



# Over expression of a Chitinase 2 gene from Chinese Wild Strawberry improves resistance to anthracnose disease in transgenic *Arabidopsis thaliana*

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## Abstract

Strawberry (*Fragaria × ananassa* Duch.) is one of the most widely cultivated fruit crop. Anthracnose caused by *Colletotrichum* spp. is a devastating disease of strawberry, causing large-scale strawberry losses worldwide. Chitinases act as defence proteins and are crucial for plant response to pathogens. Here, we isolated a class V Chitinase gene (designed as *FnCHIT2*, GenBank accession number MN709779) from Chinese wild diploid strawberry *Fragaria nilgerrensis* Schlecht (*F. nilgerrensis*), a species that exhibits high tolerance to anthracnose. Gene expression analysis showed that *FnCHIT2* expression was highly induced after *Colletotrichum gloeosporioides* inoculation and salicylic acid (SA) treatment. Subcellular localization analysis revealed the presence of *FnCHIT2* in the plasma membrane. Recombinant *FnCHIT2* protein was successfully expressed in *E. coli* Rosetta (DE3). Furthermore, we transformed *FnCHIT2* into Col-0 wild type *A. thaliana* to perform functional analysis and evaluated the functions of *Colletotrichum higginsianum* and *Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000). *FnCHIT2* overexpression in *A. thaliana* showed enhanced resistance to *C. higginsianum* and *Pst* DC3000. Enhanced disease resistance of *FnCHIT2* transgenic plants to *C. higginsianum* was correlated with *pathogenesis-related gene 1* (*AtPRI*) and *plant defensin 1.2* (*AtPDF1.2*) gene expression levels. These results provide evidence that *FnCHIT2* may play an important role in response to fungal pathogens in strawberry. Our study provides an important theoretical reference for future strawberry resistance breeding.

**Keyword** Strawberry · Anthracnose · *FnCHIT2* · Signal transduction pathways

## Abbreviations

*F. nilgerrensis*      *Fragaria nilgerrensis* Schlecht  
*C. gloeosporioides*      *Colletotrichum gloeosporioides*  
SA      Salicylic acid

JA      Jasmonic acid  
*A. thaliana*      *Arabidopsis thaliana*  
*Pst* DC3000      *Pseudomonas syringae* Pv. tomato DC3000  
*C. higginsianum*      *Colletotrichum higginsianum*  
PR1      Pathogenesis-related gene 1  
PDF1.2      Plant defensin 1.2  
PCR      Polymerase chain reaction  
qRT-PCR      Quantitative real-time PCR  
IPTG      Isopropylthio β-D- thiogalactoside  
PVDF      Polyvinylidene difluoride  
TBST      Tris-Buffered Saline Tween-20  
PEG      Polyethylene glycol  
ROS      Reactive oxygen species  
hpi      Hours post inoculation  
dpi      Days post inoculation  
NBT      Nitro blue tetrazolium

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## Introduction

Cultivated strawberry [*Fragaria* × *ananassa* Duch. (2n = 8x = 56)] is one of the most economically important horticultural crop throughout the world. However, fruit yield and quality are limited by a range of biotic and abiotic stresses. Anthracnose caused by *Colletotrichum* spp. is one of the most devastating fungal diseases in strawberry (Karimi et al. 2019). According to previous studies, *C. acutatum*, *C. gloeosporioides*, and *C. fragariae* are causative agents of strawberry anthracnose. However, some recent studies have reported that several new *Colletotrichum* spp. can cause anthracnose in strawberry (Chung et al. 2020). China has diverse germplasm resources of the genus *Fragaria*, including some diploid strawberries, such as *F. nilgerrensis*, *F. hayatai*, *F. mandshurica*, and *F. chinensis*. This diploid strawberry germplasm have been utilized for strawberry breeding programmes based on numerous characteristics such as distinct aroma and resistance to fungal diseases (Zhang et al. 2020a, b). *F. nilgerrensis* is a wild strawberry that is widely distributed in Southwest China this diploid strawberry has been exploited as a breeding material. For example, the special characteristic of *F. nilgerrensis* (aroma) was introduced into *Fragaria* × *ananassa*. This process added to the genetic diversity of cultivated strawberry (Noguchi et al. 2002). In addition to its special aroma, this wild strawberry also has high resistance levels against abiotic (drought and heat) and biotic (anthracnose and pests) stresses (Zhang et al. 2016). Understanding the mechanisms of disease resistance and characterizing genes involved in resistance in *F. nilgerrensis* can greatly accelerate strawberry disease resistance breeding.

