
The *Blumeria graminis* effector BgtH12 suppresses wheat immunity and acts as a virulence factor

Xiuli Xin¹, Boyu Yang², Mengying Guo¹, Weiwei Yuan¹, Chiye Yang¹, Ruoyu Hao¹, Yuhang Li¹, Jiale Liu¹, Mengqian Du¹, Ming Qin¹, Qiang Li¹, Wenzhi Lan³, Baotong Wang^{1*}, and Peng Cheng^{1*}

¹ State Key Laboratory for Crop Stress Resistance and High-Efficiency Production, College of Plant Protection, Northwest A&F University, Yangling, Shaanxi 712100, China

² College of Life Sciences, Northwest A&F University, Yangling, Shaanxi 712100, China

³ Institute of Future Agriculture, Northwest A&F University, Yangling, Shaanxi 712100, China

*Corresponding authors: B. T. Wang; E-mail: wangbt@nwsuaf.edu.cn and P. Cheng; E-mail: pengcheng@nwfafu.edu.cn

X. L. Xin, B. Y. Yang, and M. Y. Guo contributed equally to this work.

Abstract

The obligate biotrophic fungus *Blumeria graminis* f. sp. *tritici* (*Bgt*), the causal agent of wheat powdery mildew, secretes effector proteins to suppress plant immunity and establish infection. Here, we characterize BgtH12, a conserved small secreted protein that is highly expressed during early infection (peaking at 48 hpi). Functional analysis using host-induced gene silencing (HIGS) with two independent fragments achieved 54–82% knockdown of *BgtH12* transcripts, resulting in significantly reduced fungal sporulation, hyphal length, and branching. Stable transgenic RNAi lines confirmed these findings, with fungal biomass reduced to 0.6–0.7-fold of the wild-type control. Conversely, stable overexpression of *BgtH12* in wheat enhanced susceptibility, increasing fungal biomass 4- to 7-fold and accelerating hyphal growth. Histological examination revealed that BgtH12 promotes infection by suppressing early host defense responses: overexpression lines exhibited reduced reactive oxygen species (ROS) accumulation at 24 and 48 hpi, while RNAi lines showed enhanced ROS production. In *Nicotiana benthamiana*, BgtH12 suppressed BAX-triggered cell death, and delivery into wheat cells via the type-III secretion system (T3SS) attenuated callose deposition and the ROS burst, indicating broad-spectrum suppression of pattern-triggered immunity. Subcellular localization assays in wheat protoplasts and *N. benthamiana* leaves showed that BgtH12 associates with the cell periphery. Together, these results establish BgtH12 as a critical virulence factor that suppresses basal immunity to promote *Bgt* colonization.

Keywords: *Blumeria graminis* f. sp. *tritici*, effector, pattern-triggered immunity

(PTI), host-induced gene silencing (HIGS), wheat powdery mildew

Introduction

Wheat powdery mildew, caused by the obligate biotrophic fungus *B. graminis* f. sp. *tritici* (*Bgt*), is a devastating and widespread disease that causes substantial yield losses in major wheat-growing regions worldwide (Glawe 2008; Dean et al. 2012). Sustainable management of this disease requires a deeper understanding of the molecular dialogue between the pathogen and its host, particularly the strategies employed by *Bgt* to evade or suppress plant immunity.

Plants perceive potential pathogens through a two-tiered innate immune system (Jones and Dangl 2006). The first layer, pattern-triggered immunity (PTI), is activated upon recognition of conserved microbial-associated molecular patterns (MAMPs) by cell-surface pattern recognition receptors (PRRs) (Boller and Felix 2009). This recognition triggers a suite of defense responses, including a rapid burst of ROS (Qi et al. 2017), callose deposition at the cell wall, and transcriptional reprogramming (Boller and Felix 2009; Dodds and Rathjen 2010). To establish infection, successful pathogens secrete a diverse array of effector proteins into the host apoplast or directly into host cells to suppress PTI, resulting in effector-triggered susceptibility (ETS) (Jones and Dangl 2006). In a classic gene-for-gene resistance model, plants have evolved intracellular nucleotide-binding leucine-rich repeat (NLR) receptors that recognize specific pathogen effectors, activating a stronger second layer of defense termed effector-triggered immunity (ETI), which is often associated with a hypersensitive

response (HR) or localized programmed cell death (Jones and Dangl 2006; Dodds and Rathjen 2010). This interaction represents a continuous evolutionary arms race, driven by the pathogen's need to deploy effectors that suppress both PTI and ETI, and the host's ability to detect them (Cook et al. 2015; Win et al. 2012).

Biotrophic fungi like *Bgt* are predicted to secrete hundreds of effector proteins, many of which are small, lack conserved domains, and are rapidly evolving - hallmarks of proteins involved in host manipulation (Rovenich et al. 2014; Lo Presti et al. 2015).

While a few *Bgt* effectors have been characterized as avirulence (Avr) factors recognized by specific host resistance (R) genes, such as *AvrPm2* (Praz et al. 2017) and *AvrPm3* (Bourras et al. 2019), the vast majority of its predicted secretome remains functionally uncharacterized. Candidate effectors are typically prioritized from large-scale *Bgt*-wheat transcriptome or secretome analyses that capture genes induced during early infection (Müller et al., 2018), but only a handful have been followed up functionally. These uncharacterized effectors are hypothesized to function primarily as virulence factors that subtly manipulate host cellular processes to promote compatibility (Lo Presti et al. 2015; Bourras et al. 2018). A key and common strategy for these virulence effectors is to dampen the initial PTI response, creating a permissive environment for the establishment of specialized infection structures like haustoria during the critical early stages of infection (Koeck et al. 2011; Pedersen et al. 2012). However, the precise mechanisms by which *Bgt* effectors achieve this immunosuppression remain poorly understood. Recent studies have begun to characterize additional *Bgt* effectors with diverse immunosuppressive

activities (Huai et al. 2026; Yan et al. 2025), highlighting both the diversity of *Bgt* effector repertoires and the gaps that remain. Many fundamental questions persist: Do individual effectors target single, specific nodes in the immune signaling network, or can they act at multiple points to achieve a more comprehensive shutdown? Furthermore, what are the molecular mechanisms by which effectors manipulate distinct host immune pathways? Effectors that interfere with receptor complexes at the host cell surface may directly block PAMP perception, whereas those operating inside the cell may target downstream signaling components or transcriptional regulators (Deslandes and Rivas 2012; Böhm et al. 2014; Petre et al. 2015).

Here, we report the functional characterization of a novel *Bgt* effector, BgtH12. We demonstrate that BgtH12 is a small, secreted protein strongly induced during early infection. Comprehensive functional analysis reveals it is a potent suppressor of core PTI-associated responses, including programmed cell death (PCD), ROS burst, and callose deposition. Subcellular localization assays in wheat protoplasts and *N. benthamiana* leaves show that BgtH12 likely associates with the cell periphery. Using an integrated approach combining cell biology, physiological assays, and robust loss-of-function/gain-of-function genetics in wheat, we establish BgtH12 as a key virulence effector that suppresses basal immunity to promote *Bgt* colonization. This work identifies a new player in the wheat-*Bgt* interaction and provides a foundation for understanding how effectors manipulate host immune pathways in biotrophic pathogens.

Results

BgtH12 is an early-expressed, secreted effector conserved across *Bgt* races

BgtH12 was identified from the *Bgt* genome based on criteria typical of fungal effectors: it encodes a small protein (190 aa) with a predicted N-terminal signal peptide for secretion and lacks transmembrane domains or recognizable conserved domains (Fig. 1). The gene corresponds to locus BGT96224V316_LOCUS2603 (protein ID VCU41362.1, GenBank LR026987.1) in the *Bgt* 96224 reference genome assembly (Müller et al. 2018). BLAST searches against three additional *Bgt* isolates (E09, E15, and A13) detected a single ortholog with >95% nucleotide identity, indicating that BgtH12 is a single-copy gene in the *Bgt* lineage. Secondary structure prediction revealed that BgtH12 is composed of a mixture of alpha-helices, beta-strands, and random coils, with no extended disordered regions (Fig. 1A). PCR analysis confirmed that BgtH12 is present across five geographically diverse *Bgt* races (E09, E15, E18, A13, and A44), suggesting it is conserved across diverse *Bgt* races (Fig. 1B). Critically, qRT-PCR analysis of the infection time-course revealed that BgtH12 expression is strongly induced during early infection, peaking at 48 hours post-inoculation (hpi) - a key window for the establishment of the biotrophic interface (Fig. 1C). SignalP-5.0 identified a putative cleavage site between amino acids 16 and 17, confirming the presence of an N-terminal signal peptide (Fig. 1D, left panel). Consistent with the selection criteria, TMHMM analysis predicted no transmembrane domains (Fig. 1D, right panel). To verify the secretory function of the predicted signal peptide (cleavage site between aa 16–17), we performed a yeast secretion assay using

the YTK12 strain. In Figure 2A, yeast cells transformed with the empty vector (pSUC2) failed to grow on YPRAA medium (lacking sucrose) and did not exhibit invertase activity in the 2,3,5-triphenyltetrazolium chloride (TTC) assay (clear solution), serving as the negative control. In contrast, yeast expressing BgtH12 displayed growth comparable to the positive control (Avr1b) on YPRAA medium, indicating successful secretion of invertase. Furthermore, the TTC assay resulted in a distinct red coloration for the BgtH12 transformant, confirming the production of reducing sugars due to functional invertase secretion (Fig. 2B). It was demonstrated that the predicted N-terminal signal peptide of BgtH12 is fully functional in directing protein secretion. These data establish BgtH12 as an early-expressed, and secreted effector protein presented in diverse *Bgt* races.

Host-induced gene silencing of BgtH12 impairs fungal virulence

To establish the requirement of BgtH12 for fungal pathogenicity, we employed host-induced gene silencing (HIGS) mediated by Barley stripe mosaic virus (BSMV). Two independent fragments targeting the N-terminal and C-terminal coding regions of BgtH12 (designated BSMV:BgtH12-a and BSMV:BgtH12-b, each 200 bp in length) were cloned into the BSMV:γ vector (Fig. 3A). BSMV:PDS served as a positive control for virus infection, causing photobleaching symptoms 10 days post-inoculation (dpi), while BSMV:γ (empty vector) served as a negative control. Ten days after BSMV inoculation, leaves showing good viral symptoms were marked and challenged with *Bgt* conidia (Fig. 3B). Silencing efficiency was assessed by

qRT-PCR at 24, 48, 72, and 96 hours post-inoculation (hpi). Compared to the BSMV:γ control, BgtH12 transcript levels in the silenced plants ranged from 18% to 46% across the four time points, confirming effective gene silencing (Fig. 3C). At 10 dpi, macroscopic observation revealed that leaves silenced for BgtH12 supported significantly reduced fungal sporulation, colony size, and hyphal thickness compared to the BSMV:γ control (Fig. 3D). To quantify developmental defects, infected leaves were collected at 24, 48, 72, and 96 hpi, stained with aniline blue, and examined microscopically. Hyphal length was significantly shorter in both BSMV:BgtH12-a and BSMV:BgtH12-b treatments compared to BSMV:γ, with pronounced differences at 48, 72, and 96 hpi (Fig. 3D, E). Similarly, hyphal branching frequency was significantly reduced in the silenced samples at 24, 48, and 72 hpi (Fig. 3F). These results demonstrate that silencing BgtH12 compromises fungal growth and development, indicating that BgtH12 is required for full *Bgt* virulence.

