ORIGINAL ARTICLE

CRISPR/Cas9-mediated mutagenesis of *Clpsk1* in watermelon to confer resistance to *Fusarium oxysporum* f.sp. *niveum*

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Abstract

Key message **CRISPR/Cas9-mediated editing of *Clpsk1* enhanced watermelon resistance to *Fusarium oxysporum*.**

Abstract The clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system has proven to be an effective genome-editing tool for crop improvement. Previous studies described that Phytosulfokine (PSK) signalling attenuates plant immune response. In this work, we employed the CRISPR/Cas9 system to knockout *Clpsk1* gene, encoding the PSK precursor, to confer enhanced watermelon resistance to *Fusarium oxysporum* f.sp. *niveum* (*FON*). Interactions between PSK and *FON* were analysed and it was found that transcript of *Clpsk1* was significantly induced upon *FON* infection. Meanwhile, application of exogenous PSK increased the pathogen growth. Then, one sgRNA, which targeted the first exon of *Clpsk1*, was selected for construction of pRGEB32-CAS9-gRNA-Clpsk1 expression cassette. The construct was then transformed to watermelon through *Agrobacterium tumefaciens*-mediated transformation method. Six mutant plants were obtained and three types of mutations at the expected position were identified based on Sanger sequencing. Resistance evaluation indicated that *Clpsk1* loss-of-function rendered watermelon seedlings more resistant to infection by *FON*. These results indicate that CRISPR/Cas9-mediated gene modification is an effective approach for watermelon improvement.

Keywords *Clpsk1* · CRISPR/Cas9 · Disease resistance · *Fusarium oxysporum* f.sp. *niveum* · Watermelon

Introduction

Watermelon [*Citrullus lanatus* (Thunb.) Matsum and Nakai] is an economically important fruit crop worldwide. Global production of watermelon suffers from various biotic or abiotic challenges. *Fusarium oxysporum*, caused by *Fusarium oxysporum* f.sp.*niveum* (*FON*), is one of the most devastating diseases affecting watermelon in the world. *FON* progresses along xylem vessels resulting in hollow and dried-out stems (Zhang et al. 2015). Severe infection of *FON* causes approximately 30–80% yield loss or even worse (Lü et al. 2011; Martyn and Netzer 1991). The majority of the commercialized watermelon varieties are susceptible to *FON*. Developing *FON*-resistant watermelon varieties are the most economical and effective approach to address this issue, however, the resistance breeding was difficult via traditional breeding due to limited *FON*-resistant germplasm (Tian et al. 2018).

Phytosulfokine (PSK), a disulfated pentapeptide plant hormone, is described to act in aspects of plant growth and development (Sauter 2015) as well as in the regulation of plant immunity (Hammes 2015). The expression of *PSK* precursor genes was induced by *Hyaloperonospora arabidopsidis* (*Hpa*) (Hok et al. 2011) and *B.cinerea* (Zhang et al. 2018) infection. *PSK2* and *PSK4*-overexpressing Arabidopsis exhibited more susceptible to downy mildew infection (Rodiuc et al. 2016). Down-regulation of *psk3* significantly reduced sporulation rate of *Hpa* in Arabidopsis (Rodiuc et al. 2016) but enhanced the susceptibility of tomato to *B.cinerea* (Zhang et al. 2018). Moreover, PSK signalling

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was also found to be involved in plant immunity during their interaction with bacterial pathogen (Igarashi et al. 2012), biotrophic and necrotrophic pathogens (Mosher et al. 2013), and soil-borne pathogens (Rodiuc et al. 2016; Shen and Diener 2013), demonstrating the novel roles for PSK signalling in plant-pathogen interactions.

