Phytopathology Research

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A virus-derived small RNA targets the rice transcription factor ROC1 to induce disease-like symptom

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Abstract

Virus-derived small interfering RNAs (vsiRNAs) in plants can target host transcripts to induce symptom development. Southern rice black-streaked dwarf virus (SRBSDV) is the most damaging rice-infecting virus, which causes severe stunting and poorly developed roots in rice plants, thereby posing a serious threat to rice production. In this study, we show that a vsiRNA (vsiR-S9-18), generated from SRBSDV genome segment 9, targets the transcription factor ROC1 in rice. SRBSDV infection triggered the production of vsiR-S9-18 and resulted in the downregulation of *ROC1* in rice plants. Transient expression of vsiR-S9-18 in rice protoplasts and tobacco leaves further demonstrated the specific association of vsiR-S9-18 with *ROC1*. Moreover, the *ROC1*-knockout rice plants displayed shortened roots, similar to the phenotype of root growth inhibition caused by SRBSDV infection. We propose that vsiR-S9-18 inhibits root elongation via *ROC1*, which has been proven to regulate root growth. This finding extends our understanding of the role of vsiRNA in viral disease development and is helpful for the development of new antiviral approaches.

Keywords: SRBSDV, vsiRNA, *ROC1*, Symptom formation

Background

Plants can protect themselves from viral infection through RNA silencing. Virus-derived small interfering RNAs (vsiRNAs) trigger this antiviral immunity, which results in the specific silencing of viruses (Ding and Voinnet 2007). When viruses invade plant cells, primary vsiRNAs are produced by Dicer-like (DCLs) cleavage of double-stranded viral replication intermediates or single-stranded viral RNA with secondary structures. In eukaryotes, including plants, RNA-dependent RNA polymerases (RDRs) can convert the targeted viral transcripts into dsRNAs, which are the substrates for DCLs processing, to generate secondary vsiRNA. Secondary vsiRNAs play an enhanced role in RNAi, as well as in signal transduction (Himber et al. 2003; Molnar et al. 2011).

Similar to the regulatory mechanism of miRNAs, vsiRNAs induce disease symptoms by silencing physiologically important host genes (Li et al. 2016; Lan et al. 2018). Two pioneering works report that cucumber mosaic virus (CMV) Y-sat-derived siRNAs are involved in the development of yellowing symptom via downregulating the expression of the tobacco magnesium protoporphyrin chelatase subunit I (CHLI) (Shimura et al. 2011; Smith et al. 2011). The vsiRNAs derived from barley yellow dwarf virus (BYDV) can also target the host chlorophyll biosynthesis gene (CHLG) to induce decreased production of chlorophyll, resulting in wheat leaf yellowing symptoms (Shen et al. 2020). A rice stripe virus (RSV)-derived vsiRNA can target eukaryotic translation initiation factor 4A (*eIF4A*) to cause leaf-twisting and stunting

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symptoms in rice plants (Shi et al. 2016). Chinese wheat mosaic virus (CWMV)-derived small interfering RNA (vsiRNA-20) can regulate the mRNA accumulation of wheat *Vacuolar (H+)-PPases* (*TaVP*) to suppress cell death and maintain a weak alkaline environment in the cytoplasm to enhance CWMV infection in wheat (Yang et al. 2020). Taken together, these findings highlight the important role of vsiRNAs in virus-plant interactions.

Southern rice black-streaked dwarf virus (SRBSDV), a member of the genus *Fijivirus* in the family *Reoviridae*, has caused severe viral disease outbreaks in China and other Asian countries (Zhou et al. 2013). The SRBSDV genome contains 10 segments of double-stranded RNA, ranging in length from 1.8 kb to 4.5 kb. It encodes 13 proteins, among which P1–P4, P8, and P10 are structural proteins and others are nonstructural proteins (Zhou et al. 2008; Wang et al. 2010). Rice plants infected with SRBSDV exhibit severe symptoms, including dwarf, dark green leaves, and root dysplasia (Zhou et al. 2008, 2013). However, it remains unclear how viral infection causes these symptoms. Our previous studies showed that rice plants infected with SRBSDV produce many vsiRNAs, and we analyzed the distribution of vsiRNAs in the viral genome. Meanwhile, we also predicted that these vsiRNAs may target host genes to regulate their expression.