Overexpression of *BgtH12* in wheat enhances susceptibility to powdery mildew

To determine if BgtH12 is sufficient to enhance disease susceptibility, we generated stable transgenic wheat lines overexpressing BgtH12 under the control of the maize ubiquitin promoter. T₀ seeds from eight independent transformation events were planted (10 seeds per pot), and positive transgenic lines were identified by PCR screening of genomic DNA. Seven positive OE lines (L2–L8) were obtained from the T₀ generation (Fig. S1A).

Seven days after inoculation with *Bgt* isolate E09, both L2 and L5 lines exhibited

markedly enhanced disease symptoms compared to Fielder, producing more abundant spores and larger colonies (Fig. 4A). Quantification of fungal biomass by qRT-PCR revealed that the relative fungal DNA content in the OE lines was approximately 4- to 7-fold higher than in Fielder (Fig. 4B). Relative BgtH12 expression in both transgenic lines L2 and L5 were significantly higher than it in fielder (Fig. 4C). These results indicate that overexpression of BgtH12 significantly enhances susceptibility to powdery mildew.

To determine whether BgtH12 overexpression suppresses host reactive oxygen species (ROS) accumulation, we performed DAB staining at 24 and 48 hpi.

Microscopic examination revealed that the ROS area at infection sites was markedly smaller in L2 and L5 compared to Fielder (Fig. 4D). Quantification of ROS area from 40 infection sites confirmed that the OE lines exhibited significantly reduced ROS accumulation (Fig. 4E), suggesting that BgtH12 promotes fungal infection by suppressing the early oxidative burst.

To further examine the effect of BgtH12 overexpression on fungal development, we performed histological analysis at 24, 48, 72, and 96 hpi. Aniline blue staining revealed that fungal growth was accelerated in both OE lines compared to Fielder (Fig. 4F). Statistical analysis (Student's t-test) showed that hyphal branching number, hyphal length, and colony area were significantly greater in L2 and L5 than in Fielder at all time points except 24 hpi (Fig. 4F).

RNAi-mediated silencing of BgtH12 enhances resistance to powdery mildew

To obtain resistant wheat lines, we generated stable transgenic RNAi lines targeting BgtH12. From the T₀ generation, seven positive RNAi lines (L1–L6 and L8) were identified by PCR (Fig. S1B). After propagation to the T₁ generation, lines L4 and L5 were confirmed as homozygous positives (Fig. 5A). Seven days after inoculation with *Bgt* isolate E09, both RNAi lines displayed significantly reduced disease symptoms compared to Fielder, producing fewer spores and smaller colonies (Fig. 5A). Fungal biomass quantification revealed that the relative fungal DNA content in the RNAi lines was approximately 0.6- to 0.7-fold that of Fielder (Fig. 5B), indicating that silencing BgtH12 significantly enhances resistance to powdery mildew.

Consistent with the enhanced resistance phenotype, DAB staining at 24 and 48 hpi revealed that ROS accumulation at infection sites was markedly higher in L4 and L5 compared to Fielder (Fig. 5C). Quantification confirmed that the ROS area was significantly greater in the RNAi lines (Fig. 5D), suggesting that BgtH12 functions at least in part by modulating the ROS pathway to promote susceptibility.

Histological analysis at 24, 48, 72, and 96 hpi revealed that fungal development was visibly slower in the RNAi lines compared to Fielder (Fig. 5E). Hyphal branching number showed no significant difference at 24 and 48 hpi but was significantly lower in L4 and L5 than in Fielder at 72 and 96 hpi. Hyphal area and hyphal length were significantly reduced in the RNAi lines at 48, 72, and 96 hpi.

Together, the complementary loss-of-function (HIGS and stable RNAi) and gain-of-function (stable OE) experiments provide robust, dose-dependent genetic evidence that BgtH12 is a critical virulence factor that promotes *Bgt* infection by

suppressing host basal immunity, particularly the early oxidative burst.

BgtH12 functions as a broad-spectrum suppressor of plant immunity

To further characterize the immunosuppressive activity of BgtH12, we tested its ability to inhibit ETI-associated PCD triggered by the pro-apoptotic protein BAX (Fig. 6). Full-length BgtH12 completely suppressed BAX-induced necrosis, comparable to the known suppressor Avr1b, whereas the MgCl₂ buffer control developed clear necrosis (Fig. 6A–C). To determine whether the signal peptide is required for this activity, we performed the same assay with BgtH12^{ΔSP} (Fig. 6D–F). BAX alone induced strong cell death, while GFP and MgCl₂ did not. BgtH12^{ΔSP} alone also failed to induce cell death, and co-infiltration with BAX resulted in necrosis comparable to BAX alone (Fig. 6E–F), indicating that BgtH12^{ΔSP} could not suppress BAX-triggered PCD. These results demonstrate that BgtH12 depends on signal peptide-mediated secretion to suppress programmed cell death, indicating that it either functions extracellularly or requires trafficking through the secretory pathway to achieve functional localization.

We next assessed its impact on pattern-triggered immunity (PTI) in wheat. Delivery of BgtH12 into wheat leaf cells via the bacterial type-III secretion system resulted in a marked reduction in pathogen-induced callose deposition compared to the dsRed control, with significantly fewer callose deposits per unit area (Fig. 7A, B). Furthermore, when leaves were first infiltrated with *P. fluorescens* EtHAn delivering BgtH12 (or dsRed as a control) and then challenged with *Bgt*, the ROS burst was

significantly attenuated at both 24 and 48 hpi in BgtH12-expressing leaves relative to the EtHAN:dsRed control (Fig. 7C, D).

Together, these data demonstrate that BgtH12 is a broad-spectrum suppressor of plant immunity, capable of inhibiting both ETI-associated PCD and core PTI responses.

Subcellular localization of BgtH12

To investigate the subcellular localization of BgtH12, we expressed a GFP-tagged version of the mature effector lacking its signal peptide (BgtH12^{ΔSP}-GFP) in wheat mesophyll protoplasts. (Fig. 8A). Compared to the uniform cytosolic and nuclear distribution of the eGFP control, BgtH12^{ΔSP}-GFP exhibited strong GFP signals with a distribution pattern similar to the GFP control. In contrast, full-length BgtH12-GFP showed weak and diffuse fluorescence, with no obvious peripheral or nuclear enrichment. To confirm this localization pattern in an intact plant system, we transiently expressed BgtH12^{ΔSP}-GFP in *N. benthamiana* leaves via *Agrobacterium tumefaciens* infiltration using the pBIN vector. At 48 h after infiltration, BgtH12^{ΔSP}-GFP displayed a signal pattern similar to the control, consistent with the results obtained in wheat protoplasts (Fig. 8B).

To validate the integrity of the fusion proteins, we performed Western blot analysis using an anti-GFP antibody on total protein extracts from *N. benthamiana* leaves. Untransformed leaves (WT) and free GFP served as negative and positive controls, respectively. Both BgtH12^{ΔSP}-GFP and full-length BgtH12-GFP migrated as

predominant bands at ~55 kDa, with no detectable free GFP (~25 kDa), confirming that the fusion proteins remained intact (Fig. 8C). Furthermore, plasmolysis assays were performed in *N. benthamiana* leaf epidermal cells co-expressing BgtH12-GFP constructs with the plasma membrane marker TaWP16-mCherry. Upon treatment with 0.8 M mannitol, high-magnification views of the cell wall–plasma membrane boundary (red-boxed insets) revealed that the GFP signal of full-length BgtH12-GFP was retained in the apoplastic space between the retracted plasma membrane and the cell wall, co-localizing with TaWP16-mCherry (Fig. 8D, a–b). In contrast, BgtH12^{ASP}-GFP showed no detectable fluorescence in the apoplastic space (Fig. 8D, d–e). Bright nuclear GFP signals were observed for both constructs (Fig. 8D, c, f). Fluorescence intensity quantification using ImageJ confirmed the differential peripheral enrichment between full-length BgtH12-GFP and BgtH12^{ASP}-GFP (Fig. 8D, right panel). Together, the retention of BgtH12-GFP in the apoplastic space and the functional loss of BgtH12^{ASP} demonstrate that BgtH12 likely acts extracellularly to suppress host plant immunity.

Discussion

BgtH12 is a validated virulence factor in the *Bgt* effector repertoire

Obligate biotrophic pathogens such as *Bgt* deploy an arsenal of effector proteins to modulate host physiology and suppress immunity (Lo Presti et al. 2015; Müller et al. 2018). These effectors are often lineage-specific, rapidly evolving, and lack conserved domains, making their functional characterization a priority for

understanding compatible interactions (Rovenich et al. 2014; Bourras et al. 2019). In this study, we identified and characterized BgtH12, a previously unstudied effector from *Bgt* that is conserved across multiple races and strongly induced during early infection.

The functional importance of BgtH12 is strongly supported by complementary loss-of-function and gain-of-function genetic analyses. HIGS of *BgtH12* using two independent fragments led to significantly reduced fungal growth, hyphal branching, and sporulation, coupled with enhanced host ROS accumulation. Stable transgenic RNAi lines corroborated these findings, reducing fungal biomass to 0.6–0.7-fold of the wild-type control. Conversely, stable overexpression of BgtH12 in wheat enhanced susceptibility, with 4- to 7-fold increases in fungal biomass and accelerated hyphal growth, alongside suppressed ROS production. This dose-dependent relationship between BgtH12 expression level and disease outcome provides robust genetic evidence that BgtH12 is a bona fide virulence determinant. The consistency between transient T3SS assays and stable transgenic phenotypes reinforce the reliability of our conclusions—an important point given the inherent difficulties of functional genetics in obligate biotrophs (Koeck et al. 2011; Müller et al. 2018).

Recent studies have begun to expand the functionally characterized *Bgt* effector repertoire. BgtE-20069a localizes to the cytoplasm and nucleus and suppresses PCD to promote *Bgt* virulence (Yan et al. 2025), while Bgt5481 exhibits context-dependent activity, acting as a PAMP in nonhost plants but contributing to virulence in wheat, with apoplastic localization when expressed with its native signal peptide (Huai et al.

2026). The identification of BgtH12 adds to this emerging picture and highlights that *Bgt* employs a repertoire of effectors with distinct expression kinetics, subcellular destinations, and immunosuppressive outputs to achieve successful colonization.

BgtH12 broadly suppresses plant immune signaling

Our functional data establish BgtH12 as a suppressor of multiple layers of plant defense. Its ability to inhibit BAX-triggered PCD in *N. benthamiana* suggests it can interfere with a conserved cell-death execution pathway, a feature shared by many virulence effectors (Win et al. 2012). In wheat, BgtH12 significantly attenuated two hallmark PTI responses - ROS burst and callose deposition (Dodds and Rathjen 2010). Importantly, these responses were suppressed whether triggered by a heterologous bacterial system (*P. fluorescens* EtHAn) or by *Bgt* itself, implying that BgtH12 targets a shared signaling node downstream of multiple immune receptors, rather than a specific receptor-ligand pair (Boller and Felix 2009). This broad-spectrum immunosuppression phenotype is consistent with BgtH12 being expressed during the early infection window (peaking at 48 hpi), when the pathogen must overcome a battery of host-recognized patterns to establish the biotrophic interface (Koeck et al. 2011; Pedersen et al. 2012). BgtH12 thus represents a conserved effector that contributes to *Bgt* virulence by suppressing host immune responses at multiple levels, including PTI-associated callose deposition and ROS burst.

Integration into the *Bgt* effector landscape

While several avirulence (Avr) effectors such as AvrPm2 (Praz et al. 2017) and

AvrPm3 (Bourras et al. 2019) have been identified in *Bgt*, the majority of its predicted secretome consists of uncharacterized candidates presumed to act as virulence factors (Müller et al. 2018). BgtH12 falls into this latter category. Its sequence divergence and lack of known domains highlight the rapid evolution and functional innovation within pathogen effector repertoires (Rovenich et al. 2014; Lo Presti et al. 2015). Rather than being recognized by a host resistance protein, BgtH12 appears to operate below the detection threshold, promoting effector-triggered susceptibility (ETS) (Jones and Dangl 2006). Its early and strong induction during infection aligns with a role in establishing the biotrophic interface, a critical window where suppressing initial host alarms is paramount (Koeck et al. 2011; Pedersen et al. 2012).