Chitinases (EC3.2.1.14) are lytic enzymes that are present in a wide range of organisms from microorganisms to animals and higher plants (Dong et al. 2017). Chitinase catalyses the hydrolysis of  $\beta$ -1, 4 linkages of N-acetyl-D-glucosamine in chitin (Singh et al. 2015) and is the second most abundant biopolymer in nature after cellulose. Chitin is a major structural component of the cell walls of many pathogenic fungi and insect skeletons (Grover et al. 2012). Chitinases are strongly induced in plant responses to bacteria, fungi, and viruses (Grover et al. 2012). These proteins have an important role in plant defence against pathogen attack (Abeles et al. 1971; Punja and Zhang 1993). Hence, understanding exactly how chitinases are involved in plant immune systems and the roles they play is important for the study of plant resistance mechanisms (Dong et al. 2017). Previously, many scientists have reported the involvement of the chitinase gene in fungal disease resistance mechanisms. For example, overexpression of the chitinase gene *EuCHIT2* from *Eucommia ulmoides* oliver (*E.*

*ulmoides*) enhanced resistance to *Erysiphe cichoracearum* DC. In tobacco plants (Dong et al. 2017). Overexpression of a barley chitinase class-II gene in sugarcane enhanced resistance against red rot (Tariq et al. 2018). Furthermore, overexpression of a potato class I chitinase gene in transgenic tea enhanced resistance to blister blight (Singh et al. 2015). Transformation of barley antifungal genes chitinase and ribosome-inactivating protein-induced fungal resistance in black gram (Chopra and Saini 2014). Constitutive expression of a class II chitinase gene (*Zjchi2*) from zoysia grass significantly enhanced antifungal activity in transgenic zoysia grass compared with wild-type plants (Kang et al. 2017). Overexpression of an endo-chitinase gene from barley in potatoes decreased susceptibility after inoculation with *Alternaria solani* compared to the control (Khan et al. 2017). The pepper chitinase gene *ChiIV3* acted as an antifungal protein and as a receptor for unidentified chitin in plants to trigger cell death and defence signalling against *Phytophthora capsici* infection (Liu et al. 2017). Studies have also demonstrated that genes encoding proteins that belong to chitinase families in plants are involved in abiotic-stress responses, such as osmotic, salt, cold, wounding, and heavy metal stresses (Brotmana et al. 2012). In melon, heat shock-induced resistance increased chitinase-1 gene expression (Widiastuti et al. 2012). In *A. thaliana*, *Athot2* encodes an endo-chitinase-like protein that is essential for tolerance to heat, salt, and drought stresses (Kwon et al. 2007). According to some studies, abiotic and biotic stress conditions can elevate chitinase levels in *P. ginseng* (Brotmana et al. 2012; Pulla et al. 2011). In addition, thirty-two chitinase genes were identified in *Ammopiptanthus nanus* and 3 chitinase genes were strongly induced by low temperature and osmotic stress (Cao et al. 2019). The potential roles of chitinase genes in other plants species justify the need for functional analysis of chitinase genes in strawberry.

Two strawberry genes (class II chitinase) were partially characterized and were induced after inoculation with *Colletotrichum fragariae* or *Colletotrichum acutatum* (Akhan et al. 2004). The *ch5B* gene encodes a chitinase from *Phaseolus vulgaris*. This gene was transformed along with *gln2* and *ap24* into strawberry (cultivar Pájaro), and transgenic plants displayed high levels of resistance to grey mould disease (*Botrytis cinerea*) (Vellicce et al. 2006). A chitinase gene from *Lycopersicon chilense* was transformed into 'Joliette' strawberry, and the transgenic strawberry exhibited increased resistance to *Verticillium dahliae* compared to non-transgenic plants (Chalavi and Tabaeizadeh 2003). However, characteristics and functional analysis of the chitinase gene from Chinese wild strawberry has not been reported.

