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RESEARCH ARTICLE

Silence of five *Fusarium graminearum* genes in wheat host confers resistance to Fusarium head blight

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Highlights

- Targeting five key fungal genes through both SIGS and HIGS enhanced wheat resistance to Fusarium head blight.
- HIGS transgenic lines delivered strong FHB resistance without compromising plant growth or yield.

Abstract

Fusarium head blight (FHB), mainly caused by fungus *Fusarium graminearum*, is a devastating wheat disease worldwide, leading to reduced yield production and compromised grain quality due to contamination by mycotoxins, such as deoxynivalenol (DON). Manipulating the specific gene expression in microorganisms through RNA interference (RNAi) presents an opportunity for new-generation double-stranded RNA (dsRNA)-based formulations to combat a large number of plant diseases. Here, we applied both spray-induced gene silencing (SIGS) and host-induced gene silencing (HIGS) to target five virulence-related and DON-synthesized genes in *F. graminearum*, including protein kinase gene *Gpmk1*, zinc finger protein gene *FgChy1*, transcription factor *FgSR*, DON synthesis gene *TRI5* and the cell-end marker protein gene *FgTeaA*, aiming to effectively control FHB in wheat. Direct spraying of individual or combined small interfering RNA (siRNAs) from the fungus showed reduced expression of target genes and suppressed pathogenic symptoms during *F. graminearum* infection in wheat leaves, with the combination of all five siRNAs demonstrating superior resistance. Furthermore, we generated transgenic wheat lines expressing chimeric RNAi cassettes targeting these five genes, and two independent lines exhibited strong resistance to FHB and Fusarium crown rot, and the reduced DON accumulation. Notably, the HIGS transgenic lines did not adversely impact plant growth and yield traits. Collectively, our findings support that SIGS and HIGS represent effective strategies targeting key pathogenic genes for bolstering disease resistance in crops.

Keywords: *Fusarium graminearum*, FHB, resistance, HIGS, SIGS, gene expression, wheat

1. Introduction

Various microbial pathogens, viruses, and pests are estimated to be responsible for causing plant yield losses, accounting for more than 30% of global agricultural production (Alexander *et al.* 2017; Keulemans *et al.* 2019). To manage fungal diseases of plants and crops, several methods including cultural practices, planting disease-resistant cultivars, and biological controls, have been employed in agricultural system. However, fungicide applications currently remain the primary means of disease management due to the limited availability of effectively resistant genes and the time-consuming nature of the crop breeding process (El-Baky and Amara 2021). Nevertheless, the annual increase in fungicide application results in environmental harm to humans and ecosystems. Additionally, the unprecedented rise in the emergence of fungicide-resistant and multidrug-resistant

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fungal populations has further complicated the situation (Fisher *et al.* 2018; Sang *et al.* 2018a, 2019; Schnitkey 2018). It is necessary to develop new methods for controlling pathogenic fungi to ensure effective crop protection.

Fusarium fungal species cause diseases in staple crops such as maize, wheat, rice, barley and soybean (Dean *et al.* 2012; Sang *et al.* 2018b; Wang *et al.* 2019). Fusarium head blight (FHB), caused predominately by *F. graminearum* complex, is one of the most devastating diseases of wheat and barley. FHB leads to reduced grain yield and grain contamination with mycotoxin such as deoxynivalenol (DON) and phytoestrogen zearalenone, threating to both animal feed and human consumption (Dean *et al.* 2012). FHB epidemics have been frequently reported in global wheat-producing regions over the past decade, with an anticipated increase due to the climate change (Zhu *et al.* 2019; Ma *et al.* 2020). During FHB infection, ascospores of *F. graminearum* initially attach to the surface of wheat spikelets, germinating to produce germ tubes that colonize floral organs. Attached germ tubes develop into specialized hyphae known as runner hyphae (RH). The RH grows epiphytically on the surface of wheat spikelets and differentiates into multicellular infection cushions (IC) to initiate infection (Boenisch and Schäfer 2011; Mentges *et al.* 2020). Eventually, infection hyphae of *F. graminearum* rapidly penetrate throughout the spikelet, spreading into adjacent spikelets through the vascular bundles of the rachilla and rachial node. This infection leads to premature head necrosis, resulting in significant yield losses. Additionally, *F. graminearum* can produce various mycotoxins and secondary metabolites in wheat tissues during infection, with DON and its derivatives accounting for the largest fraction, further accumulating these toxins in grains (Bai and Shaner 2004; Chen *et al.* 2019). Moreover, extensive accumulation of DON can enhance *F. graminearum* virulence and accelerate infection in the wheat rachis (Bai *et al.* 2002; Jansen *et al.* 2005). Therefore, repressing DON accumulation in plants emerges as an alternative measure to improve the resistance to FHB.

In recent decades, diverse candidate genes involving in the regulation of pathogen growth, development, pathogenicity, and DON biosynthesis and their encoded proteins, including protein kinases, transcription factors and G proteins, have been characterized in *F. graminearum*. These regulators participate in MAPK signal pathways, transcriptional networks, GPCR (G-protein-coupled receptors) signaling, and cytoskeleton organization, significantly advancing our comprehension of the *F. graminearum*–wheat interaction (Jiang *et al.* 2018, 2019; Chen *et al.* 2019; Cao *et al.* 2021; John *et al.* 2021; Xu *et al.* 2022). *Fusarium graminearum Gpmk1* gene (FGSG_06385), encoding a mitogen-activated protein kinase, is a crucial component of the MAPK pathway for invasive growth. Deletion of *Gpmk1* results in the loss of pathogenicity and the reduction in DON production during infection (Urban *et al.* 2003). The Gpmk1-mediated MAPK kinase cascade phosphorylates multiple regulators in *F. graminearum*, thereby governing its development and virulence (Ramamoorthy *et al.* 2007; Gu *et al.* 2015). Moreover, GPCRs-mediated fungal signaling pathways are important for initial colonization and subsequent switches of plant infection (Jiang *et al.* 2019; Xu *et al.* 2022). Characterization of 105 *GPCR* genes in *F. graminearum* reveals that five of them (*GIV1–GIV5*) are up-regulated during infection of plant and required for the pathogen virulence (Jiang *et al.* 2019). Interestingly, Giv1 likely acts as the upstream of the Gpmk1 MAPK pathway to regulate the IC formation, contributing to pathogenic infection (Hu *et al.* 2014; Jiang *et al.* 2018, 2019). In addition, a novel zinc finger protein, FgChy1 (FGSG_07281), has been identified to be involved in polarized growth, conidiogenesis, septation, sexual reproduction, pathogenicity, and DON production by regulating microtubule morphology in *F. graminearum*. The *fgchy1* mutant exhibits curved and directionless hyphal growth, along with disrupted cytoskeletal organization, resulting in the loss of polarity and virulence (Cao *et al.* 2021). Transcription factor also play a pivotal role in controlling virulence in phytopathogenic fungi (John *et al.* 2021). A novel transcription factor FgSR (FGSG_01176) is essential for mycelial growth by regulating ergosterol biosynthesis in *F. graminearum*. The phosphorylated of FgSR interacts with the SWI/SNF complex and enhances its transcriptional activity, which directly targets to the promoter of ergosterol biosynthesis genes, such as *FgCYP51*. Notably, FgSR functions in virulence by modulating DON biosynthesis and fungal responses to phytoalexins (Liu *et al.* 2019). Therefore, these genes implicated in *F. graminearum* growth, development, and pathogenicity represent promising targets for crop disease control and plant protection.