Our plasmolysis assays and quantitative fluorescence data demonstrated that full-length BgtH12-GFP accumulates at the apoplast and co-localizes with the plasma membrane marker. In contrast, weak signal observed in wheat protoplasts implies a functional or physical association between BgtH12 and the host cell wall. Together with the functional loss of BgtH12^{ΔSP}-GFP in cell death suppression assays, these findings support the notion that BgtH12 likely acts extracellularly. We note that the localization patterns of BgtH12-GFP and BgtH12^{ΔSP}-GFP differ somewhat between wheat protoplasts and plasmolyzed *N. benthamiana* leaf cells. This is likely attributable to the inherent differences between the two experimental systems: wheat protoplasts lack cell walls and are maintained as single cells in suspension, which may affect protein secretion efficiency and retention at the cell surface. This could explain the weak fluorescence signal observed for full-length BgtH12-GFP if the protein

associates with the cell wall. In contrast, *N. benthamiana* leaf cells retain an intact cell wall and apoplastic environment, providing a more physiologically relevant context for assessing peripheral association. Despite these differences, both systems consistently demonstrate that the signal peptide is required for BgtH12 to localize to the extracellular space and associate with the cell periphery.

This study establishes BgtH12 as a well-validated virulence factor in the *Bgt*-wheat interaction. The combination of HIGS, stable RNAi, and stable overexpression provides a genetic framework that can be extended to other uncharacterized *Bgt* SSPs with similar expression profiles. More broadly, the identification of effectors such as BgtH12, BgtE-20069a (Yan et al. 2025), and Bgt5481 (Huai et al. 2026) begins to reveal a repertoire of *Bgt* effectors with distinct subcellular destinations and immunosuppressive outputs, suggesting that functional diversification underlies *Bgt*'s success as an obligate biotroph.

Materials and Methods

Plant materials, fungal isolates, and microbial strains

Wheat (*Triticum aestivum*) cultivars Jingshuang 16 (for *Bgt* maintenance and propagation), Avocet S (AVS) (for T3SS-mediated effector delivery in wheat), and Fielder (for stable transformation, BSMV-mediated HIGS, protoplast isolation, and EtHAn-mediated assays), as well as *N. benthamiana* (for *Agrobacterium*-mediated transient expression assays), were used in this study. All plants were grown in growth chambers under controlled conditions: 16-h light/8-h dark photoperiod, 22°C (wheat)

or 25°C (*N. benthamiana*), and 70% relative humidity. The *Bgt* races E09, E15, E18, A13, and A44 were maintained on susceptible wheat plants Jingshuang 16 under controlled growth conditions.

Escherichia coli strain DH5 α was cultured in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract, and 1% NaCl) at 37°C and used for plasmid construction. *A.* strain GV3101 was cultured in LB liquid medium at 28°C and used for transient expression in *N. benthamiana*. *P. fluorescens* strain EtHAn was cultured in King's B (KB) liquid medium (2% peptone, 0.15% K₂HPO₄, 0.15% MgSO₄, and 0.1% glycerol) at 28°C and used for T3SS-mediated delivery into wheat leaves (Upadhyaya et al., 2014). Antibiotics (kanamycin, ampicillin, rifampicin, chloramphenicol, and gentamicin) were added as required.

Identification of BgtH12

We identified BgtH12 as a candidate effector from the *Bgt* 96224 genome assembly (Müller et al. 2018), annotation version v3.16, based on criteria typical of fungal effectors. All predicted proteins were filtered for those smaller than 190 amino acids and containing a predicted N-terminal signal peptide (SignalP-5.0, probability ≥ 0.9). Proteins with transmembrane domains (TMHMM) or known functional domains (Pfam, e-value ≤ 0.05) were excluded. Among the remaining small secreted proteins (SSPs), BgtH12 (locus BGT96224V316_LOCUS2603, protein ID VCU41362.1, GenBank LR026987.1) was selected for further characterization based on its high expression level and conservation across isolates. PCR analysis confirmed that BgtH12 is present in five geographically diverse *Bgt* races (E09, E15, E18, A13, and

A44), indicating that it is a conserved gene in the *Bgt* lineage. BLAST searches against three additional *Bgt* isolates (E09, E15, and A13) detected a single ortholog with >95% nucleotide identity, confirming that BgtH12 is a single-copy gene.

Sequences analysis of *BgtH12*

Homologs of BgtH12 in other organisms were identified by BLAST search against the NCBI non-redundant database. To identify sequence variation, the coding region of BgtH12 from different *Bgt* isolates was amplified and analyzed for single nucleotide polymorphisms (SNPs).

Plasmid construction

cDNA of BgtH12 was cloned from *Bgt* race E09. To validate secretory function, the predicted signal peptide sequence was cloned into the pSUC2 vector. For subcellular localization, the BgtH12 open reading frame without the signal peptide and stop codon was fused to pJIT163-GFP (for wheat protoplasts) and pBIN-eGFP (for *N. benthamiana*), respectively.

To assess suppression of plant immune responses, the coding sequence was inserted into the pGR106 vector (Lu et al. 2003) for transient expression in *N. benthamiana*. For T3SS-mediated delivery, the target sequence was cloned into pDONR221 using BP Clonase (Thermo Fisher Scientific, USA) and then recombined into pEDV6 (Sohn et al., 2007, 2014) using LR Clonase (Thermo Fisher Scientific, USA). The resulting construct was transformed into *Pseudomonas fluorescens* EtHAN (Thomas et al., 2009), and the recombinant strain was infiltrated into wheat leaves as described (Upadhyaya et al., 2014). For BSMV-mediated host induced gene silencing

(Nowara et al. 2010), specific fragments of BgtH12 were cloned into the BSMV:γ vector (Holzberg et al. 2002; Scofield et al. 2005). BgtH12-a (nt1–291) was amplified with primers BgtH12-a-F and BgtH12-a-R; BgtH12-b (nt404–630) was amplified with primers BgtH12-b-F and BgtH12-b-R.

For stable transformation, the BgtH12 coding sequence was cloned into a binary vector pUbi-Bar (modified from pCAMBIA1300; Hajdukiewicz et al., 1994) under the control of the maize ubiquitin promoter for overexpression, and a hairpin RNAi construct targeting BgtH12 was assembled in an RNAi vector pANDA-mini (Miki and Shimamoto, 2004). Both constructs were transformed into wheat cultivar Fielder via *A. tumefaciens*-mediated transformation.

All primer sequences, fragment positions, and vector details are listed in Supplementary Table S1.

RNA extraction and transcriptional analysis

Total RNA was extracted from *Bgt* and infected wheat leaves at 0, 6, 18, 24, 48, 72, and 96 h post-inoculation (hpi) with the virulent race E09 using the HiPure Plant RNA Mini Kit (Magen, Guangzhou, China) according to the manufacturer's instructions. Only RNA samples with an A260/A230 ratio > 2.0, an A260/A280 ratio of 1.8–2.0, and a concentration > 100 ng/μl were used for subsequent analysis.

First-strand cDNA was synthesized from total RNA and used as a template for quantitative RT-PCR (RT-qPCR). The powdery mildew reference gene *Bgt-F/R* (Zeng et al. 2010) was used as an internal control. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). All experiments

were performed with three independent biological replicates.

Functional Validation of Signal Peptides

The secretion activity of the BgtH12 signal peptide was assessed using the yeast invertase secretion system. The pSUC2 vector contains an invertase gene lacking its native signal peptide and initiation codon (Jacobs et al. 1997). The signal peptide sequence of BgtH12 was cloned into the vector using EcoRI and XhoI and transformed into the invertase-deficient yeast strain YTK12 using the lithium acetate method (Oh et al. 2009). Transformants were selected on CMD-W medium (0.67% yeast nitrogen base without amino acids, 0.075% dropout supplement lacking tryptophan, 0.1% glucose, 2% sucrose, and 2% agar) at 30°C. Positive colonies were confirmed by PCR using pSUC2-specific primers. Secretion activity was assessed on YPRAA medium (1% yeast extract, 2% peptone, 2% raffinose, 2 µg/mL antimycin A, and 2% agar). For enzymatic activity assays, single colonies were cultured in CMD-W liquid medium at 30°C for 24–36 h. Cells were collected, resuspended in sterile distilled water, and incubated in 10 mM sodium acetate buffer with 10% sucrose at 37°C for 10 min. The supernatant was then mixed with 0.1% 2,3,5-triphenyltetrazolium chloride (TTC), and color development was observed after 5 min at room temperature (Yin et al. 2018).

Host-induced gene silence

BSMV constructs (BSMV:α, BSMV:β, BSMV:γ, BSMV:γ-PDS, BSMV:BgtH12-a, and BSMV:BgtH12-b) were linearized and transcribed in vitro using the RiboMAX™ Large Scale RNA Production System-T7 Kit (Promega, USA).

The second leaves of wheat seedlings at the two-leaf stage were inoculated with viral transcripts by gentle rubbing. Approximately 10 days after infection, when the third and fourth leaves exhibited stripe photobleaching symptoms, plants were inoculated with *Bgt* race E09. Leaf samples were collected at 24, 48, 72, and 96 hpi for RT-qPCR and cytological analyses. Disease symptoms were recorded at 10 days post-inoculation (dpi).

Generation of transgenic wheat

Overexpression and RNAi transgenic wheat lines were generated in the cultivar Fielder via *A. tumefaciens*-mediated transformation by the wheat transformation service group at Shandong Agricultural University (Li, G.Y. Laboratory). T₀ seeds from eight independent transformation events were planted (10 seeds per pot), and positive transgenic lines were identified by PCR amplification of the transgene from genomic DNA. After propagation to the T₁ generation, homozygous lines were confirmed by PCR and RT-qPCR.

Potential off-target effects of the RNAi sequences were evaluated by genome-wide alignment against the *Bgt* 96224 reference transcriptome using BLASTn, allowing up to two mismatches. Predicted off-target candidates were then queried against publicly available functional annotation databases (e.g., NCBI nr, Pfam) to assess their functional relevance. No predicted off-targets with high sequence similarity (>80%) were found to be functionally associated with known defense-related pathways or fungal virulence processes, suggesting that the observed resistance phenotypes in the RNAi lines are specifically attributable to silencing of

BgtH12.

Cytological observation

Wheat leaves were cut into 1-2 cm segments and decolorized in ethanol:acetic acid (1:1, v/v) for 2 days. After clearing, samples were stained with Coomassie brilliant blue for 10–20 min (Liang et al. 2019). After washing, samples were stored in 20% glycerol, and fungal structures were observed and photographed under a microscope. Hyphal length, number of branches, and infection area were quantified from at least 30 infection sites per sample using ImageJ software.

Agrobacterium*-mediated transient expression in *N. benthamiana

The recombinant plasmid was introduced into *A. tumefaciens* strain GV3101 by electroporation. *Agrobacteria* were cultured in LB liquid medium with 50 mg/mL kanamycin and 25 mg/mL rifampicin at 28°C for 48 h. Bacteria were collected and resuspended in 10 mM MgCl₂ to a final OD₆₀₀ of 0.5 and incubated at room temperature for 2 h before infiltration. One-month-old *N. benthamiana* plants were infiltrated with the bacterial suspension. For cell death suppression assays, the pro-apoptotic protein BAX was used as an inducer of PCD; the BAX-triggered cell death phenotype closely resembles the hypersensitive response (Lacomme and Santa Cruz, 1999). The *Phytophthora sojae* effector Avr1b, which suppresses BAX-induced PCD in *N. benthamiana* cells, was included as a positive control for PCD suppression (Dou et al., 2008). The same infiltration site was challenged with *Agrobacterium* carrying the BAX gene 24 h later. Symptoms were monitored and photographed 4–5 days after BAX infiltration.