In a previous study, we identified a Chitinase 2 gene through RNA-Seq analysis that was differently expressed

after inoculation with *C. gloeosporioides* in *F. nilgerrensis*. In the present study, we isolated this gene *FnCHIT2* (GH-18 family) and expressed the recombinant FnCHIT2 protein in *E. coli* and overexpressed the gene in *A. thaliana* to evaluate its potential function in resistance to *C. higginsianum* and *PstDC3000*. Based on our results, we suggest that the *FnCHIT2* gene may function as a positive regulator in the strawberry defence response to anthracnose.

## Materials and methods

### Plant materials and pathogens

Chinese wild diploid strawberry (*Fragaria nilgerrensis* Schltdl,  $2n = 2x = 14$ ) was maintained at  $23 \pm 2^\circ\text{C}$  under a 14-h (h) day/10-h night photoperiod with 75% humidity. The plants were provided by Beijing Academy of Agricultural Sciences, Beijing, China. These plants were propagated as runner plants. After propagation, plants were maintained for an additional 2 weeks before inoculation with the pathogen *C. gloeosporioides* (strain Lch-1911). *A. thaliana* wild-type (WT) and *35S::FnCHIT2* overexpression transgenic plants with a Col-0 ecotype background were used in this study. Plants were grown in a growth chamber at  $21^\circ\text{C}$ , 70% relative humidity, and a 12-h light/12-h dark cycle for 5 weeks.

### Cloning and bioinformatics analysis of *FnCHIT2*

Total RNA was extracted from leaves of *F. nilgerrensis* using the RNAPre Pure Plant Kit (Tiangen, China). First-strand cDNA was synthesized from 1  $\mu\text{g}$  of total RNA using the Primer Script™ 1st Strand cDNA Synthesis kit (TaKaRa Bio Inc., Dalian, China) according to the manufacturer's guidelines. Polymerase chain reaction (PCR) master mix (Promega, Beijing, China) was used to amplify the ORF sequence of *FnCHIT2*. PCR was performed with an initial denaturation step of  $94^\circ\text{C}$  for 90 s; 32 cycles of denaturation at  $94^\circ\text{C}$  for 30 s, annealing at  $58^\circ\text{C}$  for 30 s, and extension at  $72^\circ\text{C}$  for 90 s; and a final extension at  $72^\circ\text{C}$  for 10 min. PCR products were purified using the Universal DNA Purification Kit DNA (Tiangen Bio Inc., Beijing, China) and cloned into the pMD® 18-T vector for sequencing (Shang Ya Bio Inc., Fuzhou, China). Molecular weight (MW) and isoelectric point (pI) predictions for the deduced protein were performed using the online ExPASy proteomics server database (<http://www.expasy.org/tools/protparam.html>). Sequence alignment analysis was undertaken with DNAMAN (version 7.0), and the phylogenetic tree was generated using the CLUSTALW2 program (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>).

### SA treatment of *F. nilgerrensis*

For SA treatment, strawberry plants were sprayed with 100  $\mu\text{M}$  SA (Sigma), and 0.05% Tween-20 was used as a surfactant (Wang et al. 2006), plants were grown in a growth chamber at  $21^\circ\text{C}$ , 70% relative humidity. The leaves were harvested at 0, 6, 12, 24, and 48 h post spraying. The treatments were replicated thrice, and samples were stored at  $-80^\circ\text{C}$  (for RNA analysis).