Precise genome editing technologies are vital tools in crop improvement (Feng et al. 2013). The clustered regularly interspaced short palindromic repeats-associated protein 9 (CRISPR /Cas9) system revolutionized the genome editing which perform the target genome editing in organisms under the guidance of an engineered sequence-specific single guide RNA (sgRNA). To date, CRISPR /Cas9 system has been widely employed for germplasm improvement in plenty of plant species (Schaeffer and Nakata 2016), and has been demonstrated to be a powerful tool for improvement of disease resistance in Arabidopsis (Pyott et al. 2016), rice (Kim et al. 2019; Peng et al. 2017; Wang et al. 2016), Citrus (Wang et al. 2019) and cucumber (Chandrasekaran et al. 2016). In watermelon, an efficient CRISPR /Cas9-based gene knockout system was established (Tian et al. 2017) and was successfully utilized to generate herbicide-resistant watermelon germplasm (Tian et al. 2018). These works established a foundation to create disease-resistant germplasm in watermelon.

Here, the CRISPR/Cas9 system was employed for mutagenesis of *Clpsk1* gene in watermelon. Results showed that the CRISPR/Cas9 system successfully generated mutants at the target site, and the mutant lines displayed enhanced resistance to *FON*.

Materials and methods

Plant and pathogen materials

Watermelon line Sumi1 was used for transformation. Seeds of Sumi1 were surface-disinfected in 70% ethanol for 40 s, 10% sodium hypochlorite for 6 min and then rinsed three times with sterilized water. The sterilized seeds were then cultured in one-half strength Murashige and Skoog (MS) medium (1962) until germination in the dark at 25 °C in a growth chamber. *FON race 1* (*FON1*) was used for fungal inoculation. *FON1* was cultured on Potato Dextrose Agar (PDA) medium containing 50 mg/L streptomycin at 28 °C for 7 days. All materials used in this study were obtained from the Institute of Vegetable, Jiangsu Academy of Agricultural Sciences, Nanjing, Jiangsu province, China.

Infection of watermelon seedlings with *FON1*

FON1 spore suspension was prepared in 100 ml liquid potato dextrose agar (PDA) medium cultured at 25 °C on a rotary shaker at 150 rpm for 7 days. The concentration of *FON1* spore suspension was adjusted to 10^6 conidia ml^{-1} with sterilized water and used for plant inoculation.

Watermelon plants were grown in a growth chamber (16 h light: 8 h dark, 25 ± 1 °C, 70% humidity). 15-day-old seedlings were used for inoculation. Plants were removed gently from the growth medium (peatmoss:vermiculite:perlite 6:1:3, *v/v/v*) and washed in tap water. Roots were immersed in *FON1* spore suspension (10^6 conidia ml^{-1}) for 30 min and then replanted in the growth medium. Root samples were collected 0, 8, 12, 24, 48 h after inoculation and used for real-time quantitative PCR analysis. Three biological replicates were collected at each time-point.

Clpsk1 expression analysis by real-time quantitative PCR

Total RNA from root samples inoculated with *FON1* was extracted using the RNApure Plant kit with DNaseI (CWBiotech, Beijing, P. R. China) and reverse transcribed using the BU-Superscript RT kit (Biouniquer, Beijing, P. R. China) according to the manufacturer's instructions. qRT-PCR was performed using GeneAmp® 7300 Sequence Detection System (PE-Applied Biosystems, USA). The *18S rRNA* gene (GenBank accession no. AB490410) was used as internal control.

Vector construction

The vector pRGEB32 (Xie et al. 2015) was used for CRISPR/Cas9 expression vector construction. The sgRNAs were designed using the online tool CRISPRdirect (Naito et al. 2015). The sgRNA expression cassette driven by the AtU6 promoter and the CRISPR/Cas9 complex (pRGEB32-CAS9-gRNA-Clpsk1) were performed according to Xie et al. (2015) by Bio-cubes (Wuhan). The CRISPR/Cas9 expression vector was transferred into *Agrobacterium tumefaciens* strain EHA105 by freeze–thaw method.