Trichothecene mycotoxin DON serves as a key virulence factor and source of grain contamination. Its biosynthesis and signaling have been extensively studied in *F. graminearum* during plant infection (Chen *et al.* 2019; Bai *et al.* 2002; Jansen *et al.* 2005). A total of 15 *TRI* genes, including *TRI5* (FGSG_03537), are responsible for DON biosynthesis, distributed across three loci in the *F. graminearum* genome: a core *TRI* cluster with 12 genes, a two-gene *TRI1–TRI16* locus, and the *TRI101* locus (Alexander *et al.* 2009; Seong *et al.* 2009; Chen *et al.* 2019). A comparative transcriptome study reveals that *TRI* genes are highly induced in wheat tissues during infection, indicating a crucial role of DON in modulating host responses and establishing the infection (Brown *et al.* 2017). The gene *TRI5* encodes trichodiene synthase, catalyzing the first step in trichothecene biosynthesis by cyclizing farnesyl pyrophosphate (FPP) to trichodiene (Kimura *et al.* 2007; Alexander *et al.* 2009; Chen *et al.* 2019). Metabolite profiling of *F. graminearum* wild-type and the *tri5* mutant in infected wheat tissues provides evidence supporting the role of DON in suppressing host defense-related metabolites (Bonnighausen *et al.* 2019). The *tri5* deletion mutant, which blocks DON production (Bai *et al.* 2002), is restricted to the pathogen crossing rachis of spikelets (Jansen *et al.* 2005), indicating that DON facilitates the spread of *F. graminearum* throughout the wheat head. Thus, manipulating these genes through novel strategies could be useful in the management of FHB and grain quality.

RNA interference (RNAi) is a highly-conserved mechanism for silencing gene expression present in most eukaryotes, including plants, animals, and fungi. Spray-induced gene silencing (SIGS) and host-induced gene silencing (HIGS) are RNAi-based processes in which small RNAs, either

artificially produced or generated within the plant, repress the expression of genes from pathogens attacking the plant (Sang and Kim 2020). Both SIGS and HIGS approaches have been intensively employed to manage Fusarium pathogens in crops (Wang and Jin 2017; Qi *et al.* 2019). Koch *et al.* (2016) used SIGS to silence three *F. graminearum* genes encoding cytochrome P450 lanosterol C-14α-demethylases, successfully restricting fungal development in barley. HIGS also proves to be an effective and convenient tool for disease control in crops. Transgenic expression of double-stranded RNAs (dsRNAs) targeting three paralogous genes (*CYP51A*, *CYP51B* and *CYP51C*) involved in the ergosterol biosynthesis pathway in *Arabidopsis* and barley enhances resistance to *F. graminearum* infection (Koch *et al.* 2013). Similarly, silencing a Fusarium virulence factor gene, *chitin synthase 3b* (*CHS3b*), in wheat by HIGS shows stable and consistent resistance to FHB and Fusarium seedling blight (Cheng *et al.* 2015). Wang *et al.* (2020) also demonstrate that silencing multiple *F. graminearum* genes related to DON biosynthesis and virulence by HIGS significantly reduces DON production and enhances resistance to FHB in wheat. These suggest that SIGS and HIGS may have great potentials for disease management and engineering resistant cultivars in crops.

In this study, we employ SIGS and HIGS approaches to target five essential *F. graminearum* genes, including those encoding MAP kinase, transcription factor, DON synthesis enzyme, and microtubule-associated proteins, crucial for pathogenicity and DON production in wheat. Our data demonstrate that applying siRNA through SIGS and stably expressing dsRNA *via* HIGS in wheat effectively suppresses the expression of target genes, conferring resistance to FHB, Fusarium crown rot (FCR), and DON accumulation. Our findings provide valuable insights and a potential strategy to enhance plant disease resistance.

2. Materials and methods

2.1. FgTeaA identification and phylogenetic analysis

Through the BLAST function of NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi), the homologous proteins of TeaA (*Aspergillus nidulans*) in *F. graminearum* PH-1 were identified. At the same time, the homologs in the other five fungi and the reported homologous Tea1 protein (*Schizosaccharomyces pombe*) were also found. The phylogenetic tree was constructed using software MEGA7 and the key domain kelch repeat internal (http://smart.embl.de/) was analyzed to screen the most similar proteins to TeaA in *F. graminearum* PH-1.

2.2. Fungal material and plant infection

Fusarium graminearum strain PH-1 was cultured on potato dextrose agar (PDA). Preparation of fungal inoculum was performed as described (Jiang *et al.* 2019). Wild-type (Fielder) and transgenic wheat plants were grown in a growth chamber with a 16 h-ligh/8 h-dark photoperiod and 60% relative humidity at 22°C. For infection of wheat, ten detached second leaves of 2-week-old plants were collected and transferred in square Petri plates containing 1% water-agar. Ten microliters of conidial spore suspension (approximately 5×10^4 conidia mL^{-1}) were incubated at the central of wheat leaves. Infection symptom was recorded at 5–6 days post-inoculation (dpi) as infection area by determining the size of chlorotic lesions relative to total leaf area using ImageJ (https://www.computerbild.de/download/ImageJ-422527.html).

2.3. Spray application of siRNA on wheat leaves

The siRNA sequences of *Gpmk1* (FGSG_06385), *FgChy1* (FGSG_07281), *FgSR* (FGSG_01176), *TRI5* (FGSG_03537) and *FgTeaA* (FGSG_08703) were list in Appendix A, which were artificially synthesized from Sangon Biotech (Shanghai) Co., Ltd. The synthesized RNA oligo dosage form was freeze-dried powder, and stored at −80°C. The siRNA was freshly diluted in DEPC water to a final concentration of 10 μmol L^{-1} before spraying. Approximately 3 cm length tissues of the second leaf at the 2-week-old seedling stage was cut and transferred to square Petri plates containing 1% water-agar. Spraying of the leaves was carried out using a spray flask as described earlier (Koch *et al.* 2016). Gently poke a wound in the middle of the leaves, and subsequently each plate containing ten detached leaves was evenly sprayed with either FGSG_06385, FGSG_07281, FGSG_01176, FGSG_03537, or FGSG_08703-siRNAs, combined five siRNAs by giving three to four puffs, and then leaves were drop-inoculated with 10 μL of *F. graminearum* suspension containing 5×10^4 conidia mL^{-1}. The samples were cultivated at 22°C and observed the incidence of disease symptom after 5–6 days.

2.4. RNA extraction and gene expression analysis

To assess silencing of the target genes, mRNA expression analysis was performed using quantitative real-time PCR (qRT-PCR). Total RNA was extracted from the infected leaves or spikelets using TRIzol reagent (TaKaRa, Dalian, China, catalogue number T9108), and was quantified using the NanoDrop spectrophotometer (Thermo Scientific, USA). The first-strand cDNAs were synthesized using PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, catalogue number RR036A). qRT-PCR was performed using a Roche thermal cycler 96 using the TB Green® Premix Ex Taq™ II reagent (TaKaRa, catalogue number RR820A) as described (Xia *et al.* 2022). The PCR procedure was started at 95°C for 5 min, followed by 40 cycles with 95°C for 5 s, 58°C for 30 s, 72°C for 30 s, and heating to 95°C at a rate of 0.1°C s^{-1} with continuous reading of fluorescence. *Elongation factor 1-alpha* (*EF1-a*, FGSG_08811, *Fusarium*) and *TaTubulin* (wheat) genes served as internal control for normalization of expression value. The transcript level was calculated *via* the $2^{-\Delta\Delta Ct}$ method. Triplicates were applied for all qRT-PCR analyses in this study. Primers used in qRT-PCR are listed in Appendix A.

2.5. Generation of transgenic wheat plants

To design a chimeric hairpin RNAi construct simultaneously targeting five genes, a tandem DNA fragment containing a 200-bp partial coding region for each of the following genes: *Gpmk1* (FGSG_06385), *FgChy1* (FGSG_07281), *FgSR* (FGSG_01176), *TRI5* (FGSG_03537) and *FgTeaA*

(FGSG_08703), was assembled and inserted into the pTCK303 vector to generate a dsRNA sequence with a hairpin structure. Primers used for construction are listed in Appendix A.

The construct with RNAi cassette were transformed into *Agrobacterium tumefaciens* strain EHA105. The wheat transformation was performed as previously described (Ishida *et al.* 2015). Briefly, immature seeds, harvested approximately 14–16 days after anthesis, surface-sterilized for 1 min in 70% (v/v) ethanol, followed by 15 min in 1% (w/v) sodium hypochlorite, and finally rinsed 5 times with sterile water in aseptic conditions. Immature embryos were isolated and incubated with the EHA105 strain for 5–10 min. After 2 days co-cultivation in darkness, embryonic axes were removed with a scalpel, and the scutella were transferred onto plates. The tissues were subsequently moved to callus selection medium for 2 weeks, followed by 3–4 weeks on induction medium. Shoots were developed under continuous illumination with fluorescent lights at 25°C for 14 days. Rooted plantlets were then transferred into pots and grown in growth chambers. At least 30 independent T0 transgenic wheat lines were generated and lines from T1 and T2 generations were further confirmed by PCR using the gene-specific primers (Appendix A).