### Subcellular localization of FnCHIT2 protein

ORF regions of *FnCHIT2* were amplified using specific primers F-FnCHIT2-YFP and R-FnCHIT2-YFP (Table S1) that contained *BamH* I and *Kpn* I, and the product was subcloned into pYFPc vector. The resulting vectors were confirmed by sequencing, pm-rk CD3-1007 fusion protein as a plasma membrane-anchored marker (Nelson and Cai 2007). Four-week-old tobacco plants were used for transient expression (Wang et al. 2018). YFP fluorescence in tobacco leaves was examined by laser scanning confocal microscopy (Leica TCS SP8, Leica Microsystems, German).

### Pathogen inoculation

The *C. gloeosporioides* strain “Lch-1911” provided by Dr. Han Yongchao (Han et al. 2018) was cultured in liquid PDA medium (potato dextrose broth) at  $25^\circ\text{C}$  for 7–10 days with shaking. Then, conidia were collected by centrifugation (3000 rpm/min) and subsequently filtrated with cheese-cloth. Conidia were then re-suspended in distilled water and adjusted to  $1 \times 10^6$  conidia/mL. Five mL of suspension was sprayed with a hand sprayer over each strawberry plant (*F. nilgerrensis*), and control plants were sprayed with distilled water. Twenty uniform, healthy, and micro propagated plantlets at the stage of ten compound leaves were used in the experiment. All inoculations were performed thrice. The inoculated plants were maintained in a moist environment at  $28^\circ\text{C}$  (Suzuki et al. 2010), and the leaves were harvested at 0, 6, 12, 24, 48, and 72 h after inoculation.

*Pseudomonas syringae* pv. tomato DC3000 (*Pst* DC3000) was cultured in King's B medium containing rifampicin (60  $\mu\text{g}/\text{mL}$ ) and kanamycin (50  $\mu\text{g}/\text{mL}$ ) at  $28^\circ\text{C}$  overnight. Then, pathogens were collected by centrifugation at 3000 g, then washed with 10 mmol  $\text{MgCl}_2$  and diluted with distilled water to concentrations of  $1 \times 10^5$  and  $10^7$  cfu/mL for syringe and spray inoculations, respectively (Katagiri et al. 2002; Zhang et al. 2015). Control treatment was performed with distilled water. After inoculation, the plants were placed in a growth chamber under 16-h light ( $23^\circ\text{C}$ )/8-h dark ( $21^\circ\text{C}$ ) cycles. Leaves were collected at 48 h post-inoculation. Furthermore, the bacterium growth assays in plants were performed, as previously described (Zhang et al. 2015).

*C. higginsianum* was used to inoculate transgenic *Arabidopsis* to elucidate the biological function of *FnCHIT2* against anthracnose infection. *C. higginsianum* strain Ch-1 was provided by Dr. Zheng (Gu et al. 2019) and cultured on PDA at 25 °C in dark conditions for 7 days. Conidia were harvested from PDA culture plates by flooding the surface of culture with distilled water and filtering the suspension through cheese-cloth (Casado-Díaz et al. 2006). The spore suspension was examined with a haemocytometer and adjusted to  $1 \times 10^6$  conidia/mL. Nine pots of four-week-old *Col A. thaliana* plants were sprayed with 5 mL spore suspension with a hand sprayer. The control plants were sprayed with distilled water. For qRT-PCR experiments, the leaves were collected at 0, 6, 12, 24, 48, 72, and 120 h after inoculation. For trypan blue staining and nitroblue tetrazolium (NBT) staining, fully developed rosette leaves were drop-inoculated with 8  $\mu$ L of *C. higginsianum* spore suspension containing  $1 \times 10^6$  spores/mL (Wen et al. 2015).

