The Sclerotinia sclerotiorum-inducible promoter \( pBnGH17^D7 \) in \textit{Brassica napus}: isolation, characterization, and application in host-induced gene silencing

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Abstract

Sclerotinia stem rot (SSR), caused by \textit{Sclerotinia sclerotiorum}, is among the most devastating diseases in \textit{Brassica napus} worldwide. Conventional breeding for SSR resistance in \textit{Brassica} species is challenging due to the limited availability of resistant germplasm. Therefore, genetic engineering is an attractive approach for developing SSR-resistant \textit{Brassica} crops. Compared with the constitutive promoter, an \textit{S. sclerotiorum}-inducible promoter would avoid ectopic expression of defense genes that may cause plant growth deficits. In this study, we generated a \textit{S. sclerotiorum}-inducible promoter, \( pBnGH17^D7 \), from the promoter of \textit{B. napus} glycosyl hydrolase 17 gene (\( pBnGH17 \)). Specifically, 5’-deletion and promoter activity analyses in transgenic \textit{Arabidopsis thaliana} plants defined a 189 bp region of \( pBnGH17 \) which was indispensable for \textit{S. sclerotiorum}-induced response. Compared with \( pBnGH17 \), \( pBnGH17^D7 \) showed a similar response upon \textit{S. sclerotiorum} infection, but lower activity in plant tissues in the absence of \textit{S. sclerotiorum} infection. Moreover, we revealed that the transcription factor BnTGA7 directly binds to the TGACG motif in \( pBnGH17^D7 \) to activate \( BnGH17 \). Ultimately, \( pBnGH17^D7 \) was exploited for engineering \textit{Sclerotinia}-resistant \textit{B. napus} via host-induced gene silencing. It induces high expression of siRNAs against the \textit{S. sclerotiorum} pathogenic factor gene specifically during infection, leading to increased resistance.

Keywords: \textit{Brassica napus}, host-induced gene silencing, inducible promoter, \textit{Sclerotinia sclerotiorum}, TGA7, TGACG motif.

Introduction

Due to the world’s growing population and changing climate, powerful tools are needed to engineer desirable agronomic traits in crops that increase productivity. Compared with conventional breeding, genetic engineering enables the introduction, removal, or modification of desired genes in a specific crop with minimal modifications to the crop genome (Dong and Ronald, 2019). Three different types of promoters are typically employed to control transgene expression in plant genetic engineering: constitutive promoters, inducible promoters, and tissue-specific promoters. In plants, constitutive promoters such as the \textit{Cauliflower mosaic virus} (CaMV) \( 35S \) and maize \textit{ubiquitin} promoters are most commonly used (Benfey and Chua,
However, constitutive promoters drive transgene expression throughout all stages of plant development, in most plant tissues, and under all conditions, making it challenging to control specific temporal and spatial expression of transgenes (Holtorf et al., 1995; Sunilkumar et al., 2002). In addition, constitutive promoters trigger a continuously high level of transgene expression, which often leads to unnecessary nutrition consumption and plant growth deficits (Hull et al., 2000; Pino et al., 2007). In contrast to constitutive promoters, transgenes driven by tissue-specific promoters could achieve optimal effectiveness (Koellhoffer et al., 2015; Li et al., 2019). Inducible promoters that regulate transgene expression in a desired temporal and/or spatial manner lessen the incidence of unexpected adverse effects on plant growth. Therefore, tissue-specific and inducible promoters are the preferred route in plant genetic engineering.

Plants deploy a wide range of immune defense strategies against pathogens that can be exploited to confer disease resistance through genetic engineering. However, genetic immunity to disease often comes with the cost of reduced plant growth and reproduction (Ning et al., 2017; Guo et al., 2018). Yield penalties caused by enhanced disease resistance have been described in several crop species. The wheat resistance (R) gene Wsm1 conferred plant resistance to _Wheat streak mosaic virus_, but caused a relative 11–28% reduction in yield, even in unstressed conditions (Sharp et al., 2002). A similar observation was made with powdery mildew-resistant barley, in which the _mlo_ R gene-mediated resistance to _Bgh_ (powdery mildew) reduced grain yield by 4% (Jørgensen, 1992). In _Arabidopsis thaliana_, non-expresser of pathogenesis-related gene 1 (_AtNPR1_), a master immune regulatory gene, was a prime gene to be employed in disease resistance engineering, in that _AtNPR1_ conferred resistance to diverse pathogens in different plant species (Cao et al., 1998; Lin et al., 2004; Quilis et al., 2008; Wally et al., 2009). However, constitutive overexpression of _AtNPR1_ resulted in reduced plant height and yield loss in rice, limiting its potential application in broad-spectrum resistance (Quilis et al., 2008). Thus, immune responses during genetic engineering should be precisely regulated to mitigate the cost of resistance. This can be achieved by inducing the expression of defense genes at particular times or in specific plant tissues (Karasov et al., 2017). It is believed that expression of defense genes under the control of pathogen-inducible promoters could maintain the balance between plant growth and disease resistance during genetic engineering.

To date, a number of natural pathogen-inducible promoters have been isolated and identified in plants, including: the _Magnaporthe grisea_-inducible promoters OsR2329, OsR2184, and OsPBZ1 in rice (Sasaki et al., 2007); the _Phytophthora sojae_-inducible promoter _PcCMPG1_ in _Petroselinum crispum_ (Kirsch et al., 2001); the _Bgh_- and _Rhyhchosporium sclacil_-inducible promoter _HvGER1c_ in barley and wheat (Himmelbach et al., 2010); the _Uniculma nectar_-inducible promoter _VpSTS_ in grapevine (Xu et al., 2010); the _Xanthomonas axonopodis_-inducible promoter _NtpPPP1_ in _Citrus sinensis_ Osbeck (Zou et al., 2014); and the _Erwinia amylovora_-inducible promoters _Nstr246C_, _Ntsg24_, and _StPgs1_ in pear and apple (Malnoy et al., 2003, 2006). However, research on pathogen-inducible promoters in _Brassica_ crops is limited.