2.6. Evaluation of FHB phenotyping

Wheat spikelets at the early anthesis stage in the greenhouse were used for FHB evaluation as previously described (Bai *et al.* 2001). *F. graminearum* strain PH-1 was used as the inoculum for FHB infection. Ten microliters of conidial spore suspension (approximately 5×10^4 conidia mL^{-1}) were injected into a floret of the top spikelet within a spike and covered with plastic bags to maintain 100% relative humidity for 2 days, initiating fungal infection. Subsequently, after removing the plastic bags, the humidity in the growth chambers was adjusted to 70% to facilitate further FHB development. Evaluation of FHB symptom spread within a spike (type II resistance) involved in counting symptomatic spikelets at 11, 15, 19, and 21 dpi. FHB severity for each line was calculated based on the number of infected spikelets per spike. Statistical analysis involved investigating at least 10 spikes from each genotype plant. All phenotyping experiments were conducted with at least three independent repeats, consistently displaying similar trends in FHB severity.

2.7. Evaluation of Fusarium crown rot (FCR) symptom

FCR evaluation was carried out in the laboratory referred to Liu *et al.* (2010). Seedlings were inoculated when the length reach between 0.5 and 1.0 cm by immersing in a suspension of PH-1 spores (containing 1×10^6 spores mL^{-1}) for 1–2 min. Ten FCR-inoculated seedlings were then wrapped and grown in a single piece of moist paper towel in a plastic container at 22°C and 60% humidity with a 16-h period in growth chambers. Resistance evaluation was conducted by measuring the length of deep brown lesions at the base of the stem.

2.8. Measurement of DON content

The spikelets of transgenic and wild-type plants after inoculation with *F. graminearum* for 3 days were collected for DON content determination with the DON ELISA test kit (Huaan Magnech Bio-Tech, China, HEM1896-2) using an indirect competitive enzyme-labeled immunoassay according to the manufacturing processes.

2.9. Evaluation of agronomic traits

For agronomic traits analysis between WT and transgenic plants, ear and grain morphology were recorded using Canon 800d camara. Effective tillering number was calculated as the number of branches with head from individual plant. Spike length, spikelet number per spike, and plant height per plants, grain length and width were measured by ImageJ after taking photos. Thousand grain weight was evaluated from random seeds from individual lines.

2.10. Statistical analysis

All experiments were conducted at least three times with technical and biological replicates at an appropriate sample size, which showed similar results. No statistical methods were used to predetermine sample size. All experiments involving measurements/quantifications and imaging were repeated at least three times with similar results. Data were analyzed by Microsoft Excel, GraphPad Prism 9.0 or ImageJ were routinely used to show individual data points for each experimental observation, and bar graphs contain individual data points for each experimental replicate. A two-tailed Student's *t*-test was used to evaluate significant variations, as appropriate, using Microsoft Excel. Values of $P<0.05$ were considered statistically significant. For all figure legends, 'n' represents the number of sample size.

3. Results

3.1. Selecting target genes associated with *F. graminearum* pathogenicity

Previous studies have revealed that *Gpmk1* (FGSG_06385), *FgChy1* (FGSG_07281), *FgSR* (FGSG_01176), and *TRI5* (FGSG_03537), encoding the MAP kinase, zinc finger protein, transcription factor, and trichodiene synthase, respectively, are indispensable for pathogenicity and DON accumulation during the *F. graminearum*–wheat interaction (Table 1) (Urban *et al.* 2003; Alexander *et al.* 2009; Liu *et al.* 2019; Cao *et al.* 2021). Hence, these genes were selected as target genes for further investigation into expression silencing. Additionally, microtubules, serving as transport tracks, play important roles in hyphal tip growth in fungi. In yeast, Tea1, a kelch internal repeat protein, acts as an end marker and is necessary for microtubular organization to regulate the cell fission (Mata and Nurse 1997). In filamentous fungus *Aspergillus nidulans*, TeaA is the homolog of yeast Tea1, which localizes at the tip membrane depending on microtubules. Conversely, TeaA is required for microtubules to converge at a single point at the tip of the hypha, regulating hyphal development and polarized growth (Takeshita *et al.* 2008, 2013). The role of TeaA-mediated microtubular organization in virulence has also been demonstrated in other fungi, such as *Magnaporthe oryzae*, *Pyricularia oryzae* and *Colletotrichum orbiculare* (Sakaguchi

et al. 2010; Qu *et al*. 2022; Rogers *et al*. 2024). However, the homolog of TeaA and its function in *F. graminearum* remains unknown. We used the animo acid sequence of TeaA from *Aspergillus nidulans* and Tea1 from yeast to identify the homolog in *F. graminearum* genome using BlastP. In total, we identified 9 members in the reference genome PH-1. Among them, FGSG_08703, named *FgTeaA*, encoding the cell-end marker protein gene, exhibited a close evolutionary relationship with TeaA and Tea1, while the other 8 members in *F. graminearum* were grouped into another two different clades from TeaA and Tea1 (Appendix B). Similar to the TeaA and Tea1, FgTeaA contained kelch internal repeat domains, but only one member of *F. graminearum* in another two clades had this motif property (Appendix B). We thus identified FGSG_08703 (FgTeaA) as the homolog of TeaA and included this encoding gene in our list for further SIGS and HIGS (Table 1).

3.2. SIGS targeting five genes confers resistance to *Fusarium* infection in wheat leaves

To assess the potential of the identified five genes in enhancing resistance to *Fusarium* infection, we artificially synthesized each siRNA targeting FGSG_06385, FGSG_07281, FGSG_01176, FGSG_03537, and FGSG_08703, respectively (Appendix A), and evaluated the silencing efficiency through SIGS. To avoid the unstable siRNA uptake efficiency in spikelets, characterized by poor permeability due to the presence of lemma and palea compounds containing cutin and wax, we initially conducted SIGS assays on young leaves at the seedling stage to assess the effects of *F. graminearum*-caused diseases and the repression of selected target gene expression. After detaching wheat leaves, we pretreated the tissue by spraying 10 μmol L^{-1} siRNA on the surface, followed by drop-inoculation of a suspension of *F. graminearum* conidia on the leaves. At 6 dpi, necrotic lesions were observed at the inoculation sites of leaves sprayed with DEPC water (mock). In contrast, the treatment with the five siRNAs displayed significantly reduced *Fusarium* infection symptoms, evident by considerably smaller lesions (Fig. 1-A and B, Student's *t*-test, $P<0.05$). Specifically, *Gpmk1*-, *FgChy1*-, *FgSR*-, *TRI5*- and *FgTeaA*-siRNA treatments led to an average reduction in infected areas by approximately 70–80%, compared to the mock spraying (Fig. 1-B). Remarkably, the combined treatment using mixed all five siRNAs for SIGS exhibited a more potent inhibition of necrotic lesions than both siRNA alone and mock treatments, resulting in a decrease of over 90% compared to the mock (Fig. 1-A and B), highlighting the functional roles of these target genes in the virulence of *Fusarium*.

The observed disease symptom phenotype suggests that the sprayed siRNAs are likely taken up by wheat leaves, subsequently forming dsRNA to silence gene expression through the RNAi machinery. We further examined the expression of the five target genes in wheat leaves of SIGS assays. qRT-PCR demonstrated that individual siRNA sprays significantly repressed the expression of each gene, with *Gpmk1*-siRNA and *TRI5*-siRNA exhibiting stronger silencing effects on the targets compared to others (Fig. 1-C). Moreover, spraying of mixed five siRNAs resulted in the repressed expression level of all genes in wheat tissues after infection (Fig. 1-D), showing a similar trend to the individual siRNA sprays, with *Gpmk1* and *TRI5* being strongly repressed (Fig. 1-C and D). Overall, these findings establish a robust correlation between the resistance phenotypes and the silenced expression of the target genes by SIGS.