Rice outermost cell-specific gene 1 (*ROC1*) is a GL2-type homeobox transcription factor expressed in the protoderm. The expression of *ROC1* in the outermost cells is established shortly after fertilization, much earlier than protoderm differentiation. *ROC1* expression could be induced in newly produced outermost cells when a callus-cutting experiment was performed (Ito et al. 2002). Although there is no evidence that *ROC1* can regulate the development of rice roots, its expression pattern and mode of function suggest that it may be involved in the processes.

In this study, we identified and verified a rice host gene *ROC1*, which is the target of the SRBSDV-derived vsiRNA, vsiR-S9-18. When vsiR-S9-18 was transiently expressed in vivo, the *ROC1* mRNA level was downregulated. Interestingly, the *ROC1*-knockout plants exhibited a shortened root length, similar to the phenotype of stunted root induced by SRBSDV infection, suggesting that vsiR-S9-18 might contribute to stunted root development through targeting the host *ROC1*.

Results

Identification of vsiR-S9-18 in SRBSDV-infected rice plants and the prediction of its target gene

Based on the small RNA sequencing data from SRBSDV-infected rice plants, we obtained a long list of small RNAs, 1.72% of which were derived from the viral genome (Li et al. 2018). One vsiRNA derived from the ninth segment of the SRBSDV genome, referred to as vsiR-S9-18, was predicted to target the 3′ UTR of a rice gene *LOC_Os08g08820* (Fig. 1a). *LOC_Os08g08820* encodes ROC1, a transcription factor belonging to the class IV homeodomain leucine zipper (C4HDZ) family (Zalewski et al. 2013).

Accumulation of vsiR-S9-18 and its target gene *ROC1* in SRBSDV-infected rice plants

To detect the accumulation of vsiR-S9-18, we used stem-loop reverse transcription quantitative PCR (RT-qPCR) to examine the dynamic change in vsiR-S9-18 during SRBSDV infection. The results showed that the accumulation of vsiR-S9-18 increased gradually as the viral infection progressed (Fig. 1b). Next, we explored the expression of the target gene of vsiR-S9-18 and found that the expression of *ROC1* was significantly decreased in SRBSDV-infected plants at 40 days post-inoculation (dpi) compared with that in mock-inoculated controls (Fig. 1c). To explore the expression pattern of *ROC1* in rice tissues, RT-qPCR was performed, and the results showed that *ROC1* was most highly expressed in stem tissues (Fig. 1d).

vsiR-S9-18 downregulates the expression of *ROC1*

To investigate how vsiR-S9-18 regulates *ROC1* during SRBSDV-host plant interaction, an amiRNA vector, pACTIN:vsiR-S9-18, was constructed using the rice miR528 precursor as a backbone. We then constructed 35S:LUC-3′UTR^{ROC1} by ligating the 3′ UTR of ROC1 to the 3′ end of the luciferase gene. ROC1-M contained synonymous substitutions in the vsiR-S9-18-targeting site in 3′ UTR and was used to construct 35S:LUC-3′UTR^{ROC1-M} (negative control). Plasmid 35S:LUC-3′UTR^{ROC1} or 35S:LUC-3′UTR^{ROC1-M} was co-infiltrated into *N. benthamiana* leaves with pACTIN:00 (empty vector, EV), or pACTIN:vsiR-S9-18, followed by in vivo fluorescence imaging. It was shown that both 35S:LUC-3′UTR^{ROC1} and 35S:LUC-3′UTR^{ROC1-M} were successfully expressed in *N. benthamiana* leaves, while vsiR-S9-18 expression significantly inhibited fluorescence signals produced by LUC-3′UTR^{ROC1} but not by LUC-3′UTR^{ROC1-M} (Fig. 2a, b). These results indicate that vsiR-S9-18 can specifically regulate 3′ UTR-mediated *ROC1* decay in plant cells.