As a major oil crop worldwide, oilseed rape (_Brassica napus_) provides vegetable oil for humans and edible fodder for animals. However, the growth of oilseed rape is constantly threatened by _Sclerotinia stem rot_ (SSR). This disease, caused by the broad-host-range fungal pathogen _Sclerotinia sclerotiorum_, leads to severe reduction in seed yield and quality worldwide. SSR resistance in _B. napus_ is a quantitative trait, determined by multiple minor quantitative trait loci (QTLs) (Wu et al., 2016a). However, none of the QTLs has been cloned, limiting their utilization in SSR resistance breeding. Reverse genetic analysis of SSR resistance has been conducted, and several defense genes have been identified (Ding et al., 2021).

Genetic engineering for SSR resistance using the identified defense genes is a promising strategy for controlling SSR. However, overexpression of defense genes may cause plant growth deficits (Ning et al., 2017). Therefore, the regulation of defense gene expression precisely controlled by _S. sclerotiorum_-inducible promoters is an optimal strategy to generate SSR-resistant varieties with stable _B. napus_ yields. While two synthetic promoters containing pathogen-related cis-acting elements have been described that respond to _S. sclerotiorum_ (Shokouhifar et al., 2011a, b). To date, _S. sclerotiorum_-inducible promoters in plants have not been identified or explored.

Here, we identified an _S. sclerotiorum_-inducible promoter derived from _B. napus_ and highlighted its potential application in agriculture. The crucial promoter region and the core cis-elements that respond to _S. sclerotiorum_ were investigated by 5′-deletion analysis and site-directed mutagenesis. The transcription factor (TF) that directly binds to this crucial promoter region was verified by yeast one-hybrid (Y1H) assay, dual-luciferase assay (dual-LUC), and EMSA. Finally, this promoter was used to engineer SSR-resistant _B. napus_ and tested for its specificity. This research highlights the potential of a _S. sclerotiorum_-inducible promoter to facilitate precise genetic engineering of SSR-resistant _B. napus_ and potentially other crops.

Materials and methods

**Plant materials, abiotic stress and phytohormone treatments, and _S. sclerotiorum_ inoculation**

_B. napus_ line J9712 was kindly provided by Professor Yongmung Zhou (Huazhong Agricultural University, Wuhan, Hubei, China). Plants were grown in nutrient solution in the greenhouse for 4 weeks, and then the entire seedling plants were subjected to various treatments, including osmotic, cold, heat, and salt stresses, as well as to hydrogen peroxide (H₂O₂) and hormone treatments with salicylic acid (SA), abscisic acid (ABA), methyl jasmonate (MeJA), and ethephon (ETH), and _S. sclerotiorum_ inoculation. For the osmotic stress and salt stress treatments, seedlings were transferred to nutrient solutions containing 15% polyethylene glycol 6000 (PEG6000) and 200 mM NaCl, respectively, and sampled at 6 h after treatment, as described by Li et al. (2021a, b). For the cold and heat treatments, seedlings were transferred to growth chambers
with light intensity of ~300 µmol·m⁻²·s⁻¹ at 4 °C and 42 °C, respectively, and sampled 1 h and 6 h post-treatment (Li et al., 2021b). For the H₂O₂ and hormone treatments, seedlings were sprayed with 100 µM H₂O₂ (Mierek-Adamska et al., 2019), 1 mM SA (Wang et al., 2014a), 100 µM MeJA (Wang et al., 2014a), 100 µM ABA (Li et al., 2021b), and 100 µM ETH (Xue et al., 2020), and sampled at 3 h and 6 h post-treatments. For S. sclerotiorum inoculation, the S. sclerotiorum isolate SS-1 was cultured on potato dextrose agar (PDA; Becton, Dickinson and Company, Franklin Lakes, NJ, USA), as described by Wu et al. (2013). Agar plugs of 7 mm in diameter with S. sclerotiorum were used for detached leaf inoculation of unfolded leaves of B. napus line J9712, as described by Wu et al. (2021). Mock-inoculated leaves were treated with 7.0 µm diameter agar plugs. Tissues extending 10 mm beyond the inoculation site on the leaves were harvested at 3, 6, and 12 h after S. sclerotiorum or mock inoculation and stored at -80 °C. Three biological replicates were performed, and five plants were used for each biological replicate.

**Promoter isolation and promoter::GUS vector construction**

The 5′-flanking region upstream of the translation start codon of BnGH17 (BnaC01g21880D) was isolated from line J9712 using sequence-specific primers (Supplementary Table S1) that were based on reference genome sequences (Challhoub et al., 2014; Song et al., 2020). The cis-regulatory elements of the BnGH17 promoter (pBnGH17) were predicted using the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lesort et al., 2002).

For promoter deletion analysis, three deletions, D1 (−500 to −1), D2 (−848 to −1), and D3 (−1260 to −1), were generated by PCR. Four other deletions, D4 (−1784 to −1261 and −500 to −1), D5 (−1430 to −1261 and −848 to −1), and D7 (−1615 to −1427 and −500 to −1), were generated through splicing by the overlap extension PCR technique (Horton et al., 2013) with four primers pairs. (Supplementary Table S1). For site-directed mutagenesis analysis, splicing by the overlap extension PCR technique was used to introduce three mutations in the TGACG motifs of D7 at loci –1423/–1427. The primers were listed in Supplementary Table S1. To construct the promoter::GUS (β-glucuronidase) expression vectors, promoter fragments were cloned into pB1101 at EcoRI and BamH I restriction enzyme sites via homologous recombination (ClonExpress II One Step Cloning Kit, Vazyme, Nanjing, China).

**A. thaliana transformation and treatments**

Promoter::GUS recombinant plasmids were introduced into Agrobacte-
rium tumefaciens wild-type plants Columbia-0 (Col-0) were used for transformation via the floral dipping method (Zhang et al., 2006). Seeds of the T0 generation were selected on Murashige and Skoog (MS) medium supplemented with kanamycin (50 mg l⁻¹), and the positive plants were further verified by PCR. T1 and T2 transgenic plants were grown in nutrient soil and, after verification by PCR, they were transplanted for hormone treatments. S. sclerotiorum inoculation, and the GUS staining assay. All A. thali-
a-ana plants were grown in growth chambers under a 16 h light/8 h dark photoperiod (~300 µmol m⁻² s⁻¹) at 22 °C during the day and 20 °C at night, and 60% relative humidity.