For the BgtH12^{ΔSP} BAX suppression assay, the coding sequence of BgtH12^{ΔSP} was cloned into the PVX expression vector. *Agrobacterium* cultures carrying PVX-BgtH12^{ΔSP}, GFP (negative control), or the empty PVX vector were prepared as described above. The following treatments were infiltrated on the same leaf: BAX alone, GFP alone, MgCl₂ (buffer control), BgtH12^{ΔSP} alone, and BgtH12^{ΔSP} combined with BAX. Cell death phenotypes were recorded at 4–5 days post infiltration. Leaves were then cleared with ethanol to visualize necrotic lesions. Each experiment was repeated three times with consistent results.

T3SS-mediated overexpression in wheat

The recombinant plasmid pEDV6-BgtH12 was propagated in *E. coli* DH5α and then transformed into *P. fluorescens strain* EtHAn by electroporation (Sohn et al. 2007, 2014). MgCl₂ buffer and EtHAn:pEDV6 (empty vector) were used as controls. For leaf infiltration, EtHAn:pEDV6 and EtHAn:pEDV6-BgtH12 were grown in KB liquid medium with gentamicin and chloramphenicol for 24–48 h at 28°C. Bacteria were collected, washed, and resuspended in infiltration medium (10 mM MgCl₂) to an OD₆₀₀ of 1.0, then infiltrated into the second leaves of wheat cultivar AVS. Plants were maintained at 25°C. For callose observation, leaves were sampled at 48 h after infiltration, decolorized, stained with aniline blue, and examined under a fluorescence microscope. For ROS detection, leaves were inoculated with *Bgt* conidia 48 h after infiltration, sampled at 24 and 48 hpi, and stained with DAB (Thordal-Christensen et al. 1997).

Subcellular localization, Western blot, and plasmolysis assays

Subcellular localization. Wheat mesophyll protoplasts were isolated from seedling leaves as described by Yoo et al. (2007). Leaf strips (0.5-1 mm) were prepared and immediately transferred into enzyme solution, vacuum-infiltrated for 30 min, and incubated until the solution turned green. Protoplasts were collected, washed with pre-cooled W5 solution, and resuspended in MMG solution. PEG-calcium-mediated transformation was performed using 10-20 μ g of plasmid DNA. Transformed protoplasts were incubated overnight at 23-25°C, and GFP fluorescence was observed under a confocal microscope. The recombinant plasmid pBIN-BgtH12^{ΔSP}-eGFP was introduced into *A. tumefaciens* strain GV3101 by electroporation. Agrobacteria were cultured, resuspended in 10 mM MgCl₂ to OD₆₀₀ of 0.5, and infiltrated into leaves of one-month-old *N. benthamiana* plants. GFP fluorescence was observed under a confocal microscope at 48 h after infiltration.

Western blot analysis. Total proteins were extracted from *N. benthamiana* leaf tissues expressing BgtH12^{ΔSP}-GFP. Protein extracts from untransformed *N. benthamiana* leaves (WT) were included as a negative control. Samples were lysed by sonication in protein extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1× protease inhibitor cocktail), followed by centrifugation at 3,000 g for 5 min. The supernatants were mixed with 5× SDS sample buffer and boiled for 5 min. Protein samples (20 μ g per lane) were separated by 10% (w/v) SDS-PAGE and transferred onto a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 1× TBST (Tris-buffered saline with 0.1% Tween-20) containing 6% (w/v) skim milk for 30 min, then incubated with anti-GFP

monoclonal antibody (1:3,000 dilution; Clontech, Catalog No. 632381) at 4°C overnight (Jeong et al., 2022). After three washes with TBST, the membrane was incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (1:5,000 dilution; Bethyl Laboratories) at 4°C for 4 h. Signals were detected using ECL Prime chemiluminescent substrate (GE Healthcare) and captured on a ChemiDoc imaging system (Bio-Rad). Ponceau S staining was used as a loading control.

Plasmolysis assay. *N. benthamiana* leaf epidermal cells expressing full-length BgtH12-GFP or BgtH12^{ΔSP}-GFP were co-infiltrated with the plasma membrane marker TaWP16-mCherry. Samples were immersed in 0.05 M MES buffer containing 0.8 M mannitol for 30–40 min to induce plasmolysis. Subsequently, the samples were examined with a confocal laser scanning microscope (Zeiss LSM 710). GFP was excited at 488 nm and emission was collected at 495–550 nm, and mCherry was excited at 561 nm with emission collected at 580–630 nm. High-magnification images of the cell wall–plasma membrane boundary were acquired, and red boxes indicate the magnified regions shown in the corresponding insets. In the inset images, white letters and red arrowheads denote the nucleus (N) and the apoplastic space (arrowheads), respectively. For fluorescence quantification, the mean fluorescence intensity in the indicated regions was measured using ImageJ, and the values are presented alongside the corresponding images. Images were processed using ZEN software (Zeiss) and Adobe Illustrator. The redistribution of GFP signal upon plasmolysis was assessed to distinguish peripheral membrane association from

cytoplasmic localization (Serna 2005).

Statistical analysis

All experiments were performed with at least three independent biological replicates. Statistical analyses were conducted using IBM SPSS Statistics 19 software. Significant differences between groups were determined using Student's t test or analysis of variance (ANOVA), as appropriate.

Acknowledgements

We sincerely acknowledge Dr. Genying Li at Shandong Agricultural University for generating the transgenic wheat lines essential for this study. We also thank Profs. Dejun Han and Qingdong Zeng for their valuable comments during manuscript revision. Furthermore, we are grateful to Ms. Cuicui Huang from the National New Crop Variety Approval and Characterization Station for maintaining the greenhouse and experimental wheat plants under standard conditions.

Literature cited

- Böhm, H., Albert, I., Fan, L., Reinhard, A., and Nürnberger, T. 2014. Immune receptor complexes at the plant cell surface. *Curr. Opin. Plant Biol.* 20:47-54.
- Boller, T., and Felix, G. 2009. A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60:379-406.
- Bourras, S., Praz, C. R., Spanu, P. D., and Keller, B. 2018. Cereal powdery mildew effectors: A complex toolbox for an obligate pathogen. *Curr. Opin. Microbiol.* 46:26-33.
- Bourras, S., Kunz, L., Xue, M., Praz, C. R., Müller, M. C., Kälin, C., Schläfli, M., Ackermann, P., Flückiger, S., Parlange, F., Menardo, F., Schaefer, L. K., Ben David, R., Roffler, S., Oberhaensli, S., Widrig, V., Lindner, S., Isaksson, J., Wicker, T., Yu, D., and Keller, B. 2019. The AvrPm3-Pm3 effector-NLR interactions control both race-specific resistance and host-specificity of cereal mildews on wheat. *Nat. Commun.* 10:2292.
- Cook, D. E., Mesarich, C. H., and Thomma, B. P. H. J. 2015. Understanding plant immunity as a surveillance system to detect invasion. *Annu. Rev. Phytopathol.* 53:541-563.
- Dean, R., van Kan, J. A. L., Pretorius, Z. A., Hammond-Kosack, K. E., Di Pietro, A., Spanu, P. D., Rudd, J. J., Dickman, M., Kahmann, R., Ellis, J., and Foster, G. D. 2012. The top 10 fungal pathogens in molecular plant pathology. *Mol. Plant Pathol.* 13:414-430.

- Deslandes, L., and Rivas, S. 2012. Catch me if you can: Bacterial effectors and plant targets. *Trends Plant Sci.* 17:644-655.
- Dodds, P. N., and Rathjen, J. P. 2010. Plant immunity: Towards an integrated view of plant-pathogen interactions. *Nat. Rev. Genet.* 11:539-548.
- Dou, D., Kale, S. D., Wang, X., Chen, Y., Wang, Q., Wang, X., Jiang, R. H. Y., Arredondo, F. D., Anderson, R. G., Thakur, P. B., McDowell, J. M., Wang, Y., and Tyler, B. M. 2008. Conserved C-terminal motifs required for avirulence and suppression of cell death by *Phytophthora sojae* effector Avr1b. *Plant Cell* 20:1118-1133.
- Glawe, D. A. 2008. The powdery mildews: A review of the world's most familiar (yet poorly known) plant pathogens. *Annu. Rev. Phytopathol.* 46:27-51.
- Hajdukiewicz, P., Svab, Z., and Maliga, P. 1994. The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* 25:989-994.
- Holzberg, S., Brosio, P., Gross, C., and Pogue, G. P. 2002. Barley stripe mosaic virus-induced gene silencing in a monocot plant. *Plant J.* 30:315-327.
- Huai, B., Fan, X., Yuan, Z., Xie, R., Sun, Y., Niu, Y., Rui, D., Wang, Y., Zheng, H., Hao, N., Yang, Q., and Pan, Y. 2026. Dual-role effector Bgt5481 from *Blumeria graminis* f. sp. *tritici* induces plant immunity and contributes to virulence. *Plant Physiol.* kiag185.
- Jacobs, K. A., Collins-Racie, L. A., Colbert, M., Duckett, M., Golden-Fleet, M., Kelleher, K., Kriz, R., LaVallie, E. R., Merberg, D., Spaulding, V., Stover, J.,

- Williamson, M. J., and McCoy, J. M. 1997. A genetic selection for isolating cDNAs encoding secreted proteins. *Gene* 198:289-296.
- Jeong, J., Moon, B., Hwang, I., and Lee, D. W. 2022. Green fluorescent protein variants with enhanced folding are more efficiently imported into chloroplasts. *Plant Physiol.* 190:238-249.
- Jones, J. D. G., and Dangl, J. L. 2006. The plant immune system. *Nature* 444:323-329.
- Koeck, M., Hardham, A. R., and Dodds, P. N. 2011. The role of effectors of biotrophic and hemi-biotrophic fungi in infection. *Cell. Microbiol.* 13:1849-1857.
- Lacomme, C., and Santa Cruz, S. 1999. Bax-induced cell death in tobacco is similar to the hypersensitive response. *Proc. Natl. Acad. Sci. U.S.A.* 96:7956-7961.
- Liang, Y. P., Xia, Y., Chang, X. L., Gong, G. S., Yang, J. Z., Hu, Y. T., Cahill, M., Luo, L. Y., Li, T., He, L., and Zhang, M. 2019. Comparative proteomic analysis of wheat carrying Pm40 response to *Blumeria graminis* f. sp. *tritici* using two-dimensional electrophoresis. *Int. J. Mol. Sci.* 20:933.
- Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* 25:402-408.
- Lo Presti, L., Lanver, D., Schweizer, G., Tanaka, S., Liang, L., Tollot, M., Zuccaro, A., Reissmann, S., and Kahmann, R. 2015. Fungal effectors and plant susceptibility. *Annu. Rev. Plant Biol.* 66:513-545.
- Lu, R., Malcuit, I., Moffett, P., Ruiz, M. T., Peart, J., Wu, A. J., Rathjen, J. P.,