vsiR-S9-18 induces downregulated expression of *ROC1* in rice protoplasts

To further investigate the regulatory effect of vsiR-S9-18 on its target gene *ROC1* in rice cells, pACTIN:vsiR-S9-18 was transformed into rice protoplast and the expression level of *ROC1* was detected by RT-qPCR. Compared to the EV-transformed cells, overexpression of vsiR-S9-18

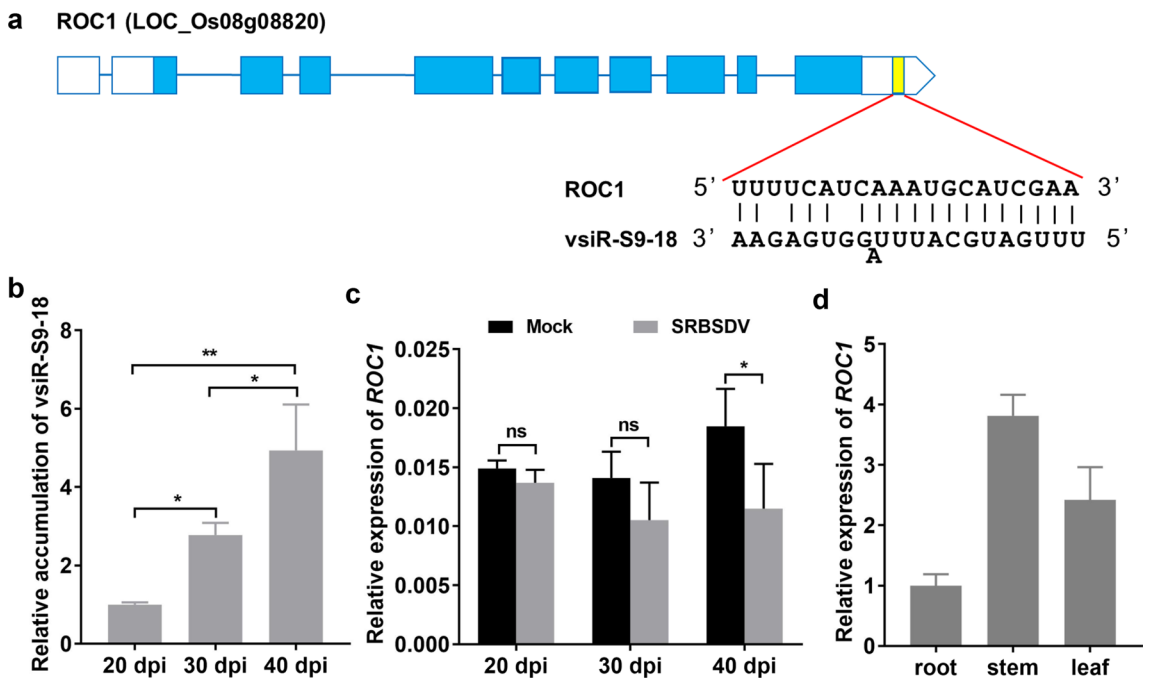

Fig. 1 Accumulation of vsiRNA and its target gene in rice plants inoculated with SRBSDV. **a** vsiRNA-S9-18 and its target gene *ROC1*. **b** RT-qPCR analysis of vsiR-S9-18 accumulation in rice plants at 20, 30, and 40 dpi. *OsU6* was used as an internal reference. **c** RT-qPCR analysis of *ROC1* expression in mock- or SRBSDV-inoculated rice plants at 20, 30, and 40 dpi. **d** Expression pattern analysis of *ROC1* in different rice tissues through RT-qPCR. The values shown are the means ± SD (n = 3). Student's t test was used for the analyses (*$P < 0.05$)

significantly reduced the expression of *ROC1* (Fig. 3), indicating that vsiR-S9-18 targets *ROC1* in rice cells.

Subcellular localization patterns of ROC1

To investigate the subcellular localization of ROC1, we fused GFP to the N-terminus of ROC1 to produce the GFP-ROC1 construct, and expressed it in *N. benthamiana* leaves. GFP-ROC1 (green fluorescence) showed a strong co-localization with H2B-RFP (a nuclear marker, red fluorescence) (Fig. 4). These data indicate that ROC1 localizes in the nucleus of plant cells, which is consistent with its function as a transcription factor.