3.3. Generation of transgenic wheat plants expressing host-induced gene silencing constructs

Our SIGS results demonstrated the high efficiency of the combined treatment with five siRNAs in repressing *Fusarium* disease symptom (Fig. 1-A and B). We thus designed and constructed a chimeric hairpin RNAi system aiming to simultaneously silence the five target genes. This system included a tandem DNA fragment comprising each 200-bp partial coding region of FGSG_06385 (*Gpmk1*), FGSG_07281 (*FgChy1*), FGSG_01176 (*FgSR*), FGSG_03537 (*TRI5*), and FGSG_08703 (*FgTeaA*), under the ubiquitin promoter to generate a dsRNA sequence with a hairpin structure (Table 1; Appendix C).

Subsequently, this construct was transformed into the FHB-susceptible wheat cultivar Fielder, to express chimeric RNAi cassette. Over 30 transgenic plants were obtained in total. We further tested the genotyping of these transgenic lines by examining the target fragments (1,000 bp) using wheat genomic DNA-based PCR approach, with the results showing that the inserted DNA was present in transgenic plants, but not in wild-type plants (Appendix C).

3.4. HIGS targeting five genes confers resistance to *Fusarium* infection in wheat leaves

We next assessed the resistance capability of multiple

Table 1 Candidate host-induced gene silencing (HIGS) genes of *Fusarium graminearum*

Gene ID (NCBI)	Protein name	dsRNA position (bp)	Annotation & function	Source
FGSG_06385	Gpmk1	18–217	Mitogen-activated protein kinase, signal transduction processes	Urban *et al.* (2003)
FGSG_07281	FgChy1	115–314	CHY-type zinc finger protein, MT morphology and polarized growth	Cao *et al.* (2021)
FGSG_01176	FgSR	620–819	Transcription factor, sterol biosynthesis regulator	Liu *et al.* (2019)
FGSG_03537	TRI5	4–203	Trichodiene synthase, the production of the DON class of mycotoxins	Alexander *et al.* (2009)
FGSG_08703	FgTeaA	672–871	Homolog of the cell-end marker protein TeaA, the convergence of MTs and proper control of MT growth	Mata and Nurse (1997); Takeshita *et al.* (2008)

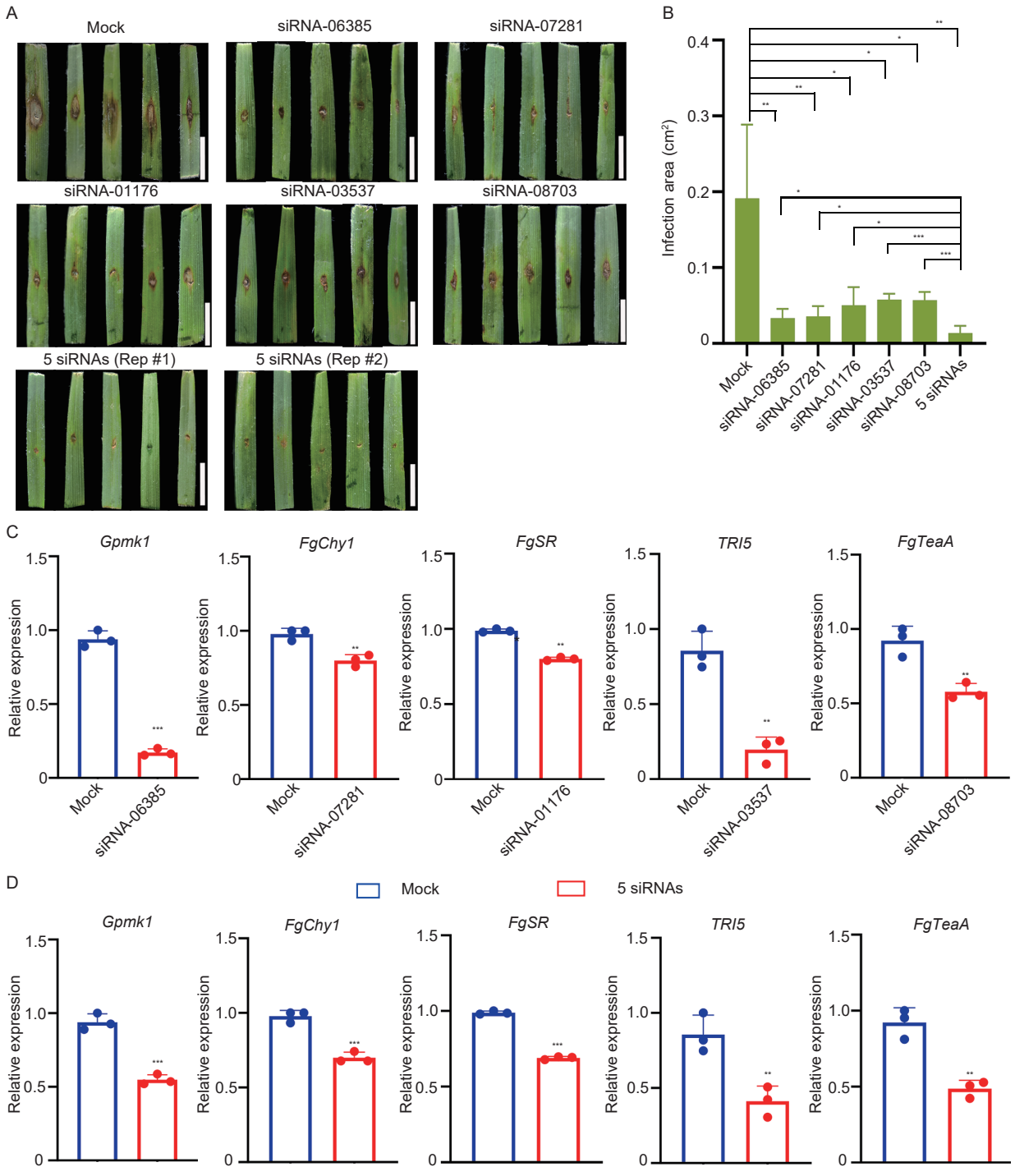


Fig. 1 SIGS-mediated suppression of *Fusarium graminearum* infection on wheat leaves sprayed with siRNAs targeting on the five pathogenic genes. A, representative images of detached 2-week-old wheat leaves spraying with the five target genes-siRNAs or mock solution. After spraying with individual siRNA (siRNA_06385, siRNA_07281, siRNA_01176, siRNA_03537, siRNA_08703) and combined five siRNAs (5 siRNAs), leaves were drop-inoculated with 5×10^4 conidia mL^{-1} and evaluated for infection symptoms at 5 days post-inoculation (dpi). Scale bars, 1 cm. B, lesion area was measured and analyzed (n=10 of each set) as mentioned in A. C, expression of five fungal genes in wheat leaves in spraying with individual siRNAs as mentioned in A (n=3 of each set). Gene-specific expression of *Gpmk1*, *FgChy1*, *FgSR*, *TRI5*, and *FgTeaA* was performed by qRT-PCR and normalized to fungal *EF1-α*. D, expression of five selected fungal genes in wheat leaves in spraying with combined 5 siRNAs as mentioned in A. Data were represented as the mean±SD of three replicates. *P*-value, Student's *t*-test (*, $P<0.05$; **, $P<0.01$; ***, $P<0.001$).

independent transgenic plants during *Fusarium* infection. Two-week-old detached leaves of transgenic wheat were drop-inoculated with 5×10^4 conidia mL^{-1} to record the infection symptom. At 6 dpi, untransformed wild-type plants exhibited evident necrotic lesions, typical symptoms of successful *F. graminearum* infection on wheat leaves (Fig. 2-A

and B; Appendix D). In clear contrast, multiple independent transgenic lines expressing the five dsRNAs showed strongly reduced infection symptoms at inoculation sites compared to the wild-type (Fig. 2-A and B; Appendix D). Statistics revealed that the average infected area of detached leaves in these transgenic lines was only one-third that of the wild-type leaves (Fig. 2-B; Appendix D). Notably, FG5-1-5 and FG5-1-6 lines demonstrated the highest resistance against *F. graminearum* (Appendix D). These data demonstrate that the expression of five dsRNAs related to pathogenicity in wheat *via* HIGS confers resistance to Fusarium infection in leaves.