Watermelon transformation

Watermelon transformation was carried out as previously described by Park et al. (2005). In brief, seeds of Sumi1 were surface-disinfected in 70% ethanol for 40 s, 10% sodium hypochlorite for 6 min and then placed on one-half strength MS medium in the dark at 25 °C for germination. Cotyledons from 3-day-old seedlings were excised to 1.5 mm × 1.5 mm pieces, followed by soaking in 10 mL MS solution containing 50 μL bacterial cell suspension (*Agrobacterium tumefaciens* strain EHA105 carrying pRGEB32-CAS9-gRNA-Clpsk1 constructs, $OD_{600} = 0.6 \sim 0.7$) for 15 min. The explants were transferred to co-cultivation medium (MS medium containing 1.0 mg L^{-1} 6-BA, 0.1 mg

L^{-1} IAA and 200 µM AS, pH 5.8) and cultured in the dark at 25 °C for 3 days. After co-cultivation, the explants were transferred to the shoot regeneration medium (MS medium containing 1.0 mg L^{-1} 6-BA, 0.1 mg L^{-1} IAA, 5 mg L^{-1} hygromycin and 400 mg L^{-1} Timentin, pH 5.8) and subcultured at 10-day intervals for 6–8 weeks. The regenerated shoots (~ 1 cm height) were transferred to the root induction medium (1/2 MS medium containing 0.1 mg L^{-1} 6-BA, 0.01 mg L^{-1} NAA, 5 mg L^{-1} hygromycin and 400 mg L^{-1} Timentin, pH 5.8) and incubated for 3–5 weeks. Gene-edited plants were confirmed by PCR using Cas9 specific primers, cas9-F: 5′-GTGCCCAGCAAGAAATTC-3′ and cas9-R: 5′-GATAGATCAGCCGCAGGTC-3′.

Identification of targeted mutant transgenic plants

Hygromycin resistant transgenic plants were analyzed by PCR using *Cas9* specific primers (same as that in "Watermelon transformation"), *hygromycin* gene-specific primers (hyg-F: 5′- CCATGTGTATCACTGGCAAACT-3′, hyg-R: 5′-TCCACTATCGGCGAGTACTTCT-3′) and *Clpsk1* gene (Cla97C01G016930 in Cucurbit Genomics Database) specific primers (Clpsk-F: 5′-CACACGCATCTCTGATCT CC-3′, Clpsk-R: 5′-CATTTCATATCAGCCGACTGATAC -3′). Watermelon genomic DNA was extracted from leaves of transgenic and non-transformed plants. PCR amplicons were directly sequenced using *Clpsk1* specific primers. Sequencing results were decoded using BioEdit software.

FON growth

The effect of exogenous PSK on fungal growth was determined. 50 µM PSK (Iris Biotech), 50 µM dPSK (ChinaPeptides) and dH$_2$O were added to PDA medium, respectively. A 5-mm fungal disk was transferred to PDA medium containing 50 µM PSK (Iris Biotech), 50 µM dPSK (ChinaPeptides) and dH$_2$O, respectively. The PDA plates were cultured for 3 days at 25 °C and the mycelium growth was measured. Three replicates were performed. Statistical analysis was performed using SAS 9.2 software (SAS Institute, Cary, NC).

Off-target analysis

Potential off-target sites were identified against watermelon genome on the CRISPR-P 2.0 Web tool (https://crispr.hzau. edu.cn/CRISPR2/) (Liu et al. 2017). Blast hits containing potential off-target sequences (less than five mismatched bases and located in CDS region) were selected (Supplementary Table S2) and amplified using specific primers (Supplementary Table S2). PCR products were subjected for Sanger sequencing.

Susceptibility of gene-edited plants to *Fusarium oxysporum*

Gene-edited plants and non-transformed plants were grown in a greenhouse at a temperature of 28 °C with a 16 h light/8 h dark cycle at Jiangsu Academy of Agricultural Sciences. 15-day-old plants were used for *FON1* inoculation using the root dip method (Chang et al. 2008). Inoculated plants were kept at greenhouse. The number of dead plants were recorded every day and continued for 21 days. Disease reaction was rated based on wilt incidence where *HR* highly resistant ($\leq 20\%$), *MR* moderate resistant ($21 \sim 50\%$), *LR* light resistant ($51 \sim 80\%$), and *S* susceptible ($> 80\%$) according to the criteria established by Martyn and Bruton (1989).