For S. sclerotiorum inoculation, 4-week-old unfolded leaves were de-
tached from A. thaliana plants, and placed on agar for leaf inoculation. Mycelial agar plugs (2 mm in diameter) punched from the margin of a 2-day-old cultures of S. sclerotiorum grown on PDA were used as the inoculum and were closely appended to the adaxial surface of leaves, ac-
cording to Wu et al. (2013). Mock-inoculated leaves were treated with 2 mm diameter agar plugs. The inoculated and mock-inoculated leaves were covered with plastic film to maintain moisture at 22 °C. At 12 h and 24 h post-inoculation (hpi), the inoculated leaves were collected for GUS staining and quantitative real-time PCR (qRT-PCR) analysis, respectively. For H₂O₂ and hormone treatments, 4-week-old A. thali-
a-ana plants were sprayed with 1 mM SA (Kovacs et al., 2015), 200 µM MeJA (Jiang et al., 2014), 7 mM ETH (Qu et al., 2015), and 100 µM H₂O₂ (Zhang et al., 2003). At 6 h and 12 h post-treatment, A. thaliana leaves were collected for GUS staining and qRT-PCR analysis, re-
spectively. GUS staining was performed on four independent transgenic lines for each treatment. For qRT-PCR analysis, three independent biological replicates were performed, each with three technical replicates.

**Histochemical GUS staining**

Histochemical GUS staining was performed as described by Jefferson et al. (1987). Briefly, samples were incubated at 37 °C overnight in GUS staining buffer containing 0.5 mg ml⁻¹ X-gluc, 0.5 mM potassium fer-
rycyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.1% Triton X-100, and 10 mM sodium phosphate (pH 7.2). After that, the samples were bleached in 75% (v/v) ethanol and photographed using a stereo-
scope with a digital camera (E4Z4W, Leica, Bensheim, Germany).

**Total RNA extraction and qRT-PCR analysis**

Total RNA from different tissues and organs of A. thaliana and B. napus was extracted according to the TRIzol method using an RNAiso reagent kit (Vazyme) according to the manufacturer’s instructions. The total RNA was reverse transcribed into first-strand cDNA with a HiScript II 1st Strand cDNA Synthesis Kit (Vazyme). qRT-PCR was carried out using AceQ Universal SYBR qPCR Master Mix (Vazyme) in an ABI Step One Plus real-time PCR system (Applied Biosystems Inc., Foster City, CA, USA). The relative expression of each gene was calculated using the 2⁻△△Ct method (Livak and Schmittgen, 2001). BnUBC9 (BnaC08g12720D) and BnUBC10 (BnaA10g06670D) in B. napus (Wu et al., 2016b), AETF-1α (A5g06390) and AtUBQ10 (A5g53300) in A. thaliana (Yang et al., 2020, Zhu et al., 2013), and SActin (SS1G_08733) and Sstub1 (SS1G_04652) (Wu et al., 2021) in S. sclerotiorum were used as reference genes. The qRT-PCR primers used are shown in Supplementary Table S1. All qRT-PCR experi-
ments were performed with three biological replicates, each with three technical replicates.

**Y1H assay**

A Y1H assay was performed as described by Ou et al. (2011). An A. thali-
a-ana TF library containing 1589 GAL4-AD-fused TFs was provided by Professor Lijia Qu (Peking University, Beijing, China). The pBnGH17 subfragment was amplified by PCR, cloned into a pHisI-1 vector, and then transformed into yeast strain YM4271 as bait. The A. thaliana TF pooled library strains and the bait clone were grown in SD-Leu and SD-His medium overnight, respectively. The pooled library strains and bait clones were mixed at equal volumes (20 µl per well) and transferred to new 2 ml 96-well plates with yeast extract peptone dextrose medium. After 1 d growth with shaking at 200 rpm at 30 °C, the mating products were diluted 10-fold with water and then the diluted mating products (10 µl per well) were plated on screening plates [SD-Leu -His+15 mM 3-amino-triazole (3-AT)] and subsequently grown for 3 d.

The point-to-point Y1H assay was carried out according to Yang et al. (2011). Briefly, the pBnGH17 subfragment was cloned into the pHIS2 vector as bait. The full-length coding sequences (CDSs) of the TGA TF genes BnTGA7 (BnaA07g33790D) and BnTGA3 (BnaC05g17700D) were amplified by PCR and cloned into the pGADT7 vector as prey. Two plasmids were co-transformed into yeast strain Y187. Transformed clones were cultured on SD/-His/-Leu/-Trp selective medium containing 50 mM 3-AT for 3 d at 30 °C. The p53HIS2 and pGAD-Rec2-53
vectors were used as positive controls, and the pHS2 and pGAD-Rec2-53 vectors were used as negative controls.

Dual-luciferase reporter gene assay

The full-length CDSs of BrnTGA7 and BrnTGA3 were cloned into the pGREEN II 62-SK vector to generate effector constructs. The pBnGH17D7 subfragment was inserted ahead of the firefly luciferase (LUC) gene in the pGREEN II 0800-LUC vector to generate a reporter construct. Then, recombinant effector and reporter constructs and the empty vector pGREEN II 62-SK were introduced into A. tumefaciens strain GV3101 (with the helper PSoup-P19 plasmid) by electroporation and used to infect Nicotiana benthamiana leaves in the light (16 h/day) at 25 °C for 2 d by Agrobacterium-mediated infiltration to induce transient gene expression (Helenus et al., 2005). The activities of the firefly luciferase (LUC) and Renilla luciferase (REN) were determined with a Dual-Luciferase Reporter Kit (Vazyme) according to the manufacturer’s instructions and detected with a microplate reader (Tecan Spark, Tecan Trading AG, Zurich, Switzerland).

EMSA

The full-length CDS of BrnTGA7 was cloned into the pGEX6p-1 vector to generate the glutathione S-transferase (GST)–BrnTGA7 fusion protein, which was expressed in Escherichia coli strain BL21. Expression and purification of the GST–BrnTGA7 protein were performed according to the manufacturer’s instructions (Transgen, Beijing, China). A 30 bp DNA sequence containing the TGACG sequence was synthesized by Beijing Qingke Biotechnology and labeled with an EMSA Probe Biotin Labeling Kit (Beyotime, Shanghai, China). EMSA was performed using a chemiluminescent EMSA kit according to the manufacturer’s instructions (Beyotime). In brief, GST–BrnTGA7 and the labeled probe were incubated at 25 °C for 20 min in a reaction system containing EMSA/gel-shift binding buffer. For the cold competition, 200-fold unlabeled probe was added to the reaction mixtures. These reaction mixtures were loaded on an 8% native PAGE gel; after transferring to a nylon membrane, cross-linking, and blocking the membrane, the reaction mixtures were hybridized with a random mixture of radiolabeled DNA probes. The autoradiographs were generated using the emulsion method.