- Bendahmane, A., Day, L., and Baulcombe, D. C. 2003. High throughput virus-induced gene silencing implicates heat shock protein 90 in plant disease resistance. *EMBO J.* 22:5690-5699.
- Miki, D., and Shimamoto, K. 2004. Simple RNAi vectors for stable and transient suppression of gene function in rice. *Plant Cell Physiol.* 45:490-495.
- Müller, M. C., Praz, C. R., Sotiropoulos, A. G., Menardo, F., Kunz, L., Schudel, S., Oberhänsli, S., Poretti, M., Wehrli, A., Bourras, S., Keller, B., and Wicker, T. 2018. A chromosome-scale genome assembly reveals a highly dynamic effector repertoire of wheat powdery mildew. *New Phytol.* 221:2176-2189.
- Nowara, D., Gay, A., Lacomme, C., Shaw, J., Ridout, C., Douchkov, D., Hensel, G., Kumlehn, J., and Schweizer, P. 2010. HIGS: Host-induced gene silencing in the obligate biotrophic fungal pathogen *Blumeria graminis*. *Plant Cell* 22:3130-3141.
- Oh, S. K., Young, C., Lee, M., Oliva, R., Bozkurt, T. O., Cano, L. M., Win, J., Bos, J. I. B., Liu, H. Y., van Damme, M., Morgan, W., Choi, D., Van der Vossen, E. A. G., Vleeshouwers, V. G. A. A., and Kamoun, S. 2009. In planta expression screens of *Phytophthora infestans* RXLR effectors reveal diverse phenotypes, including activation of the *Solanum bulbocastanum* disease resistance protein Rpi-blb2. *Plant Cell* 21:2928-2947.
- Pedersen, C., van Themaat, E. V. L., McGuffin, L. J., Abbott, J. C., Burgis, T. A., Barton, G., Bindschedler, L. V., Lu, X., Maekawa, T., Weßling, R., Cramer, R., Thordal-Christensen, H., Panstruga, R., and Spanu, P. D. 2012. Structure and

- evolution of barley powdery mildew effector candidates. *BMC Genomics* 13:694.
- Petre, B., Saunders, D. G. O., Sklenar, J., Lorrain, C., Win, J., Duplessis, S., and Kamoun, S. 2015. Candidate effector proteins of the rust pathogen *Melampsora larici-populina* target diverse plant cell compartments. *Mol. Plant-Microbe Interact.* 28:689-700.
- Praz, C. R., Bourras, S., Zeng, F., Sánchez-Martín, J., Menardo, F., Xue, M., Yang, L., Roffler, S., Böni, R., Herren, G., McNally, K. E., Ben-David, R., Parlange, F., Oberhaensli, S., Flückiger, S., Schäfer, L. K., Wicker, T., Yu, D., and Keller, B. 2017. *AvrPm2* encodes an RNase-like avirulence effector which is conserved in the two different specialized forms of wheat and rye powdery mildew fungus. *New Phytol.* 213:1301-1314.
- Qi, J., Wang, J., Gong, Z., and Zhou, J. M. 2017. Apoplastic ROS signaling in plant immunity. *Curr. Opin. Plant Biol.* 38:92-100.
- Rovenich, H., Boshoven, J. C., and Thomma, B. P. H. J. 2014. Filamentous pathogen effector functions: Of pathogens, hosts and microbiomes. *Curr. Opin. Plant Biol.* 20:96-103.
- Serna, L. 2005. A simple method for discriminating between cell membrane and cytosolic proteins. *New Phytol.* 165:947-952.
- Scofield, S. R., Huang, L., Brandt, A. S., and Gill, B. S. 2005. Development of a virus-induced gene-silencing system for hexaploid wheat and its use in functional analysis of the Lr21-mediated leaf rust resistance pathway. *Plant*

- Physiol. 138:2165-2173.
- Sohn, K. H., Lei, R., Nemri, A., and Jones, J. D. G. 2007. The downy mildew effector proteins ATR1 and ATR13 promote disease susceptibility in *Arabidopsis thaliana*. Plant Cell 19:4077-4090.
- Sohn, K. H., Segonzac, C., Rallapalli, G., Sarris, P. F., Woo, J. Y., Williams, S. J., Newman, T. E., Paek, K. H., Kobe, B., and Jones, J. D. 2014. The nuclear immune receptor RPS4 is required for RRS1SLH1-dependent constitutive defense activation in *Arabidopsis thaliana*. PLoS Genet. 10:e1004655.
- Thomas, W. J., Thireault, C. A., Kimbrel, J. A., and Chang, J. H. 2009. Recombineering and stable integration of the *Pseudomonas syringae* pv. *syringae* 61 hrp/hrc cluster into the genome of the soil bacterium *Pseudomonas fluorescens* Pf0-1. Plant J. 60:919-928. doi:10.1111/j.1365-3113X.2009.03998.x.
- Thordal-Christensen, H., Zhang, Z., Wei, Y., and Collinge, D. B. 1997. Subcellular localization of H₂O₂ in plants. H₂O₂ accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J. 11:1187-1194.
- Upadhyaya, N. M., Mago, R., Staskawicz, B. J., Ayliffe, M. A., Ellis, J. G., and Dodds, P. N. 2014. A bacterial type III secretion assay for delivery of fungal effector proteins into wheat. Mol. Plant-Microbe Interact. 27:255-264.
- Win, J., Chaparro-Garcia, A., Belhaj, K., Saunders, D. G. O., Yoshida, K., Dong, S., Schornack, S., Zipfel, C., Robatzek, S., Hogenhout, S. A., and Kamoun, S. 2012. Effector biology of plant-associated organisms: Concepts and perspectives. Cold Spring Harb. Symp. Quant. Biol. 77:235-247.

- Yan, T., Cheng, G., Zhai, Y., Wu, L., Gong, S., Yu, D., Yang, L., and Zeng, F. 2025. The putative effector BgtE-20069a, secreted from *Blumeria graminis* f. sp. *tritici*, suppresses plant immunity to facilitate wheat infection. BMC Microbiol. 25:389.
- Yin, W. X., Wang, Y. F., Chen, T., Yang, L., and Luo, C. X. 2018. Functional evaluation of the signal peptides of secreted proteins. Bio-Protoc. 8:e2839.
- Yoo, S. D., Cho, Y. H., and Sheen, J. 2007. Arabidopsis mesophyll protoplasts: A versatile cell system for transient gene expression analysis. Nat. Protoc. 2:1565-1572.
- Zeng, X. W., Luo, Y., Zheng, Y. M., Duan, X. Y., and Zhou, Y. L. 2010. Detection of latent infection of wheat leaves caused by *Blumeria graminis* f. sp. *tritici* using nested PCR. J. Phytopathol. 158:227-235.

Figure Captions

Fig. 1. Sequence features and expression pattern of BgtH12.

A, Predicted secondary structure composition. The diagram illustrates the distribution of alpha-helices (pink), beta-strands (yellow), and random coils (gray) along the protein sequence.

B, PCR amplification confirms the presence of the *BgtH12* gene in multiple *Bgt* races (E09, E15, E18, A13, A44).

C, Relative expression levels of *BgtH12* during infection of wheat by race E09 at indicated time points (0, 12, 24, 48, 72, and 96 hpi), determined by RT-qPCR and normalized to the fungal reference gene. Data are mean $\pm$ SE (n = 3). Statistical significance was determined by Student's t-test (*P < 0.05, **P < 0.01 versus 0 hpi).

D, Signal peptide prediction by SignalP-5.0 showing a cleavage site between amino acids 16 and 17 (left panel); TMHMM prediction confirming no transmembrane domains (right panel).

Fig. 2. Yeast secretion assay validates the function of the BgtH12 signal peptide.

A, Growth of yeast transformants on CMD-W medium (containing sucrose, upper) and YPRAA medium (lacking sucrose, lower). From left to right, the columns represent: the parental strain YTK12 (baseline control), the empty vector pSUC2 (negative control), BgtH12-SUC2 (experimental group), and Avr1b-SUC2 (positive control). Only the transformants with functional signal peptides (BgtH12 and Avr1b) grew on YPRAA medium.

B, TTC (2,3,5-triphenyltetrazolium chloride) assay for invertase activity. Secretion of active invertase leads to the conversion of sucrose into glucose, which reduces TTC to form a red precipitate. YTK12, pSUC2, BgtH12, and Avr1b represent the yeast strain, empty vector control, BgtH12-SUC2 fusion construct, and positive control (Avr1b-SUC2), respectively.

Fig. 3. Host-induced gene silencing of BgtH12 impairs fungal development and enhances host defense.

A, Schematic of the *BgtH12* gene structure showing the positions of the two HIGS fragments (HIGS site1, nt1–291; HIGS site2, nt404–630).

B, Disease symptoms and sporulation at 10 days post-inoculation (dpi) with *Bgt* race E09. Leaves were inoculated with BSMV:γ (control), BSMV:BgtH12-a, or BSMV:BgtH12-b, followed by *Bgt* challenge. BSMV:γ-PDS served as a positive control for virus infection (photobleaching).

C, Silencing efficiency of *BgtH12* at 24, 48, 72, and 96 hpi, measured by RT-qPCR. Transcript levels are shown relative to BSMV:γ control.

D, Aniline blue-stained fungal structures at 72 hpi at indicated time points. Bars = 20μm.

E, Hyphal length quantification.

F, Hyphal branch number quantification.

Data in C, E, and F are mean ± SD (n = 3). Statistical significance was determined by

Student's t-test (*P < 0.05, **P < 0.01 versus BSMV:γ control).

Fig. 4. Overexpression of BgtH12 enhances wheat susceptibility to powdery

A, Disease symptoms on wild-type (Fielder) and BgtH12-overexpressing transgenic lines (L2, L5) at 7 dpi with *Bgt* race E09.

B, Fungal biomass of L2 and L5 lines quantification by qPCR.

C, Relative BgtH12 expression in transgenic lines L2 and L5.

D, E, ROS accumulation at infection sites at 24 and 48 hpi. C, conidia; SH, secondary hyphae. Bars = 50 μ m.

F, Confocal micrographs and Quantification of hyphal length, branching, and infection area. Bars = 100 μ m.

Data in B, C, and F are mean $\pm$ SE ($n \geq 3$). Statistical significance was determined by Student's t-test ($P < 0.05$, $P < 0.01$, $*P < 0.001$ versus Fielder).

Fig. 5. Stable RNAi silencing of BgtH12 compromises fungal virulence and potentiates host immunity.

A, Disease symptoms on wild-type (Fielder) and BgtH12-RNAi lines (L4, L5) at 7 dpi with *Bgt*.

B, Fungal biomass quantification.

C, D, H₂O₂ detection and quantification by DAB staining at 24 and 48 hpi. C, conidia; SH, secondary hyphae. Bars = 50 μ m

E, Confocal micrographs and quantification of hyphal length, branching, and infection area at 72 and 96 hpi. Bars = 100 μ m.

Data in B, C, D, and E are mean $\pm$ SD ($n = 3$). Statistical significance was determined

by Student's t-test (* $P < 0.05$, ** $P < 0.01$ versus Fielder).

Fig. 6. Suppression of BAX-triggered cell death by BgtH12 in *N. benthamiana*.

- A**, Schematic of the transient expression assay. Six independent infiltration zones on the same leaf were assigned to three treatments (–BAX): MgCl_2 (buffer control), BgtH12, and Avr1b (positive control); and the same three treatments plus BAX (+BAX): MgCl_2 +BAX, BgtH12+BAX, and Avr1b+BAX.
- B**, Representative leaf images showing cell death phenotypes at 4–5 days post infiltration.
- C**, Chlorophyll-cleared leaf images showing necrotic lesions at 4–5 days post BAX infiltration. BgtH12 suppressed BAX-induced cell death to a level comparable to the positive control Avr1b.
- D**, Cell death assay with BgtH12^{ASP}. Treatments: BAX alone, GFP, and MgCl_2 (left), BgtH12^{ASP} alone and BgtH12^{ASP}+BAX (right).
- E**, Cell death phenotypes of the same treatments as in D at 4–5 days post infiltration.
- F**, Trypan blue staining leaves showing necrotic lesions. Necrosis was observed in BAX and BgtH12^{ASP}+BAX.