Results

PSK positively interact with *FON*

To determine whether PSK signalling functions in the watermelon immune response to *FON*, we first tested the transcript abundance of *Clpsk1* gene in roots of Sumi1 inoculated with *FON*. Expression of *Clpsk1* was slightly induced in 12 h post inoculation (hpi) and then was found gradually up-regulated with time (Fig. 1a). The maximum level of *Clpsk1* occurred 48 hpi during the compatible interaction with *FON*. We then investigated the fungal growth by applying exogenous PSK. Application of 50 µM PSK promoted the fungal growth significantly when compared with the 50 µM dPSK and the control (Fig. 1b). It was suggested PSK signalling functions as defense suppression in PTI (van Shie and Takken 2014), we further tested whether down-regulation of PSK signalling renders watermelon more resistant to *FON* in the following experiment using CRISPR/Cas9.

Vector construction for the CRISPR/Cas9 system

Considering watermelon is dicot plant, the rice U3 promoter in the CRISPR/Cas9 vector pRGEB32 was replaced with the Arabidopsis U6 promoter, and the resulting pRGEB32-AtU6 was used for watermelon CRISPR/Cas9-mediated gene editing. One target site on *Clpsk1* gene was chosen based on CRISPRdirect, including a 20-bp nucleotide sequence with protospacer adjacent motif (PAM) on its 5′-region in the first exon of *Clpsk1* (Fig. 2a). The sgRNA of the target site was subcloned into the recombinant plasmid pRGEB32-AtU6, and the resulting construct pRGEB32-CAS9-gRNA-Clpsk1 was used for watermelon transformation (Fig. 2b).
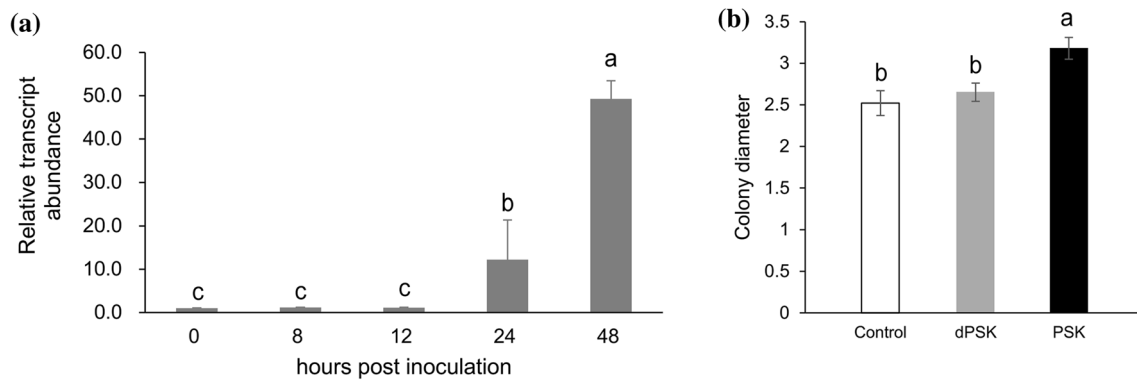
Fig. 1 Interaction between PSK signalling and *Fusarium oxysporum* f.sp. *niveum.* a Time course of transcript abundance of *Clpsk1* gene in watermelon roots infected with *FON1*. The expression level of *Clpsk1* was standardized to the level of *18S rRNA*. Different letters indicate significant differences between time points ($P < 0.05$). Three technical replicates were conducted. b Effects of exogenous PSK on the growth of *FON* colony. 100 μM PSK and dPSK were added into PDA plates before *FON* was inoculated. Colony diameter was measured 3 days post inoculation
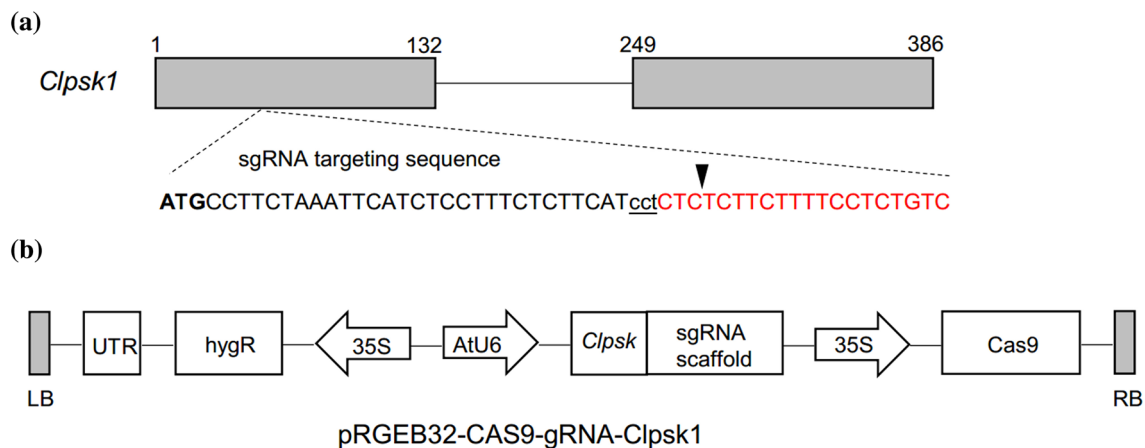