Furthermore, qRT-PCR analysis was conducted to verify the silencing of target fungal genes, revealing diverse expression patterns among the transgenic lines for the five target genes. Notably, *FgSR* exhibited the highest repression efficiency, while *FgChy1* showed the lowest. Remarkably, FG5-1-5 and FG5-1-6 lines demonstrated the most efficient silencing effect on target genes, suppressing 4 and 5 target genes, respectively, whereas other lines exhibited significant silencing effects on only 2 or 3 target genes (Appendix D). Specifically, the transcription level of *Gpmk1* was reduced by ~60% in both FG5-1-5 and FG5-1-6 lines, relative to that in non-transgenic plants. For *FgChy1*, the transcripts were significant reduced in line FG5-1-6, but not in FG5-1-5 (Fig. 2-C). The other three genes, *FgSR*, *TRI5*, and *FgTeaA*, showed half the expression level of the wild-type plants (Fig. 2-C). Thus, we chose two independent lines, FG5-1-5 and FG5-1-6, for further HIGS investigation. Overall, transcript assay results are consistent with the hypothesis of gene expression silencing in transgenic plants induced by HIGS, likely leading to impairment of the pathogen's ability to cause disease on wheat leaves.

3.5. HIGS transgenic plants exhibit the increased resistance to FHB and FCR

To further validate the efficacy of HIGS against FHB, we assessed *F. graminearum* infection and spread in wheat heads using a single-floret injection under greenhouse conditions. After observations of heads, both wild-type and transgenic plants exhibited typical FHB symptoms. However, heads of the two transgenic wheat lines showed dramatically attenuated disease symptoms compared to wild-type plants (Fig. 3-A). Statistical analysis of the infected spikelet number from 11 to 19 dpi also demonstrated a significant reduction in FHB disease symptoms in the two transgenic wheat lines expressing the dsRNA (Fig. 3-B). Among our selected target genes, besides *TRI5* functioning in DON synthesis, *Gpmk1*, *FgChy1*, *FgSR* also have been reported to regulate the DON production (Urban *et al.* 2003; Alexander *et al.* 2009; Liu *et al.* 2019; Cao *et al.* 2021). We thus measured the DON content in dsRNA expressed transgenic lines during *F. graminearum* infection, showing that the spikelets of lines FG5-1-5 and FG5-1-6 only had 68 and 57% DON production of that wild-type plants (Fig. 3-C), which is consistent with the role of these genes in *F. graminearum*-secreted DON process. These

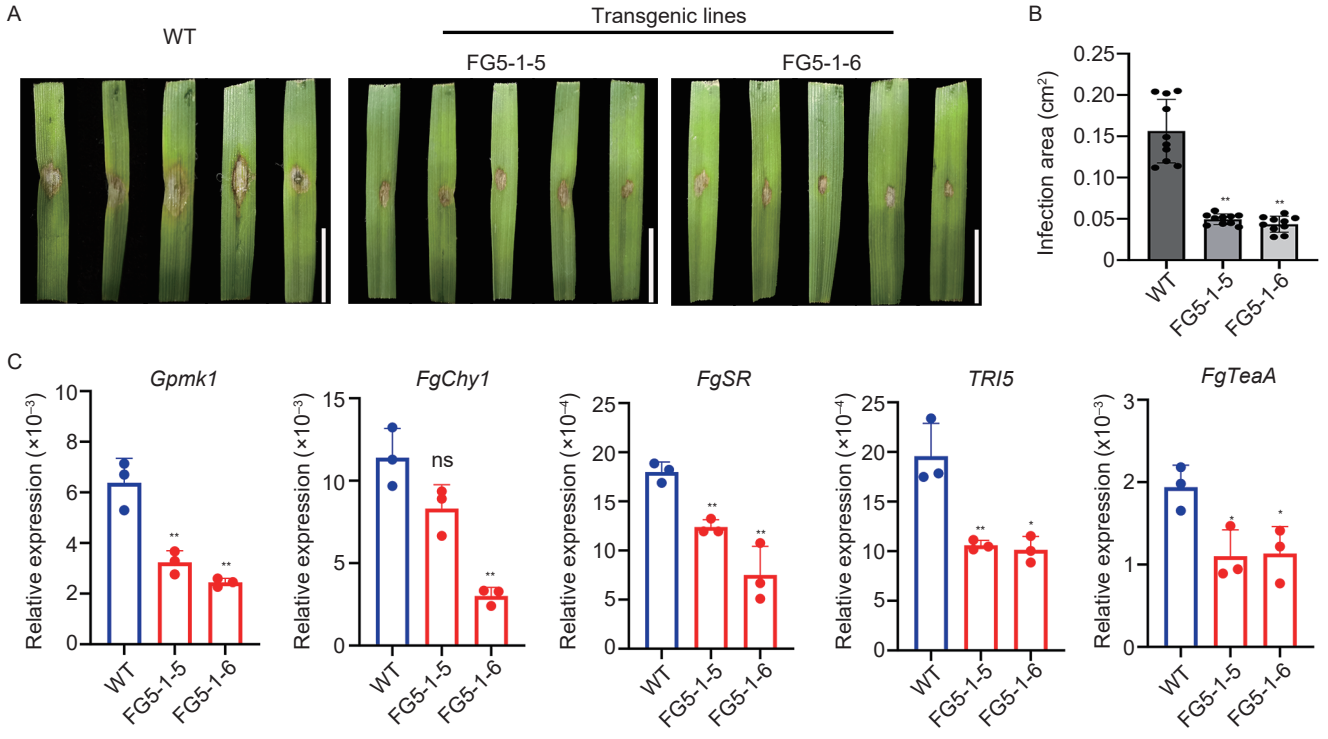


Fig. 2 Symptoms of *Fusarium graminearum* infection in transgenic wheat leaves. A, representative images of wild-type (WT) and transgenic wheat leaves in infection of *F. graminearum* at 5 days post-inoculation (dpi). Scale bars, 1 cm. B, lesion area was measured and analyzed in WT and transgenic wheat leaves (n=10 of each set). C, relative expression of *Gpmk1*, *FgChy1*, *FgSR*, *TRI5*, and *FgTeaA* in transgenic wheat leaves after fungal infection as mentioned in A (n=3 of each set). The expression value was normalized to *EF1-α* of *Fusarium*. Data were represented as the mean±SD of three replicates. *P*-value, Student's *t*-test (*, P<0.05; **, P<0.01; ns, not significant).

results demonstrate that targeting five pathogenic and DON synthesis-related genes by HIGS confers strong resistance to FHB and suppresses DON accumulation.

Next, we performed the expression of these five genes in spikelets during *F. graminearum* infection. Similar to leaves, most of the target genes were significantly repressed in the two transgenic wheat lines (Fig. 3-D). In particular, the transcriptional level of *TIR5* was reduced about 80% in transgenic plants, compared to the wild-type Fielder, while *FgTeaA* did not show a statistically significant change (Fig. 3-D). These results suggest that expression of chimeric hairpin dsRNA *in planta* effectively silences the *Gpmk1*, *FgChy1*, *FgSR*, and *TRI5* genes, possibly by directing the host plant's silencing machinery to produce the corresponding siRNAs.