Experiments were repeated three times with consistent results.

Fig. 7. BgtH12 suppresses pattern-triggered immunity responses in wheat.

- A**, Callose deposition in wheat leaves expressing EtHAN:dsRed (control) or EtHAN:BgtH12 via T3SS delivery. Leaves were stained with aniline blue at 24

and 48 h after infiltration. Arrows indicate callose deposits. Bars = 20 μ m.

B, Quantification of callose deposits per unit area. Data are mean $\pm$ SD (n = 3 independent experiments).

C, ROS accumulation at *Bgt* infection sites in wheat leaves expressing EtHAN:dsRed or EtHAN:BgtH12. Leaves were inoculated with *Bgt* 48 h after T3SS infiltration and stained with DAB at 24 and 48 hpi. C, conidia; H, hyphae. Bars = 20 μ m.

D, Quantification of DAB-stained area per infection site. Data are mean $\pm$ SD (n $\geq$ 30 sites per sample).

Statistical significance was determined by Student's t-test (*P < 0.05 versus EtHAN:dsRed control).

Fig. 8. Subcellular localization of BgtH12 in in plant cells.

A, Wheat mesophyll protoplasts expressing free GFP or BgtH12^{ASP}-GFP. Free GFP shows diffuse cytoplasmic and nuclear fluorescence, while BgtH12^{ASP}-GFP accumulates at the cell periphery and in the nucleus. Bars = 20 μ m.

B, *N. benthamiana* leaf epidermal cells expressing free GFP or BgtH12^{ASP}-GFP.

Consistent with the wheat protoplast results, BgtH12^{ASP}-GFP localizes to the cell periphery and nucleus. Bars = 50 μ m.

C, Western blot analysis of BgtH12-GFP fusion proteins using anti-GFP antibody.

Lanes: WT (negative control), free GFP (~25 kDa, positive control), BgtH12^{ASP}-GFP, and full-length BgtH12-GFP. Both fusion proteins migrated at ~55 kDa with no free GFP. Ponceau S staining served as loading control.

D, Plasmolysis assay in *N. benthamiana* leaf epidermal cells expressing full-length

BgtH12-GFP and BgtH12^{ΔSP}-GFP, together with the plasma membrane marker TaWP16-mCherry, before (upper) and after (lower) treatment with 0.8 M mannitol. Red boxes indicate the magnified regions shown in the corresponding insets, where white letters and red arrowheads denote the nucleus (N) and the apoplastic space (arrowheads). Fluorescence in the apoplastic space was observed for full-length BgtH12-GFP (a, b) but not for BgtH12^{ΔSP}-GFP (d, e). Corresponding nuclear regions showing bright GFP signals in both constructs (c, f). Fluorescence intensity values in the indicated regions are shown on the right using ImageJ. Bars = 20 μm (main panels); 5 μm (insets).

Supplementary Figure 1. Molecular identification of T₀ transgenic wheat lines.

A, PCR confirmation of the BgtH12 overexpression construct in transgenic T₀ wheat lines.

B, PCR confirmation of the BgtH12 RNAi construct in transgenic T₀ wheat lines. The primers used for identification are listed in Supplementary Table 1.