Host-induced gene silencing (HIGS) of the S. sclerotiorum endo-polygalacturonase gene (SsPG1) driven by pBnGH17D7

The HIGS construct was transformed into A. tumefaciens strain GV3101 by electroporation and then transformed into B. napus line J9712 via A. tumefaciens-mediated hypocotyl transformation as described by Liu et al. (2021a). Positive transgenic B. napus plants were selected by PCR with specific primers (Supplementary Table S1). All transgenic B. napus plants used in this study were from the T2 generation.

The resistance of transgenic B. napus plants to S. sclerotiorum was assessed by detached leaf, cotyledon, and stem inoculation, according to Wu et al. (2021). Approximately 15 plants in each of the three replicates were assessed for each line. For cotyledon inoculation, both the J9712 and T2 transgenic lines were grown in growth chambers with light intensity of 300 µmol·m−2·s−1 under a 16 h light/8 h dark photoperiod at 24 °C and 60% relative humidity. For detached leaf and stem inoculation, all plants were grown in the experimental field at Yangzhou University, Jiangsu, China. The field experiment was conducted using a randomized complete block design with three replications.

Small RNA sequencing was performed to determine the expression of target gene-specific siRNAs in HIGS transgenic plants. Sclerotinia sclerotiorum- and mock-inoculated leaves of two independent transgenic T1 lines (three plants for each treatment) were randomly selected and mixed for small RNA sequencing. Small RNA sequencing and analyses were performed as described by Wu et al. (2021).

Determination of polygalacturonase (PG) activity

To quantify PG activity in S. sclerotiorum-inoculated leaves, tissues extending 10 mm beyond the inoculation site on leaves were harvested at 24 hpi and then ground into powder with liquid nitrogen. Up to 0.1 g of each sample was used to determine the enzyme activity with the Polygalacturonase assay kit (Solarbio, Beijing, China), according to the manufacturer’s instructions. The absorbance was recorded at 540 nm using a Tecan Infinite microplate reader (Spark M200, Tecan Austria GmbH, Grodig, Austria). Enzyme activity was defined as the decomposition of polygalacturonic acid per g of sample per hour at 40 °C, pH 6.0 to produce 1 µmol of galacturonic acid.

Statistical analysis

Significance analysis was performed with Student’s t-test for comparing two independent groups (☆*<0.05 and **P<0.01) by IBM SPSS Statistics, New York, NY, USA.

Results

Screening of the S. sclerotiorum-inducible promoter in B. napus

To determine the candidate genes induced by S. sclerotiorum, we performed transcriptomic analyses of B. napus before and after S. sclerotiorum infection using RNA sequencing (Wu et al., 2016b). Among the differentially expressed genes between the S. sclerotiorum–challenged and mock-inoculated samples, the six most strongly induced genes, comprising one cysteine-rich secretory protein-, antigen 5-, and pathogenesis-related 1 protein (CAP)–encoding gene (BnaC01g04530D), two BnGH17 genes (BnaC01g21880D and BnaA01g17540D), and three legume lectin (BnLLP) genes (BnaA05g24230D, BnaC0ng78710D, and BnaC01g36130D) (Fig. 1A), were examined for their expression patterns in diverse tissues and under various stress conditions. Based on the transcriptomic database of diverse tissues of the B. napus cultivar ‘ZS11’ (http://yanglab.hzau.edu.cn/BnTIR) (Liu et al., 2021b), BnaC01g04530D was eliminated from further analysis due to its ultrahigh expression level in roots [the transcripts per million mapped reads (TPM) value was 7558; Supplementary Fig. S1]. The three BnLLP genes were highly expressed in the root and silique walls and moderately expressed in the leaf (Supplementary Fig. S1), while the expression levels of the two BnGH17 genes were extremely low in most of the tissues, except in roots (Fig. 1B; Supplementary Fig. S1). The expression patterns of BnGH17 (BnaC01g21880D) under various stress conditions and with hormone treatments were determined, and BnGH17 was not significantly induced by osmotic, cold, heat, or salt stress, or by H2O2, SA, MeJA, ABA, or ETH treatment (Fig. 1C; Supplementary Fig. S2). Consequently, the BnGH17 promoter was selected as the potential S. sclerotiorum-inducible promoter for further analysis.
The S. sclerotiorum-inducible promoter pBnGH17 D7 in B. napus

Isolation and characterization of the BnGH17 promoter

To uncover the underlying molecular mechanism of the BnGH17 promoter upon S. sclerotiorum infection, a 1784 bp promoter fragment upstream of the translational start site of BnGH17 (BnaC01g21880D) was cloned. The known cis-regulatory elements in the promoter of BnGH17 (pBnGH17) were analyzed with the PlantCARE online software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al., 2002). In addition to core promoter elements (three putative TATA boxes and one putative CAAT box) (Kadonaga et al., 1986; Ranish et al., 1999), multiple other cis-elements were mapped out in pBnGH17, including four ABA-responsive elements (ABREs) (Guiltinan et al., 1990).
two MeJA-responsive elements (one CGTCA motif and one TGACG motif) (Wang et al., 2011), two WRKY-binding sites (W boxes) (Rushton et al., 1995), one basic helix–loop–helix (bHLH)-binding site (CANNTG motif) (Blackwell and Weintraub, 1990), one ethylene-responsive element (GCC box) (Ohme-Takagi and Shinshi, 1995), and four pathogen- and salt-inducible elements (GT-1 elements) (Park et al., 2004) (Table 1). Our results indicated that pBnGH17 is probably a stress-responsive promoter.

To test its activity in vivo, pBnGH17 was fused with GUS and transformed into wild-type A. thaliana plants. Histochemical GUS staining was carried out in different tissues of A. thaliana, including 10-day-old seedlings, rosette leaves, mature roots, inflorescences, siliques with developing seeds, and stems. GUS activity was slightly detected in seedlings and mature roots, but not in other tissues (Fig. 2A–F) during different growth stages. Relative expression of the GUS gene, determined by qRT-PCR, was consistent with the observed GUS staining pattern (Fig. 2G; Supplementary Fig. S3A).