Fusarium graminearum also can infect the stem of wheat seedlings, leading to Fusarium crown rot (FCR), another devastating disease of wheat (Liu and Francis 2015). We next evaluated whether the repressed target gene expression contributes to FCR resistance. After infecting *F. graminearum* into the stem at the seedling stage, both transgenic lines FG5-1-5 and FG5-1-6 lines exhibited a significantly decreased lesion size with brown necrosis at the stem collum region,

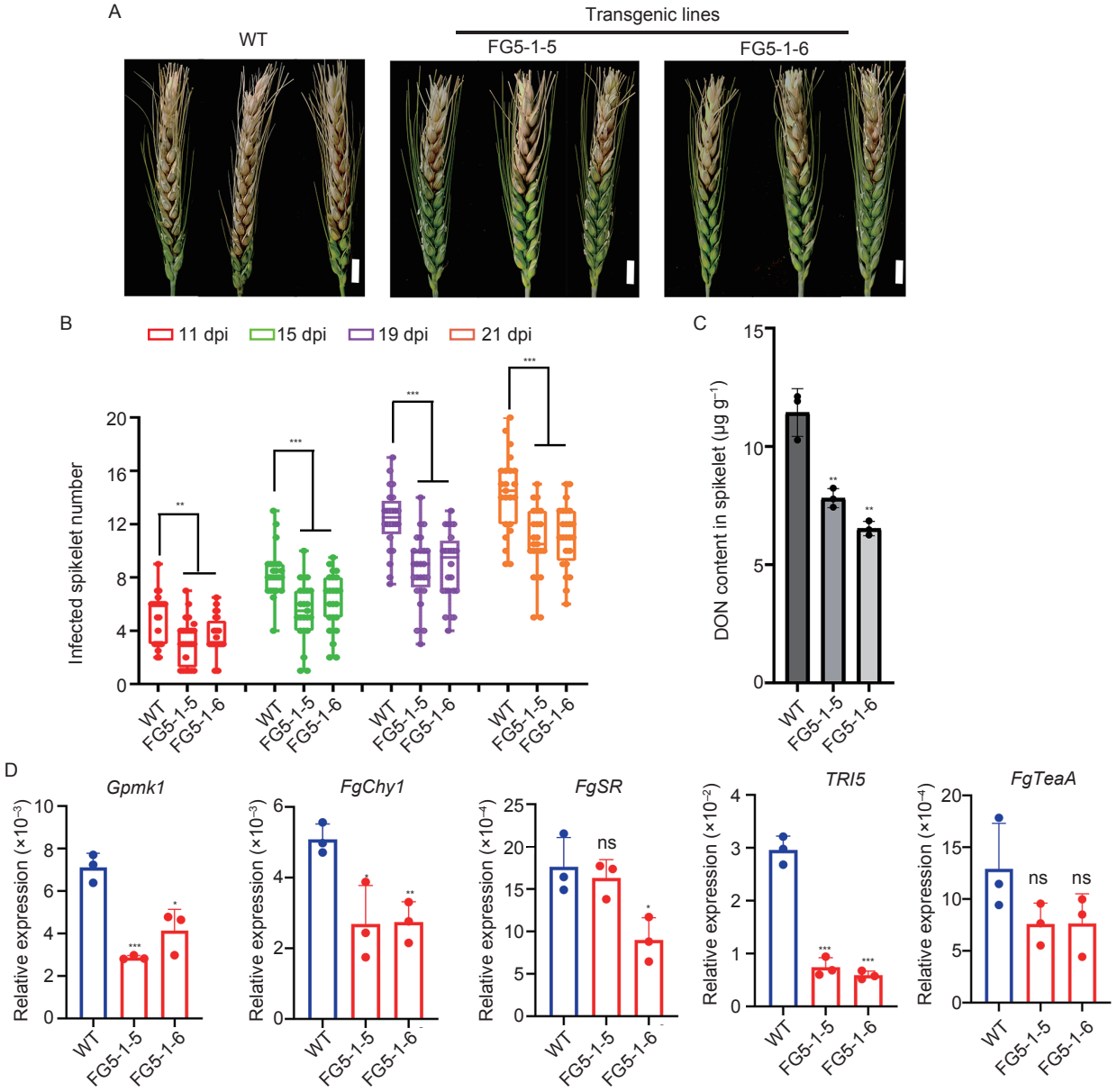


Fig. 3 Evaluation of resistance to FHB in transgenic wheat. A, representative images of wheat spikes in wild-type (WT), transgenic FG5-1-5 and FG5-1-6 lines after infection with *Fusarium graminearum* at 19 days post-inoculation (dpi). Scale bars, 1 cm. B, quantification of FHB disease index (n=18 of each set). Values were calculated from three independent experiments. C, quantification of DON content in spikelets of WT, transgenic FG5-1-5 and FG5-1-6 lines after infection with *Fusarium graminearum* at 3 dpi (n=3 of each set). D, relative expression of *Gpmk1*, *FgChy1*, *FgSR*, *TRI5*, and *FgTeaA* in wheat spikes after infection with *Fusarium graminearum* at 48 h (n=3 of each set). The value was measured by qRT-PCR and normalized to fungal *EF1-α*. Data were represented as the mean±SD of three replicates. *P*-value, Student's *t*-test (*, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant).

compared to wild-type plants (Fig. 4-A and B). Accordingly, in comparison to the infection of *F. graminearum* in wild-type plants, the transcriptional levels of selected pathogen genes, except for *FgChy1* in the FG5-1-6 line, exhibited significant decreases in HIGS transgenic lines (Fig. 4-C). Overall, transgenic wheat plants expressing dsRNA of *F. graminearum* pathogenic genes also confer to FCR resistance.

To assess whether the enhanced resistance of transgenic lines is attributed to the silencing of target genes in *F. graminearum* or the activation of host immunity, we examined the expression of three genes indicative of plant immunity pathways, *TaWRKY70* (Kage *et al.* 2017), *TaMPK3* (Rudd *et al.* 2008), and *TaPR1* (Gunupuru *et al.* 2019), in both our SIGS and HIGS assays. As shown in Appendix E, in SIGS, *TaWRKY70*, *TaMPK3*, and *TaPR1* exhibited similar transcriptional levels between mock and siRNA spraying without the infection of *F. graminearum*, suggesting that siRNA application does not trigger plant immunity. Similarly, the expression of these three genes showed no difference between wild-type and HIGS transgenic lines under normal conditions. Thus, we clarify that both SIGS and HIGS assays targeting the five pathogen genes confer resistance to FHB due to the repression of pathogen gene expression rather than the activation of host immunity. Additionally, the transcript expression of *TaWRKY70*, *TaMPK3*, and *TaPR1* was strongly induced by *F. graminearum* but exhibited a relatively low degree in siRNA-sprayed or FG5 transgenic samples compared to mock or wild-type samples (Appendix E). This suggests that SIGS and HIGS repress *F. graminearum* biomass, inducing a low plant immune response.

3.6. Expressing the dsRNA in wheat does not affect growth and yield penalty

Considering that the ectopic expression of genes in plants may have the potential to cause unpredictable effects on plant growth, we evaluated the main agronomic traits of transgenic wheat lines expressing dsRNA to further explore whether HIGS-based transgenic plants have potential applications in breeding. Under normal growth conditions, we first investigated spike traits at the heading stage, revealing no differences in spike morphology, ear length, and spikelet