Fig. 2

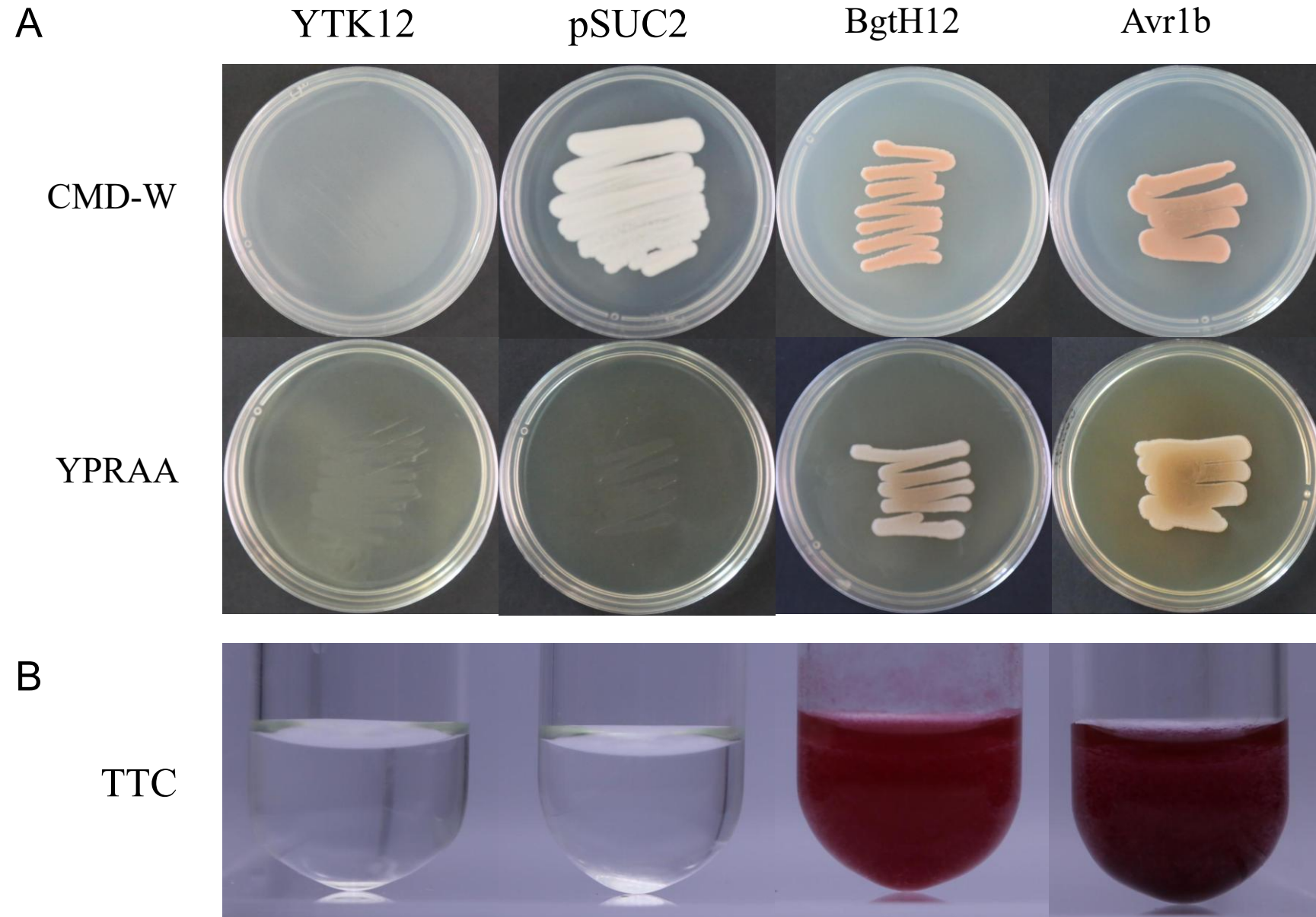


Fig. 3

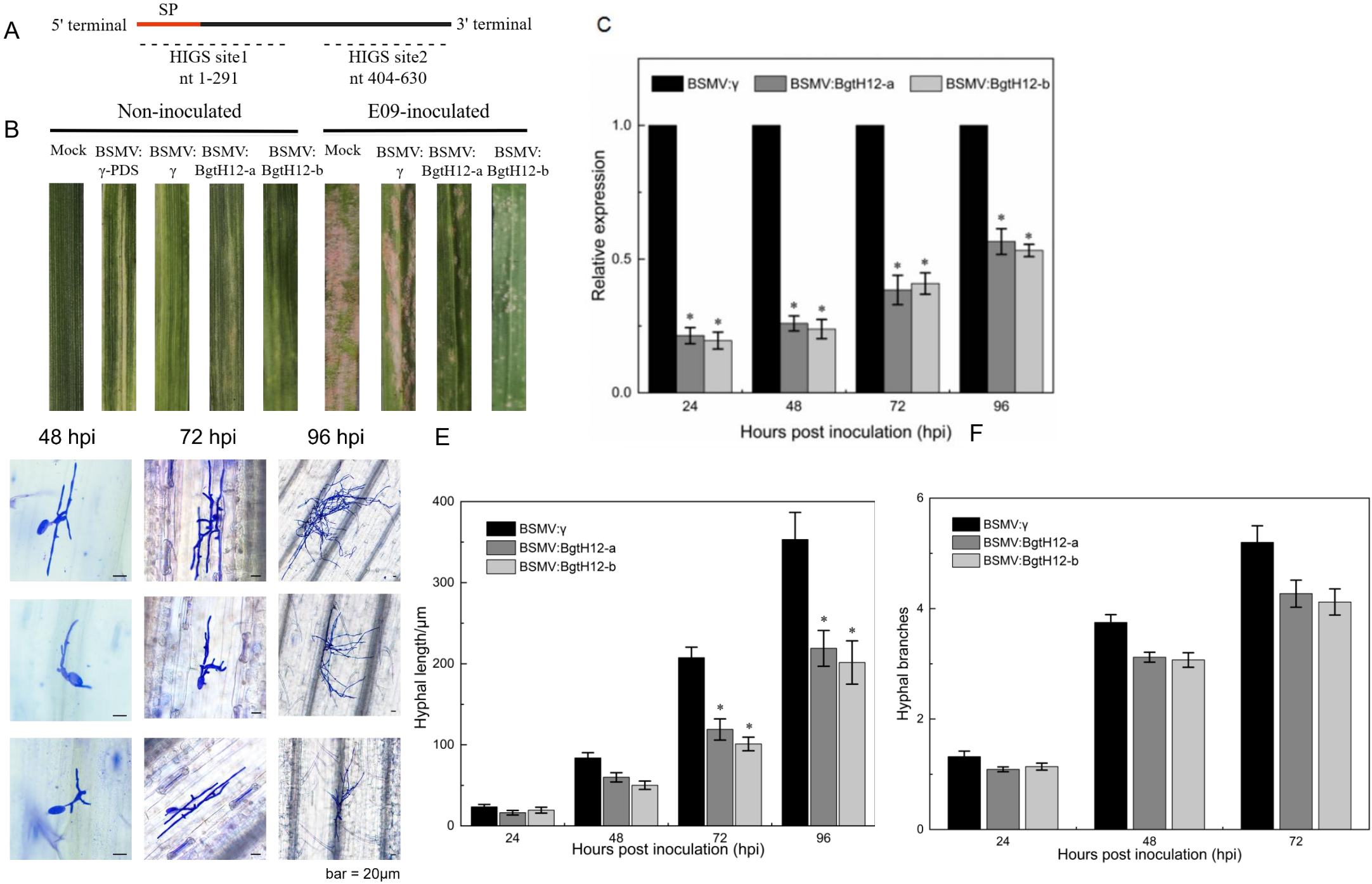


Fig. 4

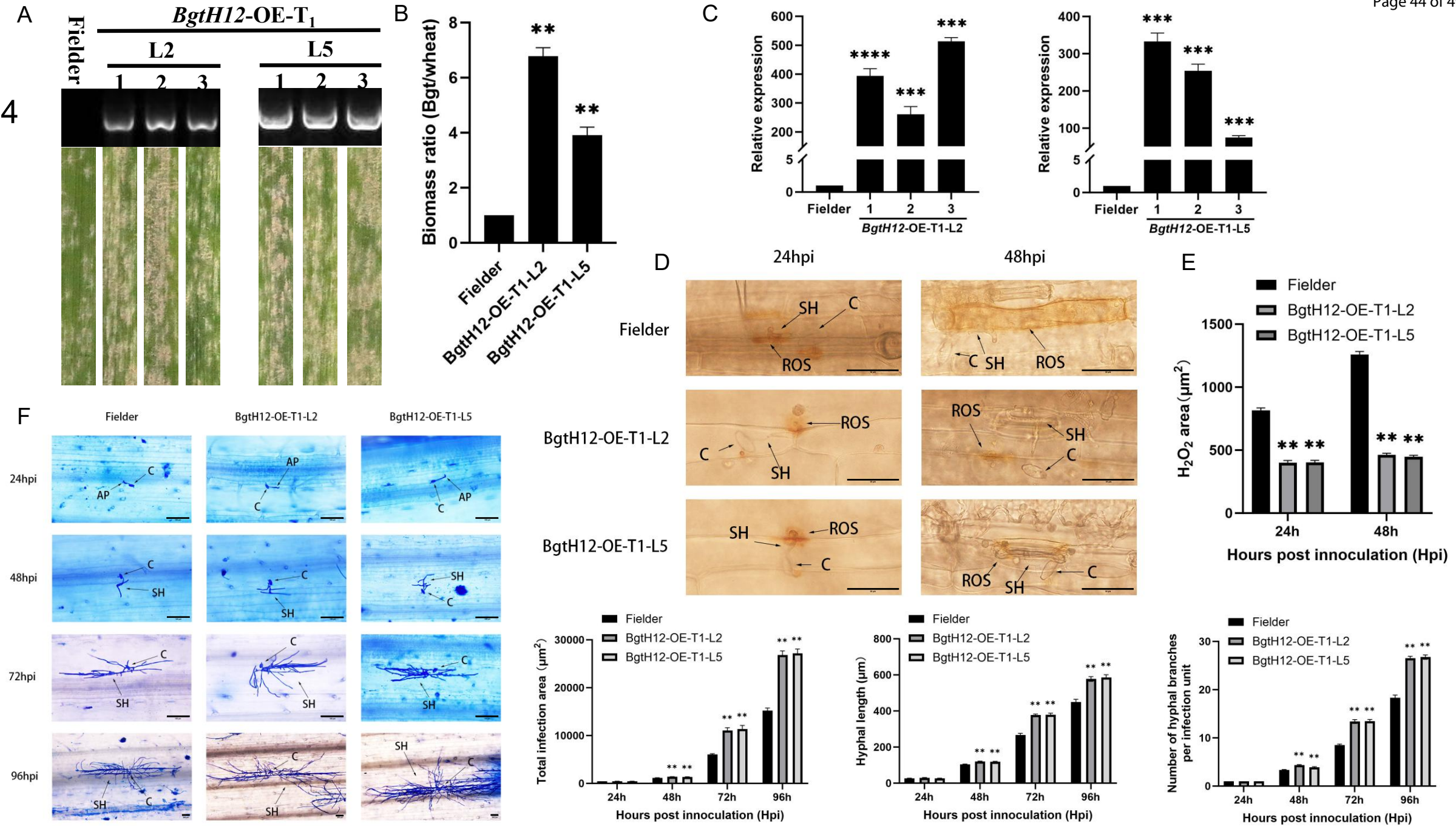


Fig. 5

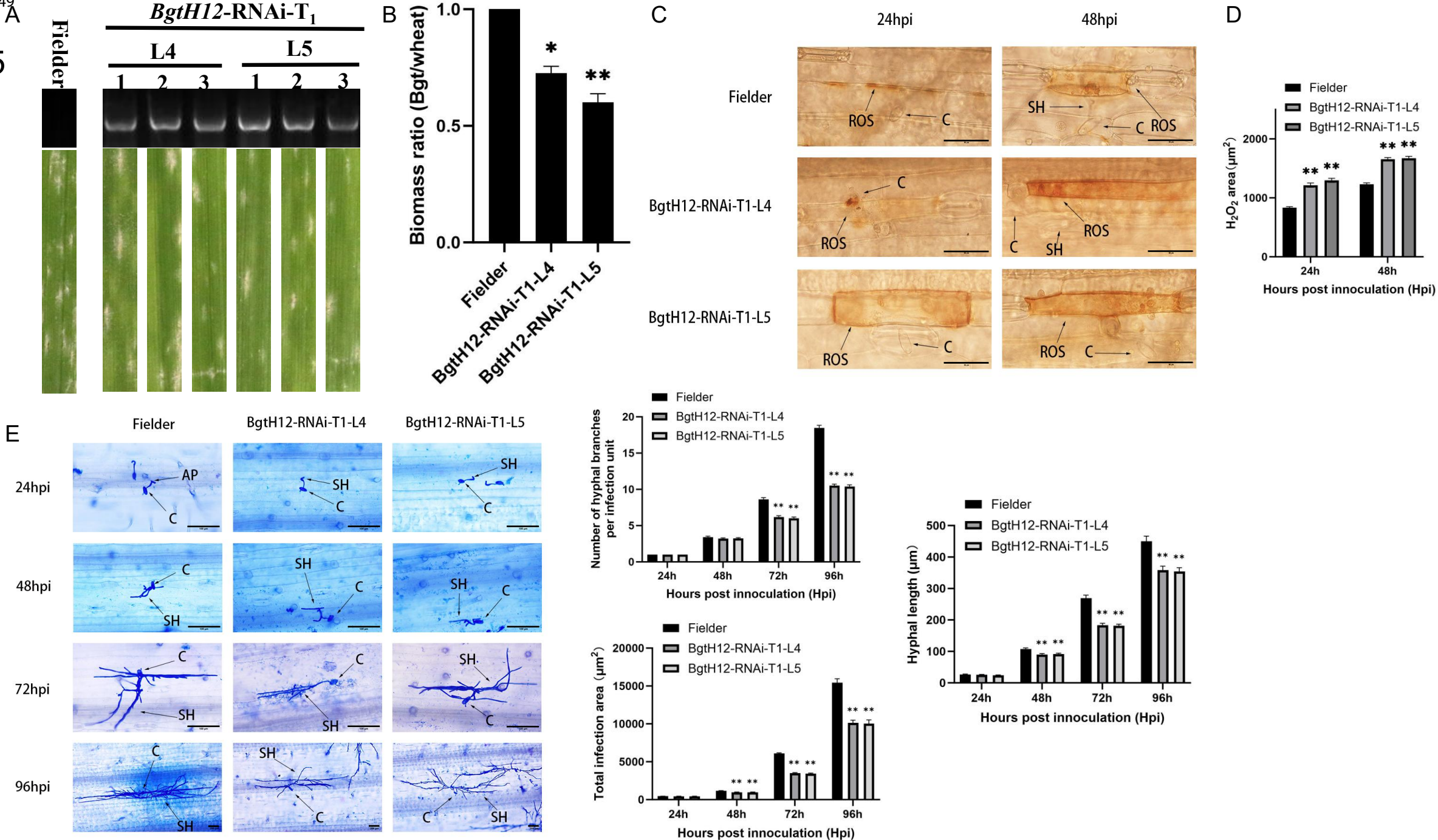


Fig. 6

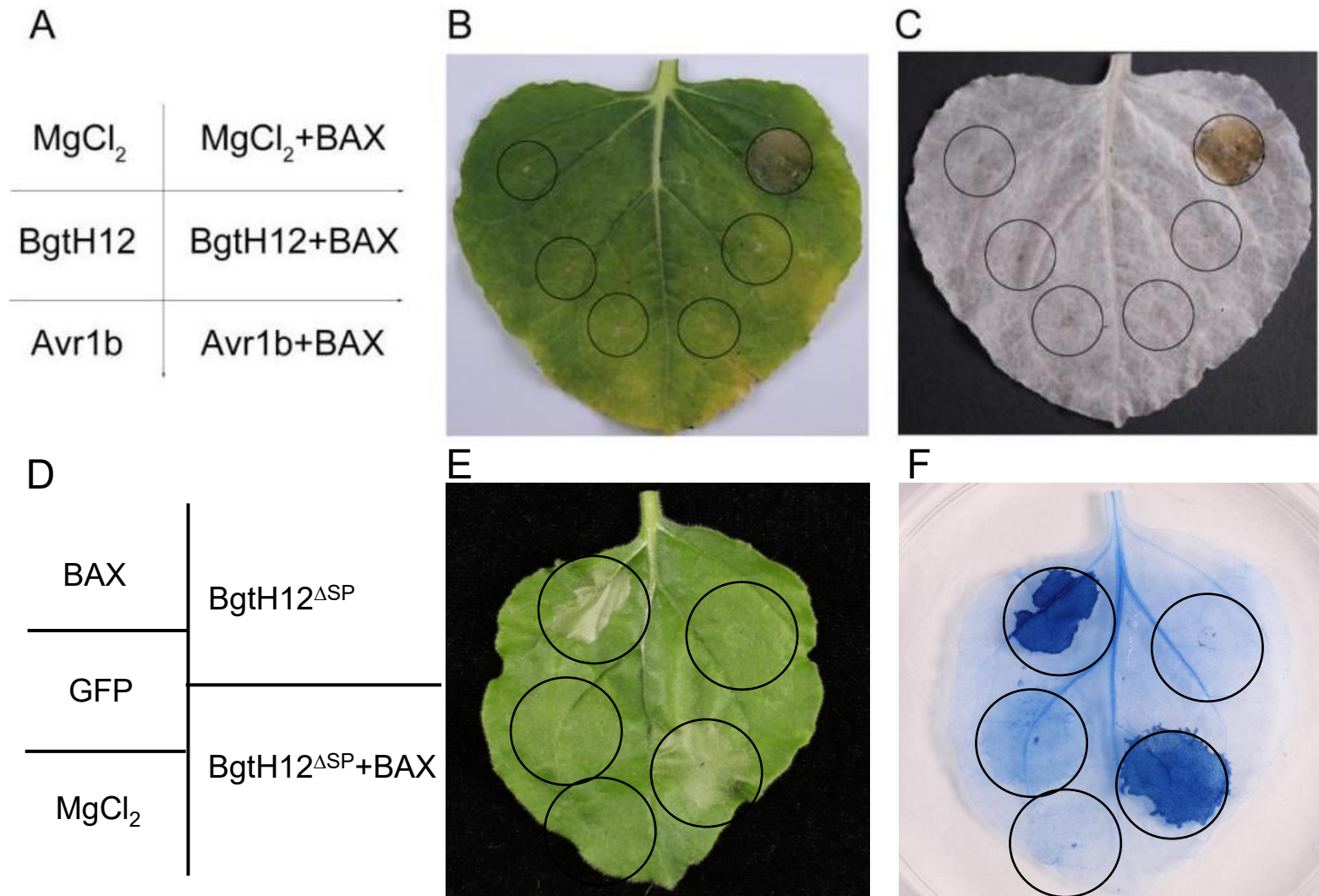
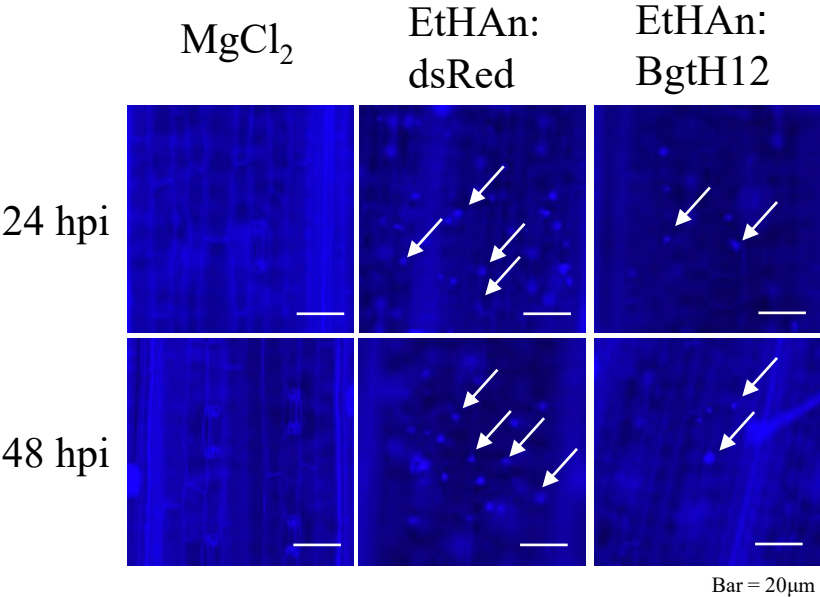
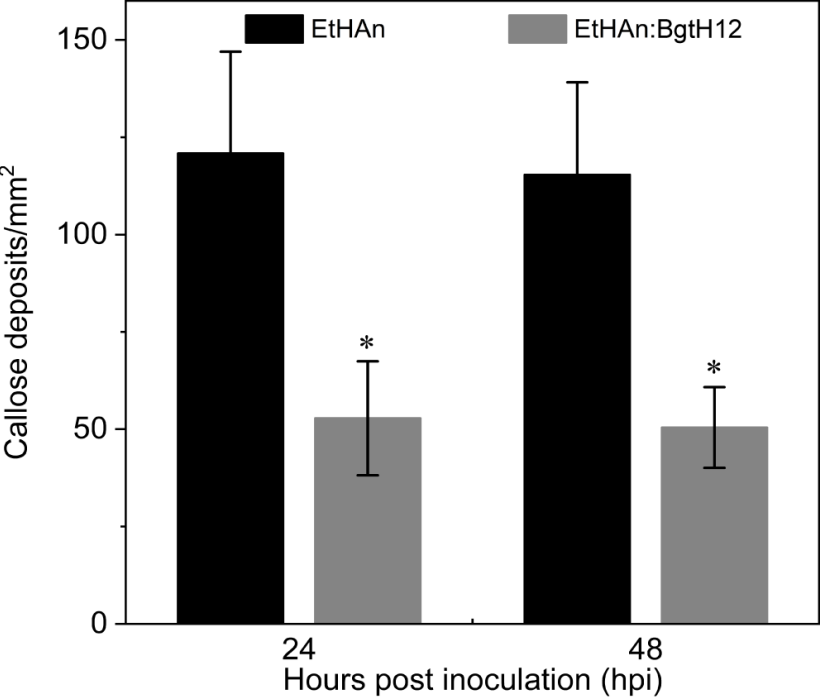