### Recombinant FnCHIT2 protein expression in *E. coli*

pMD<sup>®</sup>-18-T-FnCHIT2 plasmid was used as a template to amplify a truncated gene encoding a signal-deleted gene *FnCHIT2-1*. Gene amplification using primer (F/R) resulted in the deletion of the first 84 nucleotides in the N-terminal of the *FnCHIT2* gene, and PCR products were cloned into pMD<sup>®</sup> 18-T vector and sequenced. The truncated gene was cloned into the multiple cloning site of the prokaryotic expression vector pET32A using *Bam*H I and *Xba* I to generate recombinant vector pET32A-FnCHIT2-1 (Fig. 2S B). The recombinant plasmid was transformed to expression host *E. coli* strain Rosetta (DE3) (Yueyang Bio Inc., Beijing, China) competent cells. For induction of FnCHIT2-1 protein expression, isopropyl- $\beta$ -D-thiogalactoside (IPTG) was added to a final concentration of 0.5 mmol/L. Bacterial cells were harvested by centrifugation, and cell pellets were further separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to analyse the expression of the recombinant proteins (Laemmli et al. 1970). Ni-NTA Agarose (Qiagen) was used to purify the recombinant protein. For Western blotting, proteins were electrophoresed on 12% SDS-PAGE, transferred to the polyvinylidene difluoride (PVDF) membrane, and then blocked with the membrane 5% skim milk powder in Tris-Buffered Saline Tween-20 (TBST) for 2 h. Then, blots were incubated with primary antibodies 1 h at room temperature followed incubation with the secondary antibody (goat-anti-rabbit IgG) (Thermo Pierce Fisher, Shanghai, China) at a dilution of 1:5000 for 45 min. The protein bands were visualized upon exposure to diaminobenzidine (DAB) substrate (Tiangen Biotechnology Co., Ltd., Beijing, China). Bacterial cells were broken using a supersonic technique, and the *E. coli* lysate was centrifuged. The resulting supernatant liquid was purified with Ni-NTA

Agarose (Qiagen), and FnCHIT2 recombinant protein was detected with 12% SDS-PAGE.

### Plasmid construction and plant transformation

To generate the 35S::*FnCHIT2* construct, the ORF sequence *FnCHIT2* was amplified, and the restriction sites *Bam*H I and *Kpn* I were introduced on both ends of the sequence. Then, the FnCHIT2 coding sequence was introduced into the pCMBIA1300-HA construct under the control of the 35S promoter. The resulting construct was introduced into *Agrobacterium tumefaciens* strain GV3101 and then employed for *A. thaliana* (Col-0) transformation via the floral dip method (Clough et al. 1998). T<sub>0</sub> seeds were collected and grown on Murashige and Skoog (MS) (1962) medium (pH 5.8, 10 g/L sucrose, 8 g/L agar) supplemented with 50 mg/L hygromycin. Transgenic plants were identified by PCR amplification using gene-specific primers. Three of twenty-seven T<sub>2</sub> independent lines showed the highest resistance to *C. higginsianum* and *Pst* DC3000 inoculation and were selected for further experiments.

### Expression analysis of FnCHIT2 in strawberry and defence-related genes in *A. thaliana*

Total RNA was extracted from *C. gloeosporioides*-inoculated strawberry leaves and *C. higginsianum*-infected *A. thaliana* leaves. Briefly, 1 mg RNA was treated with amplification-grade DNase I (Invitrogen). Three biological replicates were used for each experiment. The first strand of cDNA was synthesized using the PrimeScript™ 1st Strand cDNA Synthesis Kit (TaKaRa Biotechnology, Dalian, China) following the manufacturer's instructions. Subsequently, the cDNA was diluted sixfold with the sterile water and stored at –40 °C for future use. Quantitative real-time PCR analysis was performed using SYBR Green (TaKaRa Biotechnology, Dalian, China) with the Step One Plus Real-Time PCR System (Applied Biosystems, Foster, CA, USA). PCR conditions were as follows: a denaturing step of 95 °C for 30 s and 42 cycles of denaturing at 95 °C for 5 s and annealing at 60 °C for 30 s. The *Actin* gene (Acc. No. AB116565) and *Actin1* gene (Acc. No. AT3G18780) were used as internal controls for strawberry and *A. thaliana*, respectively. The primer sequences used for qRT-PCR are listed in Table S2.

### Statistical analysis

All the experiments were performed with three technical and biological replicates. Data analysis and plotting were performed using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and SigmaPlot 12.0 (Systat, Inc., Point Richmond, CA, USA), respectively. Statistical data analysis



(Student's *t* test, \* $p < 0.05$ , \*\* $p < 0.01$ ) was performed using SPSS 16.0 (IBM Corporation, Chicago, Illinois, USA).