Fig. 2 Schematic representation of CRISPR/Cas9-mediated mutagenesis of *Clpsk1* in watermelon. a Schematic diagram of watermelon *Clpsk1* and the targeting sequence (shown in red) which is located in the first exon. The protospacer adjacent motif (PAM) is shown in underlined lower-case letters. b Construct of pRGEB32-CAS9-gRNA-Clpsk1 was used in watermelon transformation

## CRISPR/Cas9-mediated mutagenesis of *Clpsk1* in transgenic watermelon

The expression vector pRGEB32-CAS9-gRNA-Clpsk1 was introduced into watermelon through *Agrobacterium*-mediated transformation. A total of six hygromycin-resistant lines were generated. These putative transgenic lines were screened using *cas9* gene and all of them generated *cas9*-specific 480 bp amplicons (Fig. 3b). To characterize the CRISPR/Cas9-induced mutations, all positive lines were amplified by *Clpsk1*-specific primers and the target-containing amplicons were directly sequenced. Sanger sequencing results revealed that three (line 1, 2, 3) out of six T_0 transgenic plants were homozygous mutants with a two-base deletion (-CT); two (line 4, 5) were heterozygous lines with a 2-(-CT) and 6-base (-CTCTT…T) deletions, respectively; and one (line 6) were chimeric plant with a 2-(-CT) and 6-base (-CTCTT…T) deletions and 1-base insertion (+A) (Fig. 3a, c).

Off-target analysis

To evaluate whether the potential off-target effects of CRISPR/Cas9 occurred on other sgRNA positions in watermelon genome, the putative off-target mutations were detected. Three potential off-target sites, located on chromosome 7 and 11, respectively, with a PAM sequence were identified (Supplementary Table S1). Sequencing results showed that there were no mutations on these three potential sites (Supplementary Figs. S2, S3, S4).

Fig. 3 Targeted mutagenesis of watermelon Clpsk1 using CRISPR/Cas9 system. **a** Sanger sequencing of the wild type and *Clpsk1* mutant plants. Locations of the mutations were shown in underline and black arrow. **b** PCR amplification of *Cas9* and *Hyg* gene in mutant plants. *M* DL2000 DNA Marker, *WT* wild-type plant, *1~6* mutant plants, *P* positive plasmid. **c** The mutation types of *Clpsk1* in watermelon plants. Dashes indicate deleted nucleotides. Red letter means inserted nucleotide
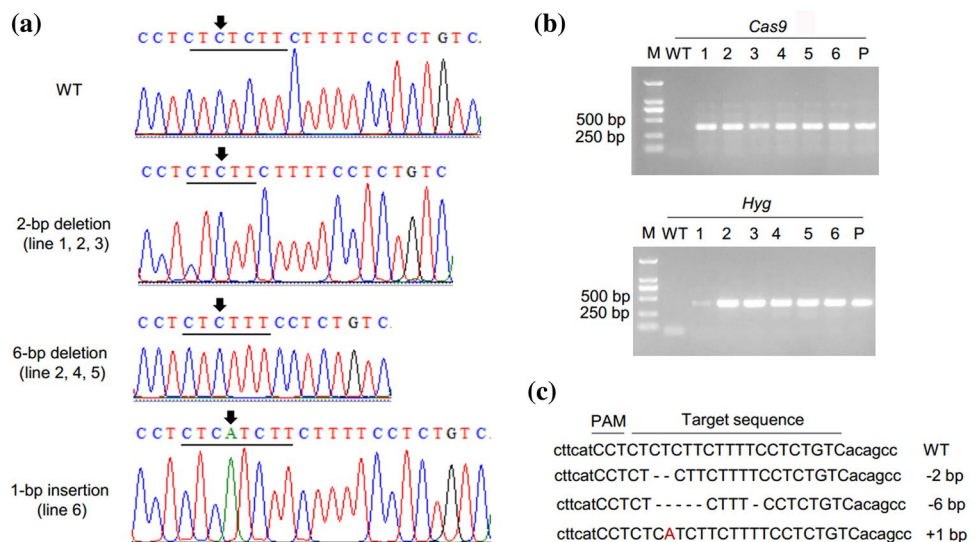
Susceptibility of mutant lines to *FON*

