than ZH17 [29,31]. We observed that the gene-editing of OsF3H04g in Nipponbare enhanced resistance to BB more than rice variety ZH17 [29,31]. Also, the different M. oryzae strains used in the two studies showed minutely increased resistance to rice blast compared with the highly increased resistance in the ZH17 background [29,31]. Herein, the gene-editing of OsF3H04g in ZH11 showed reduced resistance to BLS, while no significant impact on resistance to BB was observed (Figures 3 and S11). Thus, we consider that the loss-of-function OsF3H04g in different rice backgrounds has diverse roles in rice resistance to different pathogens.

The T-DNA vector pGA2715 carries quadruple cauliflower mosaic virus 35S enhancer elements, which are used for the generation of activation tagging lines. Usually, the insertion caused by this vector activates the expression of upstream or downstream genes flanking the insertion site in rice [64,69]. Here, we considered that the T-DNA activation tagging line osf3h04g was different from gene-editing lines. It is easy to understand that the insertion of the enhancer traps the knock-out of OsF3H04g and boosts the expression of OsS3H to accelerate the SA metabolism. The results showed that OsS3H induced hundreds of folds in osf3h04g (Figure 5). The SA level was reduced more than 10 times in osf3h04g and was reduced by about half in the OsS3H overexpression lines (Figures 2 and S4). However, we found that OsS3H had a slightly induced expression in the OsF3H04g gene-editing lines (Figure 5D), as well as a slightly induced expression compared to that previously reported in ZH17 [29]. Consistent with the results, the slightly induced expression of OsS3H decreased about 30 percent of the SA content in the OsF3H04g gene-editing lines (Figures 2 and S4). Alternatively, the PR genes, including OsPR1b, OsNPR3, and OsWRKY45, were downregulated in the osf3h04g and OsS3H overexpression lines but not in the OsF3H04g gene-editing line (Figures 2 and S7). Thus, we concluded that there are two ways to increase OsS3H expression: the enhancer trapping induction and an unidentified mechanism that slightly induces the expression. We speculate that the slight induction of OsS3H in the loss-of-function OsF3H04g rice lines may relate to the feedback of the elevated SA level when one of the SA hydroxylases is broken one explanation, and the endogenous transcriptional regulation of these SA hydroxylase family genes to balance SA metabolism in rice may be another explanation.
Rice contains four DMR6-like genes, which include OsF3H03g/OsSAH1/OsS5H2 (LOC_Os03g03034), OsF3H04g/OsSAH2/OsS5H3 (LOC_Os04g49194), OsS3H1/OsSAH3/OsS5H1 (LOC_Os04g49210), and OsSAH4/OsS5H4 (LOC_Os10g39140). OsF3H03g and OsS3H, the main S5H in rice, are negative regulators in the SA-mediated defense response of rice to diverse pathogens [27–31]. Previously, we reported that the overexpression of OsF3H04g decreased SA content and increased susceptibility to BLS [27]. However, the loss-of-function OsF3H04g also resulted in increased susceptibility to BLS in this study (Figures 1 and 3). Interestingly, in the OsF3H04g gene-editing lines, OsS3H showed an increased expression. Moreover, we also confirmed that the overexpression of OsS3H increased rice susceptibility to BLS in wild-type ZH11 rice. We found that the gene-editing of OsS3H increased rice resistance to BLS; however, the gene-editing both of OsF3H04g and OsS3H did not recover the whole resistance to BLS, nor did the OsS3H gene-editing lines (Figure 7). This may indicate that the induction of OsS3H in the OsF3H04g gene-editing lines only partially contributes to the BLS susceptibility. There may be alternative enhanced susceptibility mechanisms to be identified in the future. We observed that chemicals and M. grisea response genes, OsPibH8 [65] and OsBISAMT [66], were downregulated in the loss function of the OsF3H04g lines. Several hormone-related genes, including abscisic acid-related genes OsNCED1, OsPsbR1, and OsbHLH120; ethylene-related genes OsACO1 and OsACO2; and gibberellin-related genes OsGA2ox4, OsYSL2, and OsMYBAS1, commonly showed changed expressions (Figure 4). In addition, most of the common DEGs in the loss function of OsF3H04g lines had an unknown function. Future work may focus on the other components participating in BLS susceptibility mediated by the loss-of-function OsF3H04g in rice.

4. Materials and Methods

4.1. Plant Materials

The OsF3H04g T-DNA line, osf3h04g (PFG_3A-05627.L), was inserted with an activation tagging vector pGA2715 in Oryza sativa L. ssp. Japonica cv. DJ, which was obtained from the rice T-DNA insertion sequence database [64]. Japonica cv. ZH11 was used for generating CRISPR-cas9-mediated gene-editing and overexpression of rice lines. The OsF3H04g/OsSAH2 gene-editing rice line sah2, OsF3H04g/OsSAH2, and OsS3H/OsSAH3 double gene-editing rice line sah2&sah3 in Japonica cv. ZH17 background was provided by Dr. Xujun Chen [29]. A growth chamber with a temperature of 28 ± 2 °C and a 14/10 h cycle of light/dark was used for rice cultivation. The rice lines, including wild-type and transgenic rice, were grown in the same pot filled with soil.

4.2. Pathogen Inoculation

The Xoc strains RS105 and HGA4 were used for BLS disease measurement, and the Xoo strain PX099 was used for BB disease evaluation, according to a previous report [28]. Briefly, the OD600 = 0.5 bacterial cell cultures of Xoc and Xoo strains were resuspended in sterile water, and inoculation was performed by injection with a non-needle syringe and the leaf-clipping method, respectively. The rice growth at 6 weeks after the seeding and inoculation stage was used to perform inoculation with Xoc and Xoo, respectively.