## Results

### Characterization and expression analysis of *FnCHIT2*

A transcriptomic analysis of *F. nilgerrensis* was performed using RNA-seq and demonstrated *FnCHIT2* (GenBank accession number MN709779) expression was highly induced after inoculation with *C. gloeosporioides*. Then, we isolated the *FnCHIT2* cDNA sequence from the leaves of *F. nilgerrensis*. A 912-bp *FnCHIT2* cDNA fragment located on chromosome 3 was cloned from *F. nilgerrensis* (Fig. 1S and Fig. 2S A). The predicted open reading frame encodes a polypeptide of 303 amino acid residues (Fig. 1S) with a predicted molecular mass of 33.96 Kda and a pI value of 7.09. The predicted protein contained a glycosyl hydrolase family 18 (GH18) domain from amino acid residues 95–156 with two carbohydrate-binding sites (CBD domain) in the N-terminal. However, the predicted protein lacks the chitin-binding domain and belongs to the class V plant chitinases. The deduced amino acid sequence shows relatively high homology with GH18-chitinase-like family members of Rosacea plants. For example, FnCHIT2 has 100%, 82%, and 78.11% similarity with FvCHIT2 (XP\_004295963), RcCHIT2 (XP\_024159678), and PmCHIT2 (XP\_008223957), respectively (Fig. 1a). A phylogenetic tree was generated using putative amino acid sequences of FnCHIT2 and some counterpart GH18-chitinase-like family members from other species. FnCHIT2 clustered in the same clade as FvCHIT2 and RcCHIT2 (Fig. 1b). The responsiveness of *FnCHIT2* to *C. gloeosporioides* was analysed. In *F. nilgerrensis* strawberry, *FnCHIT2* expression was increased at 6–12 h post inoculation (hpi) (Fig. 1c) to levels greater than those of mock-inoculated plants. Upon SA treatment, *FnCHIT2* mRNA levels were increased 3.4-fold to 32.9-fold at 6 to 48 hpi, reaching a maximum level at 6 hpi and then decreasing at 48 hpi (Fig. 1d).

### Subcellular location of FnCHIT2 protein

To investigate the subcellular location of FnCHIT2 protein, the ORF of FnCHIT2 was fused with a yellow fluorescent protein (YFP) under the control of the 35S promoter. Transient expression in tobacco leaves was examined by laser scanning confocal microscopy (Leica TCS SP8, German, Leica Microsystems, German) (Fig. 2a and Fig. 2S B). Using the pm-rk CD3-1007 fusion protein plasma membrane marker and free YFP vector fluorescence of mCHERRY was observed to distribute almost uniformly along the cell wall and the cell peripheral surface (Fig. 2b). Co-transient