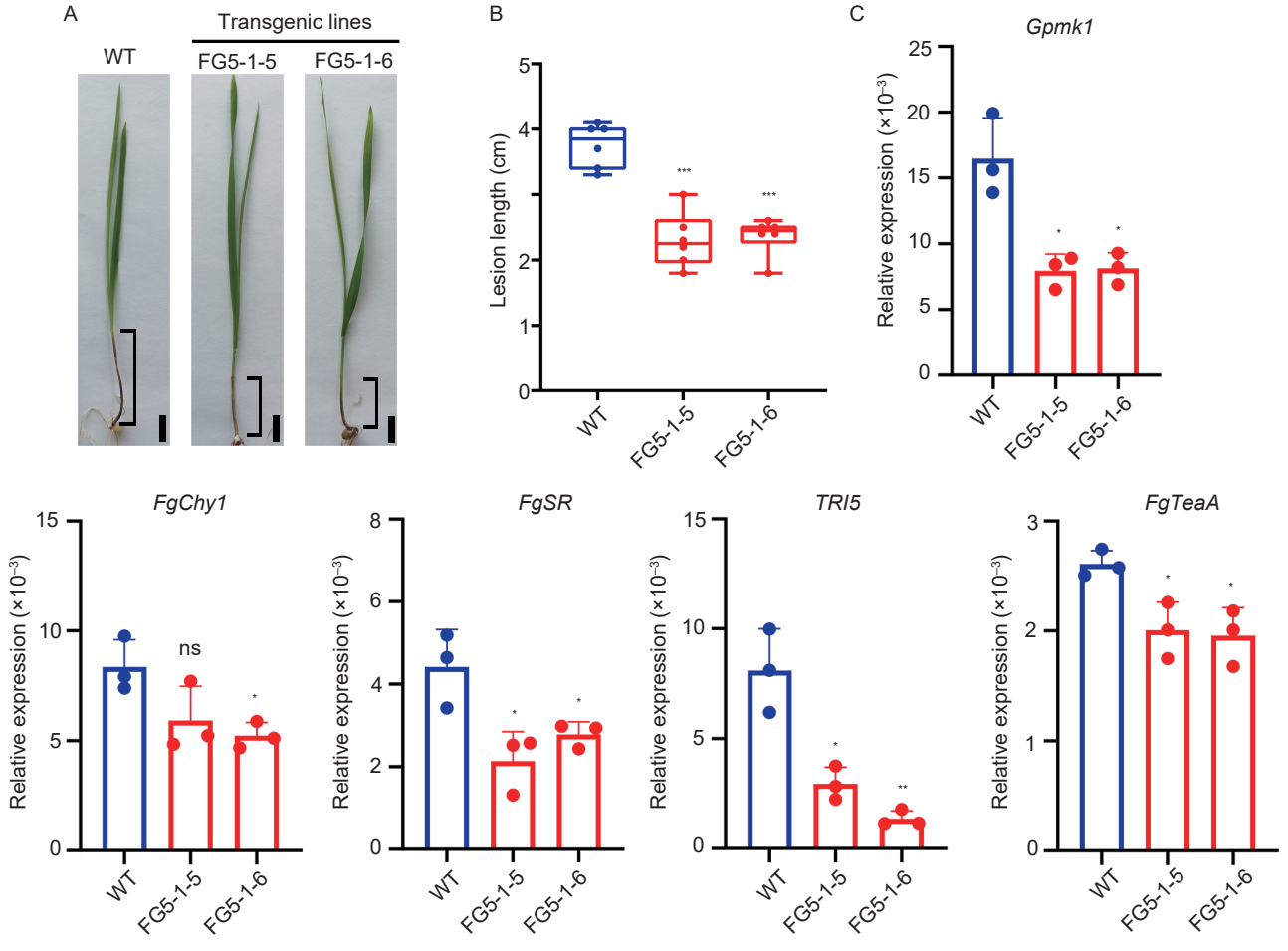


Fig. 4 Evaluation of resistance to Fusarium crown rot in transgenic wheat. A, wild-type (WT), transgenic FG5-1-5 and FG5-1-6 seedlings inoculated with the conidia of *Fusarium graminearum* (diseased tissues are indicated by brackets) for 14 days. Scale bars, 1 cm. B, quantitative measurements of stem length with Fusarium crown rot symptoms in transgenic and WT plants (n=6 of each). C, relative expression of *Gpmk1*, *FgChy1*, *FgSR*, *TRI5*, and *FgTeaA* in transgenic wheat leaves after fungal infection as mentioned in A (n=3 of each set). The expression value was normalized to *EF1-α* of *Fusarium*. Data were represented as the mean±SD of three replicates. P-value, Student's t-test (*, P<0.05; **, P<0.01; ns, not significant).

number per spike between wild-type and HIGS transgenic plants (FG5-1-5 and FG5-1-6) (Fig. 5-A–C). Regarding grain traits, we found that the grain length, grain width, and thousand-grain weight of FG5-1-5 and FG5-1-6 plants were comparable to those of wild-type plants (Fig. 5-D–G). The agronomic traits of plant height and tiller number were also similar between transgenic wheat and wild-type plants (Fig. 5-H and I), indicating that the expressed dsRNAs of five *F. graminearum* pathogenic genes are not likely to have off-target effects on regulating host genes. Together, our data suggest the promising potential of the HIGS-based transgenic strategy in improving FHB resistance without causing detrimental effects on growth, negative agronomic impacts, or yield penalties in the host.

4. Discussion

Fungal pathogens globally cause significant yield losses in crop plants. Currently, approaches such as SIGS or HIGS are being considered not only for functional genomics studies but also for the development of fungal resistant crop plants (Wang and Jin 2017; Qi *et al.* 2019; Rajam and Chauhan 2021). Both SIGS and HIGS involve silencing gene expression by

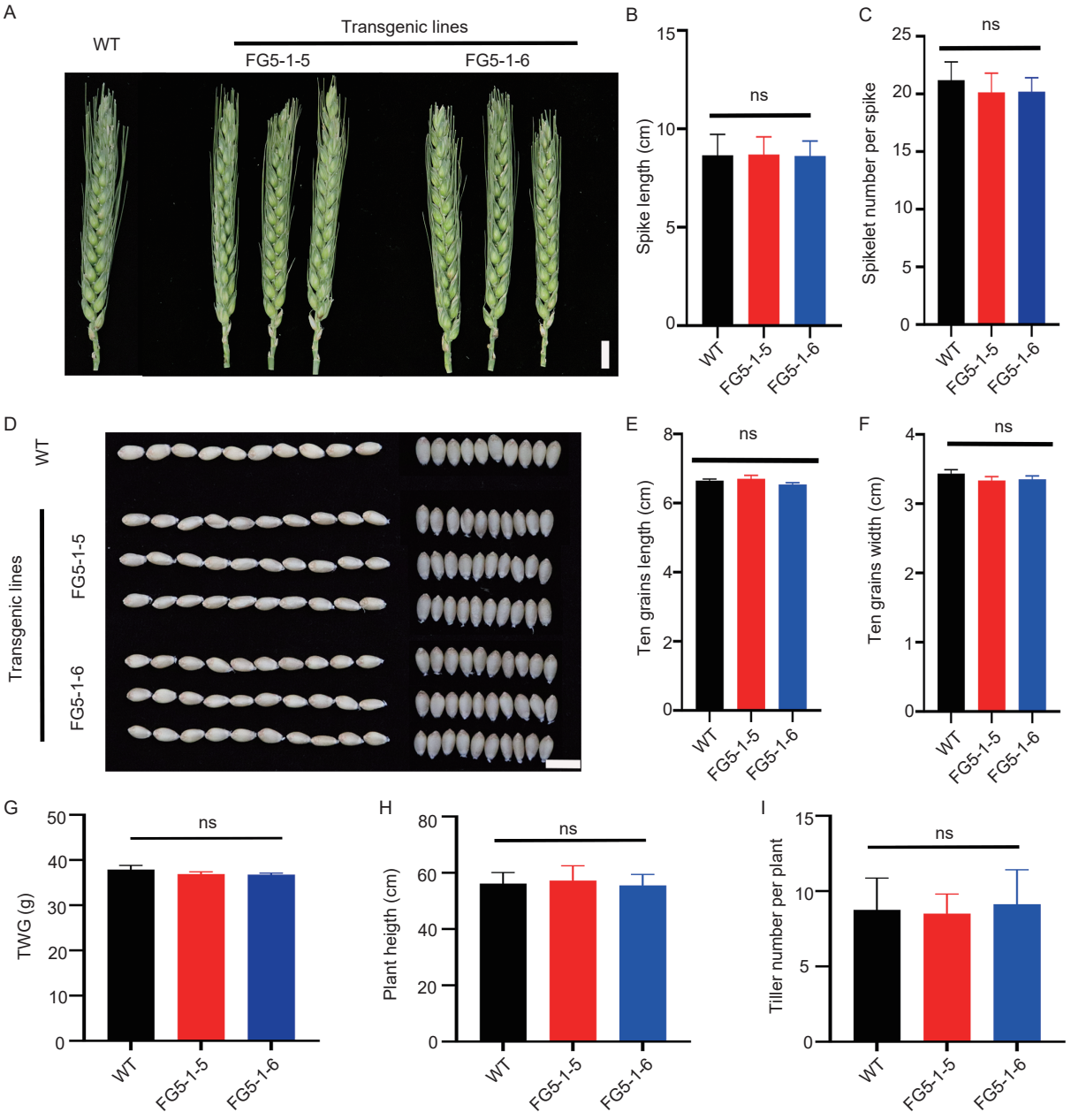


Fig. 5 Agronomic traits of HIGS transgenic wheat. A–C, representative images of ear morphology of wild-type (WT) and transgenic lines (FG5-1-5 and FG5-1-6) at grain-filling stage A. Statistical data represents the ear lengths B and spikelet number per spike C from at least 30 samples of each genotype. Scale bars, 1 cm. D–F, grain traits of WT and transgenic lines. Statistical data represents the grain length and grain width from three repeats of each genotype. G, statistical data of the thousand grain weight (TWG) between WT and transgenics (n=3). H and I, statistics on the plant height and tiller number per plant at the adult stage for WT and transgenic plants (n=9 of plant height; n=9 of tiller number). Data were represented as the mean±SD of three replicates. P-value, Student's t-test, ns indicates not significant.