SRBSDV infection induces a similar phenotype to that of *roc1* mutants in root length

To investigate the roles of ROC1 in rice development and rice resistance against SRBSDV, we generated *ROC1*-knockout mutant rice lines via CRISPR/Cas9 technology (Additional file 1: Fig. S1). The *ROC1*-knockout rice lines (*roc1-1* and *roc1-2*) were as normal as the wild type (WT) in plant height and tiller number, but their root length was significantly shorter (Fig. 5a, b). Moreover, *roc1-1* and *roc1-2* showed more severe disease symptoms than WT plants when infected with SRBSDV (Fig. 5a). After SRBSDV infection, viral RNA and coat protein accumulation were higher in *roc1* mutants than in WT plants (Fig. 5c, d). Interestingly, mock-treated *roc1* mutants and SRBSDV-infected WT plants exhibited similar root lengths (Fig. 5a, b). Therefore, it is possible that SRBSDV-derived vsiR-S9-18 targets *ROC1* to subsequently induce root dysplasia.

The locus of vsiR-S9-18 is conserved in different SRBSDV isolates

To explore whether it is conservative for SRBSDV to regulate root development in rice plants through the vsiRNA-mediated mechanism, we analyzed the locus of vsiR-S9-18 in the SRBSDV genome. Thirty-three SRBSDV isolates were collected from different locations in China and Vietnam, and the sequences of all these isolates at the locus of vsiR-S9-18 were exactly the same (Fig. 6). This finding suggests that SRBSDV-derived vsiRNA plays a conserved role in regulating the root development of rice plants.

Discussion

A variety of antiviral defenses are activated in plants in response to virus infection (Calil and Fontes 2016; Huang et al. 2020). As one of the conserved mechanisms for regulating gene expression in plants, RNAi plays an important role in plants' antiviral processes (Ding and Voinnet 2007). Plants always produce a large number of

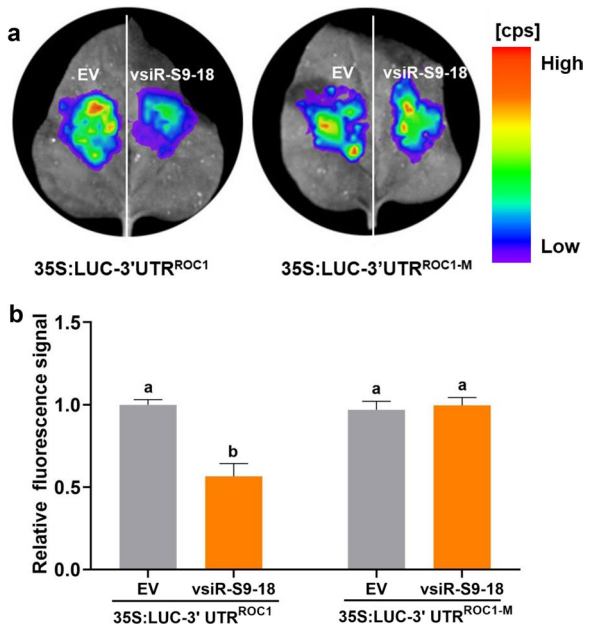

Fig. 2 vsiR-S9-18 specifically mediates mRNA degradation of ROC1 in tobacco cells. **a** Luciferase complementation imaging assay for the degradation of ROC1 in *N. benthamiana* leaves. Agrobacterium strain C58C1 harboring various combinations of the constructs was infiltrated into *N. benthamiana* leaves. At 72 h post-infiltration, luciferase activities were determined. Cps, signal counts per second. **b** Relative fluorescence signal of the samples in **a**. Values represent the mean signal counts ± SD per second. Two-way ANOVA followed by Tukey's multiple-comparison test was used for the analyses (*$P < 0.05$)