In addition, the GUS staining pattern was visible in pBnGH17::GUS transgenic A. thaliana leaves treated with S. sclerotiorum at 12 hpi, and was much stronger at 24 hpi (Fig. 2H). In contrast, GUS activity was not detectable in the leaves of the mock-inoculated controls (Fig. 2H). In this case, the relative expression of GUS detected by qRT-PCR was also consistent with the GUS staining results: GUS expression increased by nearly 200-fold at 12 hpi, and then by 400-fold at 24 hpi as compared with the mock-inoculated controls (Fig. 2I; Supplementary Fig. S3B).

The qRT-PCR results showed that the expression of BnGH17 was not induced by H2O2, SA, MeJA, or ETH treatments (Fig. 1C), even though several hormone-responsive elements were identified in the promoter region (Table 1). To further confirm this result, we examined GUS activity in pBnGH17::GUS transgenic A. thaliana after H2O2, SA, MeJA, and ETH treatments. Unsurprisingly, GUS staining was not detected at 6 h and 12 h after the H2O2, SA, and ETH treatments. While it was not detected 6 h after the MeJA treatment,
weak GUS staining was detected 12 h later (Fig. 2J), consistent with GUS expression determined by qRT-PCR (Fig. 2K; Supplementary Fig. S3C). Collectively, our analysis supported the view that pBnGH17 is a S. sclerotiorum-inducible promoter containing an S. sclerotiorum-inducible fragment.

Deletion analysis of pBnGH17 in transgenic A. thaliana

To define the cis-regulatory sequences enabling response to S. sclerotiorum infection, a series of 5’ deletions of pBnGH17 were generated (Fig. 3A). Each of these fragments was further fused with GUS and transformed into A. thaliana. Histochemical GUS staining was performed with the leaves of transgenic A. thaliana inoculated with and/or without S. sclerotiorum. Initially, we generated three sequences, D1 (–500 to –1), D2 (–848 to –1), and D3 (–1260 to –1). Unexpectedly, GUS activity was not detectable in the transgenic A. thaliana plants after S. sclerotiorum inoculation (Fig. 3B), indicating that the fragment in the −1784 to −1261 region contains a compulsory molecular component for triggering GUS expression after S. sclerotiorum infection.

To further narrow down the region of pBnGH17 that is responsible for the S. sclerotiorum infection, we generated another four deletions and linked them to putative core promoter regions, named D4–D7 (Fig. 3A). Interestingly, the D4 and D7 constructs in transgenic A. thaliana exhibited GUS activity after S. sclerotiorum infection as strong as the full-length pBnGH17 construct in transgenic A. thaliana (Fig. 3B). However, the GUS staining pattern did not develop in the plants harboring D5 and D6 after S. sclerotiorum infection (Fig. 3B), indicating that the 189 bp promoter region located between positions −1615 and −1427 in pBnGH17 is fundamentally essential for S. sclerotiorum infection responsiveness.

We next compared the GUS activity pattern between full-length pBnGH17 and D7 in different plant tissues. Consistent with plants harboring full-length pBnGH17, GUS activity of the D7 construct was undetectable in the rosette leaves (Fig. 3D), inflorescences (Fig. 3F), siliques with developing seeds (Fig. 3G), and stems (Fig. 3H) of transgenic A. thaliana under normal growth conditions. In addition, the D7 construct also abolished GUS activity that was shown in seedlings and mature roots in full-length pBnGH17 transgenic plants at the growth stage without stress (Fig. 3C, E). Hence, pBnGH17D7 was assumed to be an ideal inducible promoter to drive transgene expression during S. sclerotiorum infection.

BnTGA7 interacts with the TGACG motif in pBnGH17

To identify the putative transcription regulators that interact with pBnGH17 to mediate S. sclerotiorum-inducible gene expression, we screened a library of 1589 GAL4-fused A. thaliana TFs in a mating-based Y1H assay with the −1615 to −1427 region of pBnGH17 (pBnGH17−1615 to −1427) as bait (Ou et al., 2011). At1g77920 (AtTGA7) and At1g22070 (AtTGA3) were isolated in this high-throughput A. thaliana TF screening system. We then cloned the full-length CDS of BnTGA7 and BnTGA3 in B. napus. Using a point-to-point Y1H assay, we confirmed that both BnTGA3 (BnaC05g17700D) and BnTGA7 (BnaA07g33790D) interacted with pBnGH17 (1615 to −1427) (Fig. 4A).

We further employed a dual-LUC reporter system in N. benthamiana to examine the transcriptional activity of BnTGA3 and BnTGA7 in vivo. The pBnGH17D7 promoter–driven firefly luciferase (LUC) reporter and 35S promoter–driven Renilla luciferase (REN, the internal control) were co-introduced into the plasmid as the reporter. Plasmids with or without BnTGA3/BnTGA7 were used as the effector. The LUC:REN ratio, reflecting the transcriptional activity of the pBnGH17D7 promoter, was monitored after both the effector and reporter were transiently co-expressed in N. benthamiana. The co-expression of pBnGH17D7::LUC/p35S::REN and BnTGA7, but not BnTGA3, remarkably increased the LUC:REN ratio (Fig. 4B), suggesting that BnTGA7 activated pBnGH17D7 promoter–driven transcription in vivo.

TGA7, belonging to the TGA TF family, regulated the expression of defense genes (such as pathogenesis-related 1, PR-1) by directly binding to the TGACG motif in A. thaliana (Shearer et al., 2009). As one putative TGACG motif was identified within pBnGH17 (1615 to −1427), we subsequently tested if BnTGA7 directly binds to the TGACG motif of pBnGH17

Table 1. Putative known cis-acting elements in the BnGH17 promoter sequence

| Cis-element | Description | Motif position* | Reference |
|-------------|-------------|----------------|-----------|
| ABRE | ABA-responsive element | −1430, −1367, −1246, −517 | Guilman et al. (1990) |
| CGTCA motif | MeJA-responsive element | −493 | Wang et al. (2011) |
| TGACG motif | MeJA-responsive element | −1432 | Wang et al. (2011) |
| W-box | WRKY-binding site | −835, −237 | Rushton et al. (1995) |
| CANNTG motif | bHLH-binding site | −1688 | Blackwell et al. (1990) |
| GCC box | Ethylene-responsive element | −1219 | Ohme-Takagi et al. (1995) |
| GT-1 | Salt- and pathogen-responsive element | −862, −548, −409 | Park et al. (2004) |
| TATA box | Core promoter element | −966, −799, −131 | Ranish et al. (1999) |
| CAAT box | Core promoter element | −61 | Kadonaga et al. (1986) |