4.3. DNA Manipulation

Total plant genomic DNA was isolated through CTAB, as described in a previous report [70]. The genomic DNA of osf3h04g was used for amplification with specific primer pairs provided by SIGnAL-RiceGE (http://signal.salk.edu/cgi-bin/RiceGE, accessed on 10 June 2015) to identify the homozygous gene. To identify the OsF3H04g and OsS3H gene-editing lines, the fragments, including the gRNA target sites, were amplified with specific primers from the genomic DNA of the gene-editing lines. The primers are listed in Table S4.
4.4. RNA-seq and qRT-PCR

According to a previous report [52], 8-week-old rice leaves of DJ and osf3h04g were collected to isolate total RNA by TRlzol reagent. Two individual RNA repeats of each rice line were subjected to library construction and RNA sequencing by BGISEQ 500 (Beijing Genomics Institution, Shenzhen, China). The accession number for the raw sequence reads was submitted to the NCBI Sequence Read Archive (SRA). The significant DEGs between DJ and osf3h04g were determined by absolute log2-ratio values ≥1 and p ≤ 0.05. Gene ontology (GO) analysis for the DEGs was implemented through the software of clusterProfiler 4.0. The above total RNA of DJ and osf3h04g rice were used as templates to generate cDNA by MonScript™ RTIII All-in-One Mix with dsDNase (Monad, Wuhan, China). Subsequently, the cDNAs were performed qRT-PCR with MonAmp™ SYBR® Green qPCR Mix (Monad, Wuhan, China) by a CFX 96 system (BIO-RAD, Hercules, CA, USA). The primers used for qRT-PCR are listed in Table S4.

The RNA sequencing for the OsF3H04g gene-editing line (CR-04g-12) was performed with triple repeats as a parallel experiment with ZH11 and OsF3H04g overexpression lines according to the previous reports [27,28]. The accession number (PRJNA948698) of the raw sequence reads for CR-04g-12 was submitted to NCBI SRA. The DEGs were selected by the absolute log2-ratio values ≥1 and p ≤ 0.05 between CR-04g-12 and ZH11 (raw sequence reads of ZH11 were previously uploaded with the accession number PRJNA730674). The common and specific DEGs between osf3h04g and CR-04g-12 were identified by Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/index.html, accessed on 3 February 2021). The gene function analysis for the common DEGS was performed on Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase, accessed on 22 February 2021). Expression level validation of the common DEGs was performed through qRT-PCR, as described above.

4.5. Gene-Editing and Overexpression Rice Construction

The gRNA target sequences, including U3 (5’-GCTCATCGACCTCGCCTCGC-3’) for OsF3H04g, were designed in E-CRISP (http://www.e-crisp.org/E-CRISP, accessed on 5 April 2018). The U3- and U6a-gRNA expression cassettes for OsF3H04g were cloned into the binary vector pYLCRISPR/Cas9-MH to generate pCas9-OsF3H04g according to the previous report [71]. To construct OsS3H overexpression rice, a 1059 bp CDS fragment of the OsS3H gene was amplified from ZH11 rice cDNA and then cloned into pCXUN-HA to generate the pUN-S3H vector. To construct OsS3H gene-editing rice, the U3 target sequence (5’-GCGCGGCGATTCGCCTCGC-3’) for OsS3H was designed in E-CRISP, and then the U3-gRNA expression cassette was formed to insert into pYLCRISPR/Cas9-MH to generate the pCas9-S3H vector. To construct OsF3H04g and OsS3H double gene-editing rice, the U3- and U6a-gRNA expression cassettes carried with target sequences for OsF3H04g and OsS3H, respectively, were connected and cloned into the pYLCRISPR/Cas9-MH to generate pCas9-F3H04g-S3H vector. The abovementioned vectors were individually transformed into Agrobacterium tumefaciens EHA105 by electroporation, and then the transformants were performed. The A. tumefaciens-mediated rice transgenic operation was performed according to a previous report [72].

4.6. Determination of Salicylic Acid

The osf3h04g and the wild-type DJ, OsF3H04g gene-editing lines (CR-04g-11 and CR-04g-12), and the wild-type ZH11 were grown for 6 weeks. Then, the leaves were collected for the quantitative analysis of SA content. The abovementioned tissue was ground into powder in surrounding liquid nitrogen, and 100 mg leaves powder per repeat, and three repeats per line were used for hormone extraction. The SA content was determined using the liquid chromatography-mass spectrometry (LC-MS) system (Waters, Milford, MA, USA), according to previous reports [27,28].
5. Conclusions

In this research, we revealed that the loss function of a rice DMR6-like gene, OsF3H04g, increased rice susceptibility to BLS and BB, which is in contrast to the results of previous studies. The loss of function of OsF3H04g, mediated by the insertion of enhancer elements or by gene-editing to enhance BLS susceptibility, was mainly or partially dependent on the activation of the expression of a downstream and genomically adjacent homolog OsS3H. Our research provides an exhaustive understanding of the rice DMR6-like family genes in resistance to different pathogens, which may provide guidance in breeding for broad-spectrum resistance in rice by selectively blocking the DMR6-like genes.

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Data Availability Statement: Sequence data from this study can be found on the Rice Genome Annotation Project website (https://rice.plantbiology.msu.edu/, accessed on 13 February 2019) and NCBI (https://www.ncbi.nlm.nih.gov/, accessed on 7 December 2020) under the following accession number: OsF3H04g (LOC_Os04g49494), OsS3H (LOC_Os04g49210). Raw sequence reads of transcriptome sequencing for DJ, osf3h04g, and CR-04g-12 were performed in this study and uploaded to SRA to achieve the accession number (PRJNA983096 and PRJNA948698).

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

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