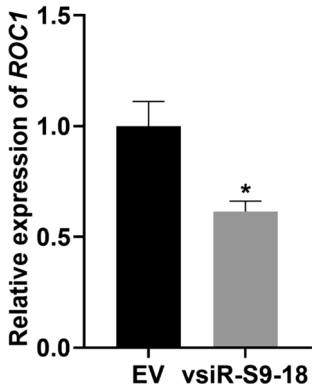

Fig. 3 RT-qPCR detection of *ROC1* expression in rice protoplasts. Total RNAs were extracted from the rice protoplasts expressing EV or vsiR-S9-18 plasmid at 16 h. The values shown are the means ± SD (n = 3). Student's t test was used for the analyses (*$P < 0.05$)

vsiRNAs when struggling with virus infection (Shi et al. 2016; Xu and Zhou 2017; Xia et al. 2018; Yang et al. 2020). In the arms race between the plant defense and virus counter-defense, vsiRNAs not only act as a plant weapon against viruses by mediating degradation of viral RNA or inhibiting transcription of viral genes, but also target host genes to facilitate virus infection. Increasing evidences have suggested that virus-derived siRNAs can silence host mRNAs to facilitate the development of disease symptoms, such as CMV- and BYDV-derived siRNAs targeting chlorophyll biosynthesis genes to generate leaf yellowing symptom, and RSV-derived siRNAs targeting eIF4A to cause leaf-twisting and stunting symptoms in plants (Shimura et al. 2011; Smith et al. 2011; Shi et al. 2016; Shen et al. 2020). It is believed that with the continuous development of next-generation sequencing technologies, more and more interactions between vsiRNAs and host genes will be revealed.

In this study, we found that the expression of the rice gene *ROC1* was downregulated by SRBSDV infection via SRBSDV-derived vsiR-S9-18. However, the transcript level of *ROC1* was only significantly reduced at 40 dpi, when the vsiRNA accumulated to a certain level. Our recent research found that the accumulation of SRBSDV increased gradually with the progress of the disease (Zhao et al. 2022). Therefore, we speculate that the SRBSDV-derived vsiRNA also increases with disease development, which has been proven by the stem-loop RT-qPCR analysis of vsiR-S9-18.

ROC1 is specifically expressed in protodermal cells in rice embryos and plays important roles in cell differentiation and stress resistance by serving as a transcriptional regulator (Ito et al. 2002; Zalewski et al. 2013). ROC4 positively regulates cuticular wax biosynthesis and the drought stress response (Wang et al. 2018). Another research study demonstrates that ROC4 promotes flowering time under long days (Wei et al, 2016). Overexpression of *ROC5* leads to adaxially rolled leaves, indicating that ROC5 negatively regulates bulliform cell fate and development (Zou et al. 2011). Moreover, it has been demonstrated that ROC5 and ROC8 interact to form a heterodimer to regulate the leaf rolling of rice plants (Xu et al. 2021). In this study, *roc1* mutants showed root dysplasia. Compared with WT plants, *roc1* mutants exhibited shorter root lengths, but had normal root numbers, suggesting that *ROC1* only affected root length and had no significant influence on the number of roots. In summary, our results showed that SRBSDV infection produced a phenotype similar to that caused by a mutation in the host gene, and this phenomenon could be related to the RNA silencing effect of vsiRNA.

Conclusions

In this study, we identified a *ROC1* gene in rice plants as the target gene of the SRBSDV-derived vsiR-S9-18. Mutation of *ROC1* in rice plants hampered root development