* Motif positions are indicated relative to the start codon ATG.
through EMSA in vitro. The GST–BnTGA7 recombinant protein was capable of binding to probes containing the TGACG motif, whereas the GST protein alone was not functional (Fig. 4C). When the labeled probe was replaced with a mutated probe, the binding was completely abolished (Fig. 4C). Moreover, the change in expression of BnTGA7 in the leaves of J9712 was detected after S. sclerotiorum inoculation. The transcript level of BnTGA7 was slightly induced at 3 hpi, reached a peak at 6 hpi, and finally recovered at 12 hpi (Supplementary Fig. S4A, B). The expression of BnGH17 was slightly induced at 3 hpi and kept increasing until 12 hpi (Supplementary Fig. S4C, D), suggesting that BnTGA7 functions upstream of BnGH17 and activates the expression of BnGH17 in response to S. sclerotiorum.

To further study the contribution of the identified cis-element, the TGACG motif, on S. sclerotiorum-inducible gene expression, the TGACG motif of pBnGH17D7 was mutated to AGGGG, and the mutated promoter fragment pBnGH17D7-Mut was fused with the GUS gene (Fig. 4D). GUS activities were completely abolished in transgenic A. thaliana lines carrying the pBnGH17D7-Mut::GUS construct after S. sclerotiorum infection (Fig. 4D), further supporting our conclusion that the TGACG motif is the core motif for S. sclerotiorum response (Fig. 4D).

Taken together, the results of the Y1H assay, dual-LUC assay, EMSA, and site-directed mutagenesis of the TF-binding site suggested that BnTGA7 directly binds to the TGACG motif of
The *S. sclerotiorum*-inducible promoter *pBnGH17 D7* in *B. napus* | 6671

**Fig. 4.** BnTGA7 directly binds to and transactivates *pBnGH17*. (A) Yeast one-hybrid assay of the binding activity of BnTGA3/BnTGA7 with *pBnGH17 D7*. Positive controls, p53HIS2 and pGAD-Rec2-53. Negative controls, pHis2 and pGAD-Rec2-53. (B) Dual-luciferase reporter assay of the interaction between BnTGA3/BnTGA7 and *pBnGH17 D7* in *N. benthamiana* leaves. The LUC/REN value of the control was set as 1 for calibration. The error bars indicate the SD. Statistical significance was determined by Student’s *t*-test (**P<0.01). (C) EMSA of the specific binding of recombinant BnTGA7 protein to the TGACG motif of *pBnGH17 D7*. Underlining signifies the TGACG motif sequence, and asterisks represent the mutated base in the TGACG motif. GST, GST–BnTGA7, labeled probe, labeled mutant probe, and 200-fold unlabeled probe were present (+) or absent (−) in each reaction. (D) Histochemical GUS staining of two independent transgenic *A. thaliana* lines (T1) harboring the mutated promoter fragment *pBnGH17 D7-Mut::GUS* fusion at 24 h post-inoculation with *S. sclerotiorum* (*S.s*). In *pBnGH17 D7-Mut*, the TGACG motif was mutated to AGGGG. Col-0 (WT) was the negative control. Transgenic plants harboring the *pBnGH17 D7::GUS* fusion were the positive control.

*pBnGH17* and activates *pBnGH17*-driven transcription after *S. sclerotiorum* infection.

**Application of pBnGH17** to engineer Sclerotinia-resistant *B. napus*

PG is one of the most crucial cell wall-degrading enzymes in *S. sclerotiorum* pathogenicity (*Amselem et al., 2011*). HIGS has been shown to induce gene silencing in pathogens by *in planta* expression of dsRNA or hairpin RNAs (hpRNAs) homologous to essential and/or pathogenicity genes of pathogens, conferring engineered plant protection from infection (*Nowara et al., 2010*). It was recently revealed that HIGS of a pathogenic factor gene (endo-polygalacturonase gene, *SsPG1*) of *S. sclerotiorum* could be an effective strategy for controlling Sclerotinia rot in *B. napus* (*Wu et al., 2021*). Therefore, we further investigated whether *pBnGH17 D7* is able to induce the expression of siRNAs in HIGS transgenic plants exclusively upon *S. sclerotiorum* infection. The HIGS target sequence of *SsPG1* was inserted into the intron-containing hairpin vector pMDC83-ihpRNAi to generate the HIGS construct, which is composed of the hygromycin phosphotransferase selection marker gene, the *pBnGH17 D7* promoter, a spacer sequence (PDK intron), and the nopaline synthase terminator (Fig. 5A).

The HIGS construct was transformed into the *B. napus* line J9712, and five independent *T1*-positive transgenic plants were obtained. To determine the expression of complementary siRNAs in HIGS transgenic plants, leaves of two transgenic *T1* lines (RNAi-4 and RNAi-7) were harvested 24 h after *S. sclerotiorum* or mock inoculation and
Fig. 5. Host-induced gene silencing (HIGS) of the *S. sclerotiorum* endo-polygalacturonase gene (*SsPG1*) in *B. napus*. (A) Schematic of the HIGS construct. The RNAi expression cassette was driven by pBnGH17D7. Sense (S) and antisense (AS) fragments were inserted between pBnGHD7 and the nos terminator (nos T), forming a sense–pyruvate orthophosphate dikinase (PDK) intron (IntPDK)–antisense cassette. *Hyg*′, hygromycin phosphotransferase selection marker gene. (B) Small RNA profiling of *SsPG1* in uninoculated leaves (mock) and inoculated leaves (*S.s*) of HIGS transgenic T1 plants. The alignment tracks were obtained by mapping the Illumina sequence reads to the 381 bp sequence of *SsPG1*. (C–D, F–G) Assessment of disease resistance in HIGS transgenic *B. napus* to *S. sclerotiorum* as determined by cotyledon inoculation (C, D) and detached leaf inoculation (F, G). Disease lesions were photographed and measured 48 h post-inoculation (hpi). Scale bars=2 cm. (E, H) The expression levels of *SsPG1* in *S. sclerotiorum*-infected *B. napus* cotyledons (E) and leaves (H). Values were normalized to the fungal endogenous control gene Sstub1 (SS1G_04652). The values are presented as the mean ±SD of three independent biological replicates. The asterisks indicate significant differences (**P<0.01, Student’s t-test**). (I) The activity of polygalacturonase (PG) was determined in *S. sclerotiorum*-infected *B. napus* leaves at 24 hpi. Error bars indicate the SDs. Statistical significance was determined by Student’s t-test (**P<0.01).
mixed for small RNA sequencing. In the mock inoculation sample, only 31 siRNAs derived from the HIGS construct were detected, accounting for 0.0003% of all small RNAs detected in this library (Fig. 5B). However, after inoculation with *S. sclerotiorum*, the abundance of the specific siRNAs (54 341) in the HIGS transgenic plants was vastly elevated, accounting for 0.5234% of the total small RNAs detected in this library (Fig. 5B). The most abundant siRNAs were 21–24 nt in length (Supplementary Fig. S5) and were distributed across the target gene region (Fig. 5B). Thus, our data supported that *pBnGH17D7* triggered high expression of specific siRNAs in HIGS transgenic plants only during *S. sclerotiorum* infection.