spraying siRNA or expressing dsRNA against specific genes of the pathogen in the host plant. In this study, we employed SIGS and HIGS against one the most devastating diseases, Fusarium head blight, in wheat, targeting five pivotal genes associated with *F. graminearum* pathogenicity. Direct SIGS application of fungal dsRNAs, individually or in combination, effectively suppressed target gene expression, mitigating pathogenic symptoms in wheat leaves. Importantly, HIGS transgenic wheat lines expressing chimeric RNAi cassettes exhibited robust resistance to *F. graminearum* associated diseases, such as FHB and FCR, along with reduced DON accumulation. Therefore, SIGS and HIGS emerge as potent strategies for enhancing wheat resistance to *F. graminearum* infection by targeting multiple key pathogenic genes.

Due to the limitation of traditional methods, such as environmental concerns from fungicide applications, in the management of crops diseases, RNAi-based gene silencing of pathogen genes is becoming a promising strategy for improving crop resistance. This tool-based approaches, such as SIGS and HIGS, have been shown to be a potent and novel alternative for controlling gene expression via small RNAs (Wang and Jin 2017; Qi *et al*. 2019; Rajam and Chauhan 2021). In such strategies, choosing of target genes is the critical step for disease control. Targeting genes involved in the growth and development of fungal pathogens can help reduce selection pressure and the fast evolution of pathogens. In our study, the targeted genes of *F. graminearum*, including *Gpmk1*, *FgChy1*, *FgSR*, and *TRI5*, have been reported functioning in *F. graminearum* growth, development, pathogenicity and DON synthesis (Urban *et al.* 2003; Alexander *et al.* 2009; Liu *et al.* 2019; Cao *et al.* 2021), which is directly associated with the FHB spread in wheat spike and DON accumulation in grains. Both SIGS and HIGS assays demonstrated that the spraying siRNA or expressing dsRNAs can repress these gene expression in host. In addition, we introduced a potential new pathogenic gene, *FgTeaA*, for SIGS and HIGS. Spraying individual siRNA or co-expressing *FgTeaA*_dsRNA with other targets in wheat plants resulted in the suppressed expression of *FgTeaA*, leading to reduced disease symptoms during *F. graminearum* infection. *FgTeaA* is considered the homolog of yeast *Tea1*, potentially controlling virulence by modulating microtubule organization (Mata and Nurse 1997; Takeshita *et al*. 2008, 2013). However, the molecular function of *FgTeaA* in fungal development and pathogenicity requires further investigation, which could be achieved through creating a null mutant in *F. graminearum* strains.

In our SIGS assays, the expression of all target genes was repressed by spraying individual or combined siRNA in wheat leaves. However, in transgenic dsRNA-expressing wheat plants, not all five target genes were repressed, and RNAi silencing efficiency varied between leaves and spikes. For instance, the expression of *FgTeaA* was repressed in transgenic leaves but not in spikes during *F. graminearum* infection, suggesting that HIGS-mediated gene silencing may depend on dsRNA expression or processing efficiency in a tissue-specific manner. In addition, gene silencing may have different efficiency in various transgenic lines. For example, the inhibition of expression of *FgChy1 and FgSR* was different in wheat leaves and spikes between line FG5-1-5 and line FG5-1-6, but the two lines show the similar phenotype in resistance to FHB. We speculate that these target genes may share overlapping effects on pathogenicity, consistent with our criteria for selecting target genes. For example, both *FgChy1* and *FgSR* have been reported to play essential roles in *F. graminearum* pathogenicity, with mutations resulting in reduced spikelet susceptibility (Liu *et al.* 2019; Cao *et al.* 2021). Therefore, the repression of multiple pathogen genes confers high levels of disease resistance, and variations in the expression of a single gene may not significantly alter the resistance threshold. Employing chimeric RNAi cassettes to silence multiple genes of HIGS, further validate that transgenics with more silenced genes exhibited stronger resistance to *F. graminearum*-associated diseases (Appendix D). We thus conclude that these five genes collectively contribute to conferring FHB resistance in our HIGS assays. Therefore, expressing chimeric RNAi cassettes targeting multiple genes is promising to ensure the high efficiency of HIGS. Among the selected target genes, *TRI5* has been reported to play a biochemical role in DON synthesis during *F. graminearum* infection in wheat heads (Maier *et al.* 2006). However, our data indicate that repressing the expression of *TRI5* also leads to decreased disease symptoms in wheat leaves. While the specific role of *TRI5*-mediated DON synthesis in leaves remains unknown, our results suggest that *TRI5* plays a broader role in *F. graminearum* pathogenicity across host tissues. This may result from the role of *TRI5* in pathogen growth and development, consistent with the observed defects in *F. graminearum* growth in *tri5* mutants, as previously described (Desjardins *et al.* 2000).

In previous studies, HIGS has shown to be a key approach in controlling disease spread in plants, utilizing genome database information of the pathogen to target pathogen-specific genes in crop plants. *Brachypodium distachyon* and wheat transgenics expressing dsRNA to silence the protein kinases gene and β-1,3-glucan synthase gene of *F. graminearum* and *F. culmorum* exhibit the reduced pathogenicity, altering the expression levels of pathogen-specific genes and thereby gaining resistance against FHB (Chen *et al.* 2016; He *et al.* 2019). Similarly, wheat transgenics lines expressing RNAi constructs against MAPK1 and cyclophilin of *Puccinia triticina* demonstrate the increased resistance against leaf rust infection (Panwar *et al.* 2018). Targeting pathogen-specific MAP kinase confers resistance against *Puccinia triticina*, *Rhizoctonia solani* and *P. striiformis*, and proving a vital role for HIGS in controlling rust and sheath blight disease in wheat and rice, respectively (Tiwari *et al.* 2017; Zhu *et al.* 2017; Panwar *et al.* 2018). In our HIGS assays, transgenic wheat lines expressing dsRNA for five target genes showed a strong resistance to *F. graminearum* associated diseases, which is consistent with our SIGS that employing combined dsRNAs exhibited a superior resistance to disease, compared to the application of individual ones. We thus propose a new strategy for combined HIGS in targeting multiple genes from different pathogens, such as *F. graminearum*, *Puccinia triticina*, or *Blumeria graminis* f. sp. *tritici*, as an effective approach to deliver broad-spectrum resistance in crops against a different range of diseases.

Importantly, one challenge of HIGS for breeding purposes is pathogen gene-specific targeting, achieved by selecting an off-target-free region to avoid unintended effects on any beneficial organism or host plant (Rajam and Chauhan 2021). Our HIGS-based transgenic wheat plants exhibited no adverse effects on agronomic traits, indicating the feasibility of this strategy in breeding for FHB-resistant wheat without yield penalties.

5. Conclusion

The results of the present study demonstrate that the potential of RNAi-based strategies, including SIGS and HIGS, in enhancing wheat resistance to FHB. Expressing dsRNA with multi-gene targeting of *F. graminearum* pathogenicity in wheat effectively suppresses target gene expression, reduces FHB symptoms and lowers DON accumulation. Further testing of these transgenic plants in field trials will be crucial to evaluate RNAi-based strategies in agricultural applications. Overall, the specificity of RNAi in gene expression silencing combined with multiple-pathogen targets of the HIGS strategy offers a promising avenue for sustainable disease management in crops.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

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