To characterize the fusarium wilt resistance phenotype, six T_0 mutant lines were self-pollinated and T_1 plants were used for susceptibility analysis. Sequencing results of the T_1 progenies showed that there was no additional mutation observed besides the existing mutations. T_1 lines with different types of mutations were inoculated with *FON* race 1 spore suspension. Non-transformed Sumi1 and Calhoun Gray were used as susceptible and resistant control, respectively. Disease reaction ratings of tested plants were calculated 21 dpi. As expected, Calhoun Gray plants showed highly resistant with 18% of the diseased plants' ratio. The ratio of non-transformed Sumi1 seedlings was 92% and defined Sumi1 as highly susceptible. While all *Clpsk1* mutant plants exhibited resistant phenotype when compared with the WT (Supplementary Fig. S1). Among which, four transgenic lines were moderate resistant with the ratio among 33~48% and one was light resistant (incidence of 68%) (Table 1). This result suggests that knock out of *Clpsk1* gene renders watermelon seedlings more resistant to *FON*.

Discussion

The CRISPR/Cas9 system has been successfully used in the generation of desired knockout mutations in the variety of crops (Ito et al. 2015; Kim et al. 2019; Tian et al. 2018; Wang et al. 2014, 2019; Zhai et al. 2019). In this work, pRGEB32-CAS9-gRNA-Clpsk1 was constructed for watermelon CRISPR/Cas9-mediated modification, and the expected mutagenesis at the target site was generated. To implement an effective editing, the OsU6 promoter for monocots was replaced with the Arabidopsis U6 promoter

Table 1 Disease reaction ratings on *Clpsk1* mutant plants infested with *Fusarium oxysporum* f.sp. *niveum*

Lines	Incidence rate[a] (%)	Reaction[b]
Calhoun Gray	18	HR
WT	84	S
C-PSK-2	33	MR
C-PSK-5	39	MR
C-PSK-6	43	MR
C-PSK-4	44	MR
C-PSK-3	48	MR
C-PSK-1	68	LR

[a]Incidence rate was calculated based on the number of dead plants inoculated with *FON* 21 dpi

[b]Disease reaction was rated based on wilt incidence where *HR* highly resistant ($\leq 20\%$), *MR* moderate resistant ($21~50\%$), *LR* light resistant ($51~80\%$), and *S* susceptible ($> 80\%$) according to the criteria established by Martyn and Bruton (1989)

which is better for dicotyledon in the pRGEB32 vector (Xie et al. 2015).

Efficient plant transformation is essential for genetic engineering. Watermelon is known for the poor regeneration potential and low transformation rate. Though, to date, a few successful cases of transgenic watermelon have been reported (Choi et al. 1994; Liu et al. 2016; Part et al. 2005; Tian et al. 2017; Yu et al. 2011), the transformation frequency is not very satisfied. The transformation rate was as low as 0.1–0.3% when CGMMV-CP gene was transformed to generate CGMMV-resistant watermelon rootstock (Park et al. 2005). While the frequency was relatively higher, approximately 9% which is highly cultivar-dependent, in the generation of ZYMV and PRSV resistance watermelon (Yu et al. 2011). Recently, Tian et al. (2017) established an efficient CRISPR/Cas9-based gene knockout system in