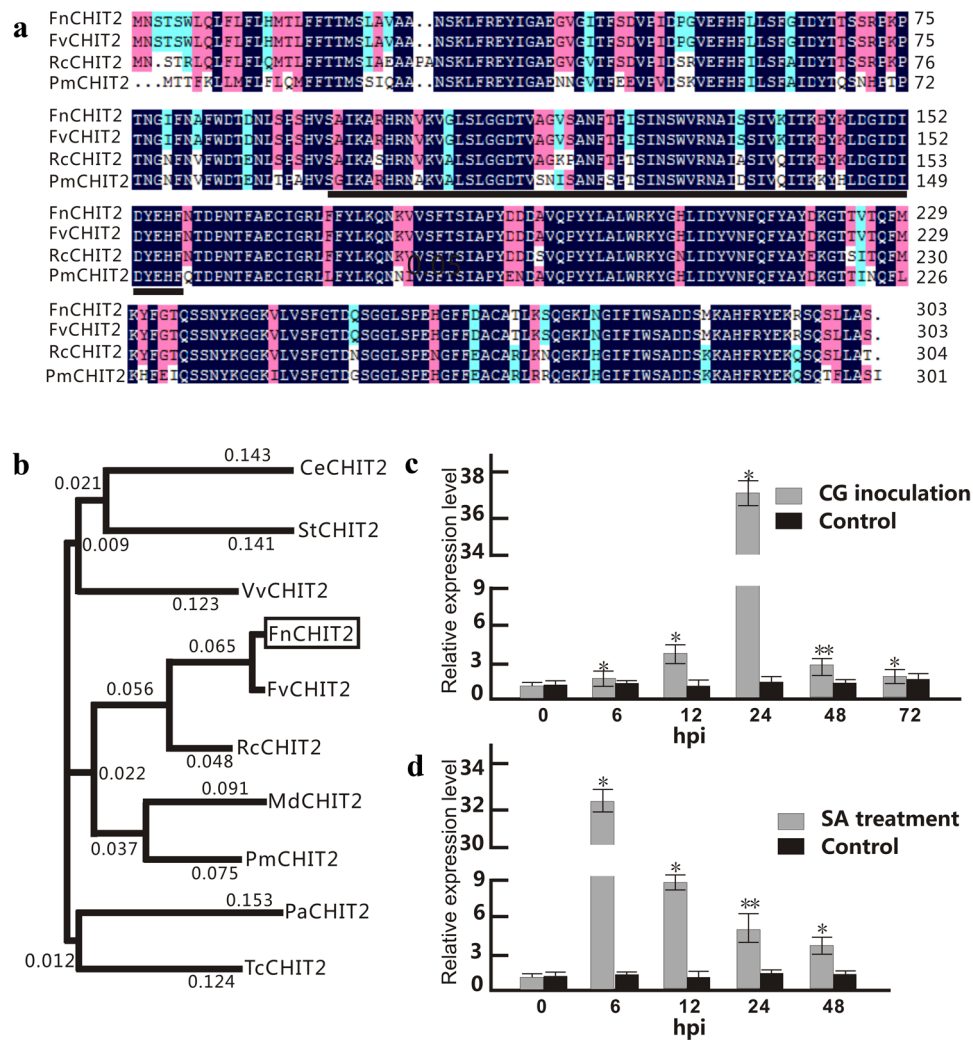
35S::YFP with plasma membrane marker pm-rk CD3-1007, the YFP signals were merged with mCHERRY signals of the plasma membrane marker (Fig. 2c), suggesting that FnCHIT2 was localized to the plasma membrane.

### Construction of recombinant plasmid pET32A-FnCHIT2-1 and expression in *E. coli*

The truncated gene *FnCHIT2-1* was inserted into the expression vector pET32A to generate the recombinant pET32A-FnCHIT2-1 construct (Fig. 3a and Fig. 2S B). The expression of recombinant protein products was detected using 12% Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the results showed that the recombinant protein with a predicted weight of approximately 32 Kda was successfully expressed in *E. coli* strain BL21 (DE3) with the induction of IPTG in the form of a fusion protein (Fig. 3b). According to Western blot analysis, FnCHIT2-1 recombinant protein has a specific reaction with anti-His-Tag rabbit monoclonal antibody (Fig. 3c). This finding suggests that the FnCHIT2-1 gene fusion protein was successfully expressed in *E. coli* BL21 (DE3). In addition, the samples contained recombinant pET32A-FnCHIT2-1 construct as demonstrated by a 32Kda band in the SDS-PAGE gel (Fig. 3c).