Next, we evaluated the SSR resistance of the HIGS transgenic lines (T₂) with cotyledon inoculation. Noticeable water-soaked lesions initially appeared on the adaxial surface of the cotyledons of J9712 at ~24 hpi and quickly extended to the abaxial surface; symptoms become even more severe after 48 hpi (Fig. 5C). In contrast, the fungus-induced water-soaked lesions appeared only on the adaxial surface at 48 hpi in most of the cotyledons of HIGS transgenic lines (Fig. 5C). In addition, 31.1% and 21.7% of the cotyledons of transgenic lines RNAi-4 and RNAi-7 were not successfully infected by *S. sclerotiorum*. The average lesion area on cotyledons of the transgenic lines was reduced by 51.8–58.2% compared with those of J9712 (Fig. 5D). The relative expression levels of *SsPG1* on the *S. sclerotiorum*-inoculated cotyledons of RNAi-4 and RNAi-7 were reduced by 81.2% and 78.1%, respectively, at 48 hpi compared with that in J9712 (Fig. 5E; Supplementary Fig. S6A), indicative of an enhanced resistance of these HIGS transgenic lines to *S. sclerotiorum* caused by target gene silencing.

To investigate whether the *S. sclerotiorum*-resistant phenotype occurs in other plant tissues, we repeated the experiments in detached leaves and stems. At 48 hpi, the lesion area on leaves of transgenic lines RNAi-4 and RNAi-7 was reduced by 26.2% and 20.1%, respectively, compared with those on the J9712 leaves at 48 hpi (Fig. 5F, G). At 7 dpi, the lesion lengths on stems of transgenic lines RNAi-4 and RNAi-7 were reduced by 25.2% and 23.1%, respectively, compared with those on the J9712 stems at 7 dpi (Supplementary Fig. S7A, B). The relative expression levels of *SsPG1* in transgenic lines RNAi-4 and RNAi-7 were reduced by 74.6% and 80.8%, respectively, at 24 hpi compared with that in J9712 (Fig. 5H; Supplementary Fig. S6B). Furthermore, the decreased expression of *SsPG1* in *S. sclerotiorum* at 24 hpi resulted in lower PG activity on the leaves of HIGS transgenic plants during infection than in the J9712 plants (Fig. 5I).

We critically compared the agronomic traits between the HIGS transgenic and wild-type plants. Our data indicated that the crop yield and the quality of the HIGS transgenic plants was not significantly influenced (Supplementary Table S2). It is hence conceivable that transgene expression driven by *pBnGH17D7* is induced after *S. sclerotiorum* infection, thereby preventing unnecessary negative impacts on plant growth and development.

**Discussion**

*pBnGH17D7* activity is highly induced by *S. sclerotiorum*

In the past two decades, QTL mapping and genome-wide association studies have uncovered the genetic architecture of SSR resistance in oilseed rape (*Ding et al., 2021*), and a considerable number of SSR resistance QTLs have been identified. However, none of them has been subjected to fine-mapping or map-based cloning, which may be attributable to the difficulty of identifying resistance phenotypes of complex plant–microbe–environment interactions. This dilemma has limited the utilization of resistance QTLs in SSR resistance breeding. Thus, genetic engineering for resistance to *S. sclerotiorum* is a promising strategy for controlling SSR. To this end, the *S. sclerotiorum*-inducible promoter is valuable for driving defense gene expression in response to *S. sclerotiorum* infection with high specificity.

To date, tissue-specific promoters and abiotic stress-inducible promoters have been identified in *B. napus*, including the seed-specific promoter *Napin* (*Sohrabi et al., 2015*), the anthr-specific promoter *Sta 44* (*Hong et al., 1997*), the flower-specific promoters *FSP046* and *FSP061* (*Li et al., 2019*), and the cold-inducible promoter *BN115* (*Sangwan et al., 2001*). However, to the best of our knowledge, *S. sclerotiorum*-inducible promoters in *Brassica* have not been reported.

Collectively, our data revealed that *pBnGH17*, especially *pBnGH17D7*, is an *S. sclerotiorum*-inducible promoter. *pBnGH17* and *pBnGH17D7* activity are induced by *S. sclerotiorum*. While *BnGH17* was expressed at a low level in most plant tissues (only root-specific expression) under normal conditions, expression was highly induced upon *S. sclerotiorum* infection, but not by other abiotic stresses or hormone treatments (Fig. 1; Supplementary Fig. S2). In addition, we confirmed that the activity of the *BnGH17* promoter in transgenic *A. thaliana* was consistent with the *BnGH17* expression pattern in *B. napus* (Fig. 2; Supplementary Fig. S3). Although the activity of full-length *pBnGH17* was observed in the roots of transgenic *A. thaliana* (Fig. 2A, C), the promoter deletion *pBnGH17D7* almost completely abolished this activity (Fig. 3C, E). These data suggested that *pBnGH17*, especially *pBnGH17D7*, is potentially useful for SSR resistance breeding.