watermelon while with 1.67% of transformation frequency. Though in their latest report for generating of herbicide-resistant watermelon variety through CRISPR/Cas9-mediated base-editing, a total of 199 transgenic plants were obtained. However, there is no information about their transformation rate. Consistent with these reports, the efficiency of transformation in the present work was 2.3% (six transgenic lines from 262 explants). Hence, an improved transformation protocol is urgently demand. Cotyledons are the predominant explants used for watermelon transformation. Vasudevan et al. (2017) developed an efficient regeneration protocol using cotyledonary node which obtained a 1.5-fold increase in shoots number compared to that from cotyledon. Though with the high regeneration rate, the transformation efficiency still needs to be determined.

Mutations in the *Clpsk1* gene in six watermelon lines were induced by the CRISPR/Cas9 technology. Types of mutation of these lines differed from one another. Off-target effect of the CRISPR/Cas9 construct targeting the *Clpsk1* gene was further examined and no off-target effects were found at potential off-target sites (Supplementary Figs. S2, S3, S4), suggesting the specificity of the target sequence selected (Hahn and Nekrasov 2019) in the present work. The CRISPR/Cas9 system has been proven to be a precise and efficient genome editing tool to create disease-resistant germplasm (Pyott et al. 2016; Kim et al. 2019; Peng et al. 2017; Wang et al. 2016; 2019; Chandrasekaran et al. 2016). CRISPR/Cas9-targeted mutagenesis of *Os8N3* conferred enhanced resistance in rice to *Xanthomonas oryzae* and the target mutations can be transmitted to subsequent generations (Kim et al. 2019) through a standard germline transmission pattern (Xu et al. 2015). Two heterozygous and three chimeric plants were obtained in the current work and will be further used to investigate the inheritance of targeted mutations in next generation and select the transgene-free mutant watermelon lines.

Particularly, phytosulfokine is thought to function as damage-associated molecular patterns (DAMPs) in plant immune response (van Schie and Takken 2014). Phytosulfokine signalling attenuates or enhances the immune response to different pathogens (Zhang et al. 2018; Rodiuc et al. 2016; Igarashi et al. 2012; Shen and Diener 2013). In the present work, *Clpsk1* loss-of-function enhanced watermelon resistance to *FON* (Supplementary Fig. S1), suggesting the negative feedback regulation of PSK signalling during its interaction with *FON*. Based on previous reports, PSK is perceived by the membrane-localized PSK receptor (*PSKR*) (Matsubayashi et al. 2006), a leucine-rich repeat receptor-like kinase receptor, then binds to the island domain of PSKR and stabilizes via recruitment of the somatic embryogenesis receptor-like kinases (SERKs) (Wang et al. 2015). To date, there are a few studies on the exploration of the PSK triggered signalling mechanisms

and pathways. Hartmann et al. (2014) elucidated that PSK signalling function requiring Ca^{2+}/CaM binding and kinase activity of PSKR1. Similar result was observed in tomato, which proved that PSK perception by PSKR1 elevated cytosolic Ca^{2+}, leading to auxin-dependent immune responses via enhanced binding activity between calmodulins and the auxin biosynthetic YUCs (Zhang et al. 2018). Alterations in SA responses were suggested to be related to an enhanced resistance to the biotrophic pathogen but loss of resistance to the necrotrophic pathogen in *Arabidopsis* (Mosher et al. 2013). Igarashi et al. (2012) suggest that PSK signalling positively regulates growth and attenuates immunity via a mechanism involving direct allocation of resources. While Shen and Diener (2013) propose a mechanism that *F. oxysporum* produces an effector that inhibits the PSY1-dependent negative feedback, which stabilizes PSY1 signalling and induces susceptibility. The above data present the possible mechanisms on PSK signalling in plant defenses to pathogen infection, which provides useful information for our further investigation of the detailed signalling mechanisms and pathways that *Clpsk1* activates in watermelon and *FON* interaction.

Author contribution statement XPY and JHX conceived and designed research. MZ, QLL and LNL performed site-directed mutagenesis and transformation. RSR and XFY performed mutant identification. GL and JX performed *FON* inoculation. MZ wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

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