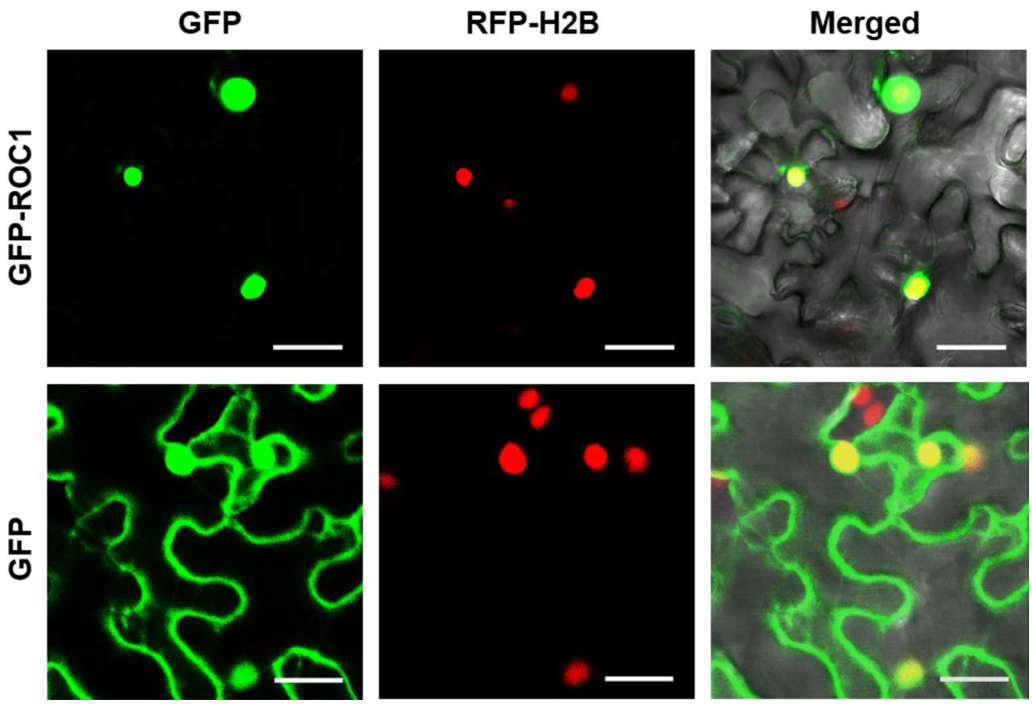

Fig. 4 Subcellular localization patterns of ROC1. GFP-ROC1 was expressed in RFP-H2B transgenic *N. benthamiana* leaves. The red fluorescence produced by RFP-H2B serves as a nuclear marker. Bar = 50 μm

and the *roc1* mutants showed a phenotype similar to that of SRBSDV-infected WT plants. These results suggest that SRBSDV-derived vsiR-S9-18 may regulate rice root development via targeting *ROC1*, which is involved in the formation of disease symptom.

Methods

Plant and virus materials

All rice plants used in this study are in the rice cv. Nipponbare background. The *roc1* mutant lines were generated using CRISPR/Cas9 technology at the Biogle Genome Editing Center, Jiangsu, China. Rice plants were grown inside a greenhouse maintained at 28–32 °C and 60 ± 5% relative humidity with natural sunlight, and *N. benthamiana* plants were grown inside a greenhouse maintained at 23 °C with a 12 h light/12 h dark photoperiod.

SRBSDV was maintained and inoculated as in our previous report (Zhao et al. 2022). Rice plants at the 3- to 4-leaf stage were inoculated with white-backed planthopper carrying SRBSDV.

Bioinformatics analysis of vsiRNAs and target gene prediction

Small RNAs with lengths of 18–30 nt were screened for bioinformatic analysis based on preprocessed raw data. To identify SRBSDV-derived vsiRNAs, clean reads were submitted to map the SRBSDV genome (accession number NC014712), and the matched small RNAs were considered as vsiRNAs. Selected vsiRNAs with reads counts over 30 were subjected to target gene prediction using psRNATarget (Dai et al. 2018).

RNA isolation and RT-qPCR

Total RNA was extracted using a Plant Total RNA extraction kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions. Quantitative PCR reactions were carried out on a CFX96 TouchTM real-time PCR detection system (Bio-Rad, Hercules, CA, USA) using the SYBR Premix Ex TaqTM II kit (Takara, Japan). Three independent biological replicates were used for each experimental group, each with three technical triplicates. For gene expression quantification, cDNA was synthesized using an oligo (dT) primer, and *EF1α* was used as an internal control for data normalization. Real-time quantification of vsiRNAs was performed using stem-loop RT-qPCR as previously described (Salone and Rederstorff 2015), and the expression level of vsiRNA was normalized to that of *U6*. Primers used for RT–qPCR are listed in Additional file 2: Table S1.

Vector construction

vsiRNA expression vector was constructed using the miR528-OE vector skeleton (kindly provided by Dr.