The potential application of *pBnGH17D7* for genetic engineering of resistance to *S. sclerotiorum*

In the past few decades, the molecular mechanism of resistance to SSR in *B. napus* has been systematically investigated. To date, several *S. sclerotiorum* resistance genes have been identified through functional genomic analysis, including *BnMKK3* (*Wang et al., 2019*); *BnMPK4* (*Wang et al., 2009*); *BnMPK6* (*Wang et al., 2020b); *BnWRKY33* (*Wang et al., 2014b; Liu et al., 2018*); *BnWRKY15* (*Liu et al., 2018*); *BnWRKY70* (*Sun et al., 2018*); *BnMKK4*, *BnWRKY28*, and *BnVQ12* (*Zhang et
Overexpression or knockout these defense-related genes might incredibly enhance resistance to *S. sclerotiorum* in *B. napus* (Liu et al., 2017; Quilis et al., 2018; X. Wang et al., 2020; Wang et al., 2020a; Zhang et al., 2021, Preprint); however, DNA mutations and alterations in the expression of these defense-related genes often negatively influenced plant growth and yield (Ning et al., 2017). Unfortunately, yield penalties caused by enhanced SSR resistance to date have not received much consideration, despite some evidence provided in studies on *A. thaliana*, rice and *N. benthamiana*. To name a few, overexpression of constitutively active *BnMKK* induced hypersensitive cell death in *N. benthamiana* leaves (Zhang et al., 2021, Preprint); constitutive expression of active *AtMPK3* resulted in a dwarf phenotype in *A. thaliana* (Genot et al., 2017); and overexpression of the *A. thaliana* gene *AtNPR1* in rice led to height reduction and yield loss (Quilis et al., 2008). Therefore, strictly controlling defense-related gene expression is gaining more and more attention. In this study, our data demonstrated that defense response. It was recently shown that overexpression of *BnNPR1* enhanced resistance to *S. sclerotiorum* in *B. napus*. These findings advanced our understanding of the TGA family in regulating PR-2 gene expression in the defense response. It was recently shown that overexpression of *BnNPR1* enhanced resistance to *S. sclerotiorum* in *B. napus* (Wang et al., 2020a), suggesting that *BnNPR1* plays a positive regulatory role in defense responses to *S. sclerotiorum* infection. Whether activation of *BnGH17* by *BnTGA7* depends on NPR1-mediated enhancement of DNA binding activity remains to be determined.

In summary, we established a new strategy for enhancing *Sclerotinia* resistance with minimal adverse effects in *B. napus*. The *S. sclerotiorum*-inducible promoter *pBnGH17*, which harbors the TGACG-motif between positions –1432 and –1427, is essential for the *S. sclerotiorum* response (Fig. 6). *BnTGA7* directly binds to the TGACG motif in *pBnGH17* and activates transcriptional expression of *BnGH17* (Fig. 6). Furthermore, *pBnGH17* was successfully utilized to drive SSR resistance regulator NPR1 (Kesarwani et al., 2007). To date, seven of 10 *A. thaliana* TGA TFs (TGA1–TGA7) have demonstrated interplay with NPR1 (Després et al., 2000; Kesarwani et al., 2007; Shearer et al., 2009). TGA1 and TGA4 (group I) control basal resistance independent of NPR1, despite their physical interaction (Shearer et al., 2012); TGA2, TGA5, and TGA6 (group II) play crucial but redundant roles in systemic acquired resistance (Y. Zhang et al., 2003; Kesarwani et al., 2007); and TGA3 and TGA7 (group III) are involved in basal resistance and the regulation of PR1 gene expression (Kesarwani et al., 2007; Chen et al., 2019). *BnGH17*, a member of the PR-2 protein group, encodes β-1,3-endoglucanase (Doxey et al., 2007). Our results suggested a role for *BnTGA7* in activating *BnGH17* expression in response to *S. sclerotiorum* infection in *B. napus*. These findings advanced our understanding of the TGA family in regulating PR-2 gene expression in the defense response.

![Diagram](http://biorender.com)

**Fig. 6.** A proposed model illustrating that the *BnGH17* promoter (*pBnGH17*) activity was regulated by *BnTGA7* upon *S. sclerotiorum* infection. *BnTGA7* directly binds to the TGACG motif of *pBnGH17* and activates expression of *BnGH17* exclusively under *S. sclerotiorum* infection. This figure was generated with BioRender (http://biorender.com).

**BnTGA7 regulates the expression of BnGH17 in response to *S. sclerotiorum* infection.**

In this study, we identified TFs that can interact with *pBnGH17* with the high-throughput Arabidopsis TF screening system (Ou et al., 2011). We clarified that *BnTGA7* directly binds to the TGACG motif of *pBnGH17* and activates the expression of *BnGH17* (Fig. 4). Moreover, *BnTGA7* was significantly up-regulated after *S. sclerotiorum* infection (Supplementary Fig. S4A, B), suggesting the potential role of *BnTGA7* in SSR resistance. TGA family members have been implicated as important plant defense regulators possibly through physical interaction with the known master immune
in *B. napus* via the HIGS technique. As *S. sclerotiorum* is a typical necrotrophic fungal pathogen with a wide range of hosts, this work will also offer reference for engineering SSR resistance into other important economic crops such as soybean, sunflower, and peanut.

**Supplementary data**

The following supplementary data are available at *JXB* online.

- Fig. S1. Tissue-specific expression pattern of BnaC01g04530D, BnaA05g24230D, BnaC01g36130D, and BnaCnng78710D.

- Fig. S2. The expression patterns of *BnGH17* under various stress conditions.

- Fig. S3. GUS expression analysis in *pBnGH17::GUS* transgenic *A. thaliana* plants.

- Fig. S4. Expression analysis of *BnTGA7* and *BnGH17* in leaves of *B. napus* after inoculation with *S. sclerotiorum*.

- Fig. S5. Length distribution of target gene-specific siRNAs.

- Fig. S6. The expression levels of *SsPG1* in *S. sclerotiorum*-infected *B. napus* cotyledons and leaves.

- Fig. S7. Assessment of the disease resistance of HIGS transgenic *B. napus* (*T*2) to *S. sclerotiorum* by the stem inoculation method in the field.

Table S1. Primers used for qRT-PCR, gene cloning, and vector construction in this study.

Table S2. Agronomic traits of HIGS transgenic lines in the *T*2 generation.

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**Author contributions**

JW and LL: designing the experiments and writing the manuscript; CL, YF, and YW: manuscript revision; YW and JW: supervision; LL, JE, PL, SR, DL, and KL: performing the experiments and data analysis; all authors read the manuscript and approved its content.

**Conflict of interest**

The authors declare no conflicts of interest.

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**Data availability**

All data supporting the findings of this study are available within the paper and within its supplementary data published online.

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