Fig. 7

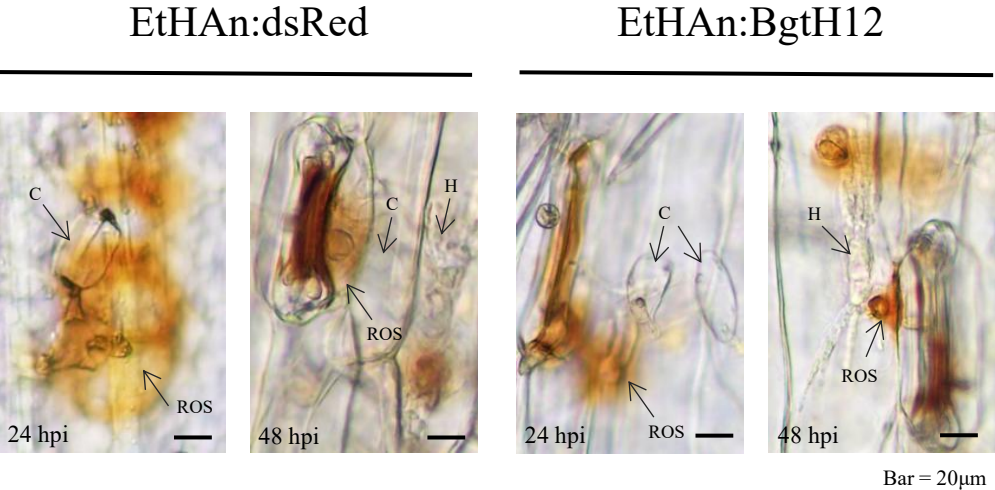
A



B



C



D

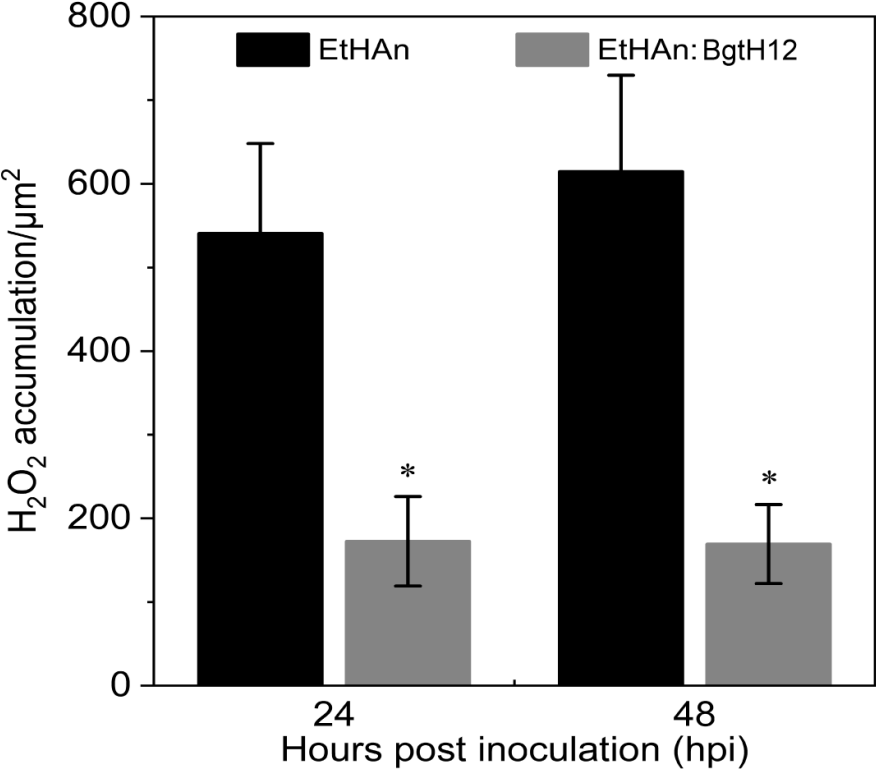


Fig. 8

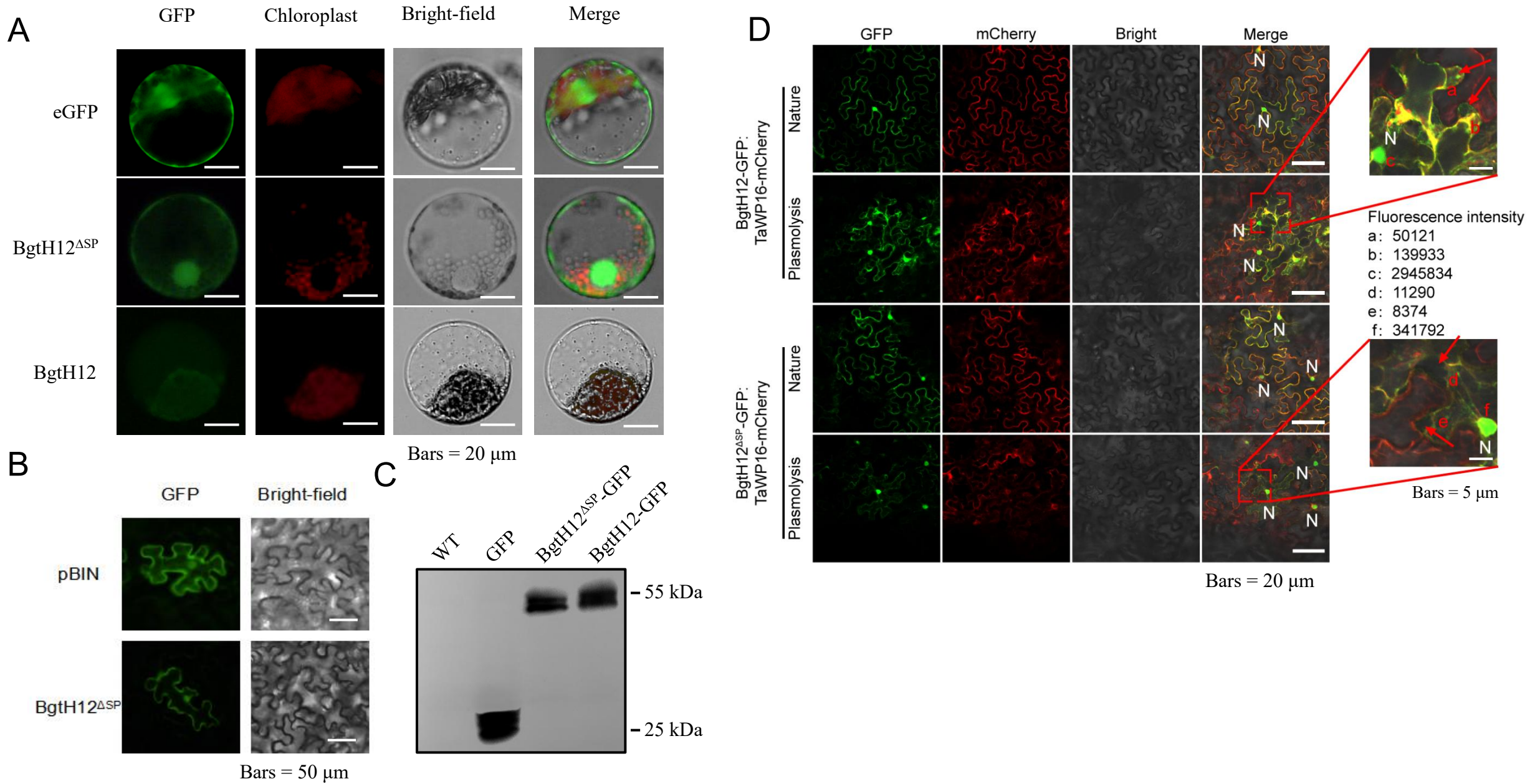
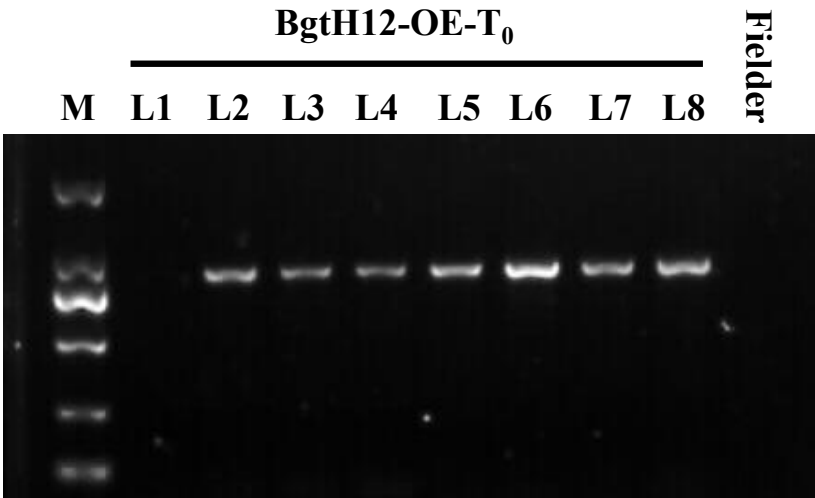


Fig. S1

A



B