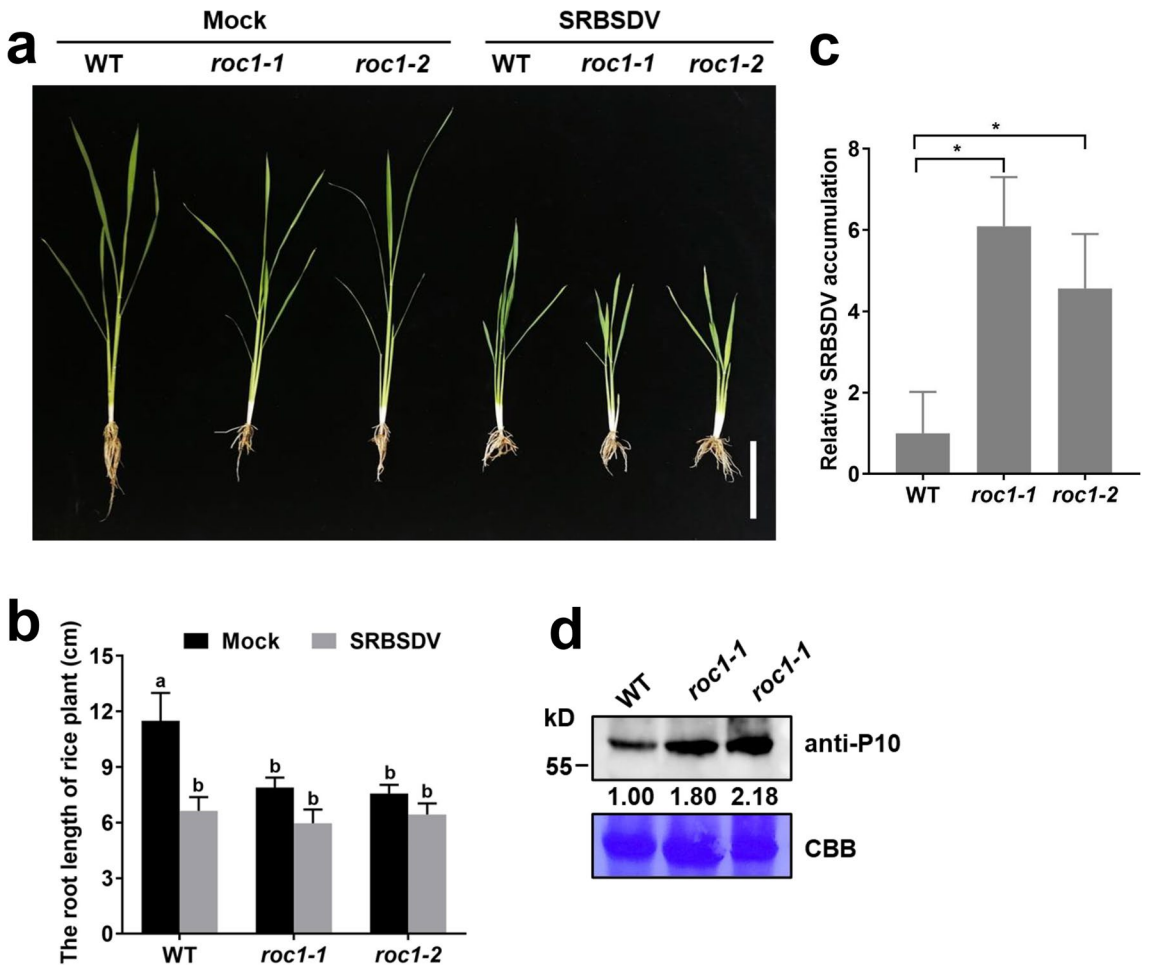

Fig. 5 Symptoms and accumulation of SRBSDV in *roc1* mutant lines and WT rice plants. **a** The symptoms caused by SRBSDV infection in WT and *roc1* plants. The plants were inoculated with viruliferous white-backed planthopper, and the photographs were taken at 20 dpi. Bars = 10 cm. **b** The average root length of WT and *roc1* rice plants. Inoculation experiments were repeated 3 times with 10 plants each. Values represent the mean root length ± SD from three independent experiments. One-way ANOVA followed by a multiple-comparisons Tukey's test was used for the analyses (**P* < 0.05). **c** RT-qPCR analysis of SRBSDV RNA accumulation in WT and *roc1* plants. Total RNAs were extracted from SRBSDV-infected leaves at 20 dpi. The values shown are the means ± SD (n = 3). Student's t test was used for the analyses (**P* < 0.05). **d** Western blot analysis of SRBSDV encoded P10 protein accumulation in WT and *roc1* plants. Total proteins were extracted from SRBSDV-infected leaves at 20 dpi and were detected by P10-specific antibodies

Xiaofeng Cao, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). The miR528 sequences were replaced by vsiRNA sequences via overlapping PCR. For construction of the ROC1 reporter vector, 35S promoter was cloned into pGWB435 using a Gateway LR reaction kit as instructed (ThermoFisher Scientific). Then, the 3'UTR sequence of *ROC1* was cloned into the *Sac*I site to generate 35S:LUC-3'UTR ^{ROC1}. The 3'UTR sequence with a mutated targeting site was cloned into the same site to generate 35S:LUC-3'UTR^{ROC1-M}. For construction of the subcellular localization vector, the coding sequence of *ROC1* was cloned into pCambia1300-GFP to generate GFP-ROC1. All these vectors were individually transformed into *Agrobacterium tumefaciens* strain EHA105, which was used to infiltrate the leaves of *N. benthamiana*.

Transfection of rice protoplasts

Rice protoplasts were isolated from 10-day-old rice seedlings and transfected as described previously (Zhang et al. 2011). The vsiRNA expression plasmids were transfected into rice protoplasts to test their effect on the target gene expression.

Fig. 6 The locus of vsiR-S9-18 in the genomes of different SRBSDV isolates

Statistical analysis

Each experiment reported in this study was repeated at least three times. SPSS 23.0 software was used to analyze the data, and the co-infiltration results with vsiRNA and LUC recombinant expression vector were analyzed by Student's t test.

Abbreviations

AGO: Argonaute protein; BYDV: Barley yellow dwarf virus; CHLI: Magnesium protoporphyrin chelatase subunit I; CMV: Cucumber mosaic virus; CWMV: Chinese wheat mosaic virus; C4HDZ: Class IV homeodomain leucine zipper; DCL: Dicer-like; eIF4A: Eukaryotic translation initiation factor 4A; EV: Empty vector; PepMV: Pepino mosaic virus; PMMoV: Peppermild mottle virus; RdRP: RNA dependent RNA polymerases; ROC: Rice outermost cell specific gene; RSV: Rice stripe virus; SRBSDV: Southern rice black-streaked dwarf virus; STV: Southern tomato virus; TaVP: Wheat Vacuolar (H+)-PPases; vsiRNA: Virus-derived small interfering RNA; WT: Wild type.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-022-00112-6.

Additional file 1: Fig. S1. Identification of *roc1* mutant rice lines generated by CRISPR/Cas9 via sequencing analysis. The red arrow points to the cleavage sites.

Additional file 2: Table S1. Primers used in this study.

Acknowledgements

We thank Dr. Xiaofeng Cao, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, for providing miR528-OE vector. We also thank Jingxian Xue for her excellent work in the growth chamber and laboratory maintenance.

Authors' contributions

GZ and TZ conceived the study. XH, FL, XZ, JC and JW performed the experiments. XH, FL, JW, XY and TZ analyzed the data and interpreted the results. XH, GZ and TZ took the lead in writing the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by the Natural Science Foundation of China (U1901204, 31871928, 32072388), the Postdoctoral Science Foundation of China (2021M691083), the Fok Ying Tung Education Foundation (171025), Guangdong Special Branch Plan for Young Talent with Scientific and Technological Innovation (2019TQ05N158), and Guangdong Provincial Innovation Team for General Key Technologies in Modern Agricultural Industry (2019KJ133).

Availability of data and materials

Not applicable.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Received: 24 January 2022 Accepted: 8 March 2022
Published online: 17 March 2022

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