### Constitutive expression of FnCHIT2 in *A. thaliana* enhances resistance to *C. higginsianum*

To investigate the biological role of *FnCHIT2*, the *FnCHIT2* cDNA sequence was cloned under the control of the CaMV 35S promoter (Fig. 4a) to create 35S:: *FnCHIT2* and over-expressed in *A. thaliana* plants. In total, 27 T<sub>2</sub> transgenic plants were obtained. Three independent T<sub>2</sub> transgenic lines (T2-6, T2-25 and T2-26) exhibiting the highest resistant against *C. higginsianum* and *Pst* DC3000 were selected for further studies. *Colletotrichum* species are hemibiotrophic fungi (Dubouzet et al. 2011). The response of Col-0 plants and three transgenic lines was noted against fungus inoculation. According to observations, all plants developed brown necrotic lesions surrounded by a yellow halo at three days post-inoculation (dpi), but more severe symptoms were observed in wild type plants (Fig. 4b). In addition, disease lesion diameters were significantly larger in wild type plants (Fig. 4c). To explore the molecular basis of the *FnCHIT2* gene resistance mechanism against *C. higginsianum* inoculation, the transcription levels of genes related to SA- (*AtPRI*) and JA- (*AtPDF1.2*) dependent disease resistance pathways were determined (Fig. 4d and e). In general, the three transgenic lines showed increased *AtPRI* expression after inoculation with *C. higginsianum* (Fig. 4d). *AtPRI* expression was significantly enhanced after inoculation in three transgenic lines, and maximum levels were reached at 12 h in T2-6 and



**Fig. 1** Sequence analysis of the deduced amino acid sequence of FnCHIT2 from *F. nilgrrensis* and related proteins as well as qRT-PCR analysis of FnCHIT2 transcript levels in strawberry leaves after inoculation with *C. gloeosporioides*. **a** Multiple sequence alignment of FnCHIT2 and related proteins from *Fragaria vesca* (GenBank accession no.XP\_004295963), *Rosa chinensis* (GenBank accession no. XP\_024159678), and *Prunus mume* (GenBank accession no.XP\_008223957). The FnCHIT2 sequence encodes a 303-amino acid protein containing one ChiA protein domain. The underlined

amino acid residues (95 to 156) indicate the ChiA protein domain. **b** Evolution analysis of FnCHIT2. The accession numbers of protein sequences used to generate the phylogenetic tree are provided in Table S1. **c** Expression analysis of FnCHIT2 in *F. nilgrrensis* leaves after inoculation with *C. gloeosporioides*. **d** Expression analysis of FnCHIT2 in *F. nilgrrensis* leaves after exogenous SA treatment. Error bars represent the standard deviation from three independent replicates. Significance levels of  $*p < 0.05$  and  $**p < 0.01$  are indicated compared with control using Student's *t* test

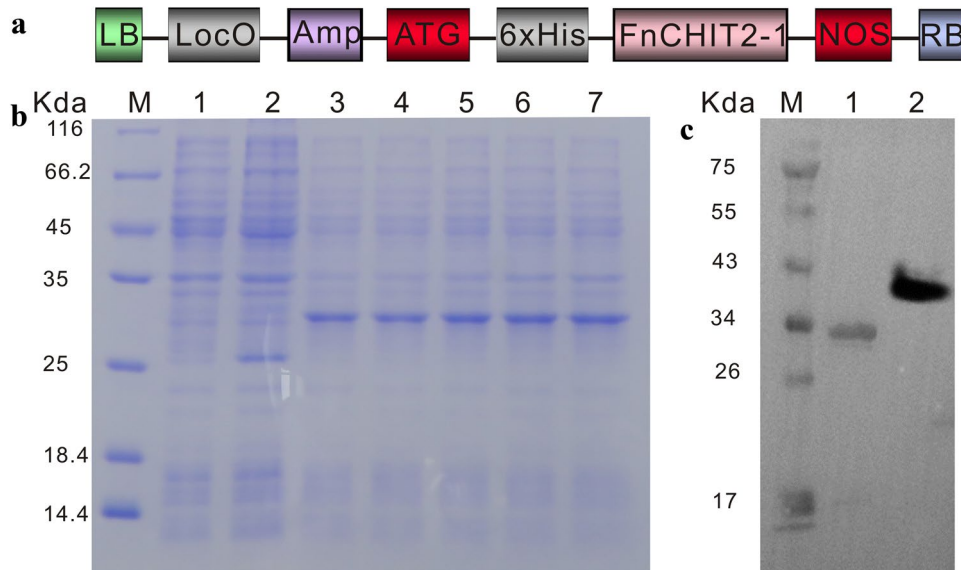
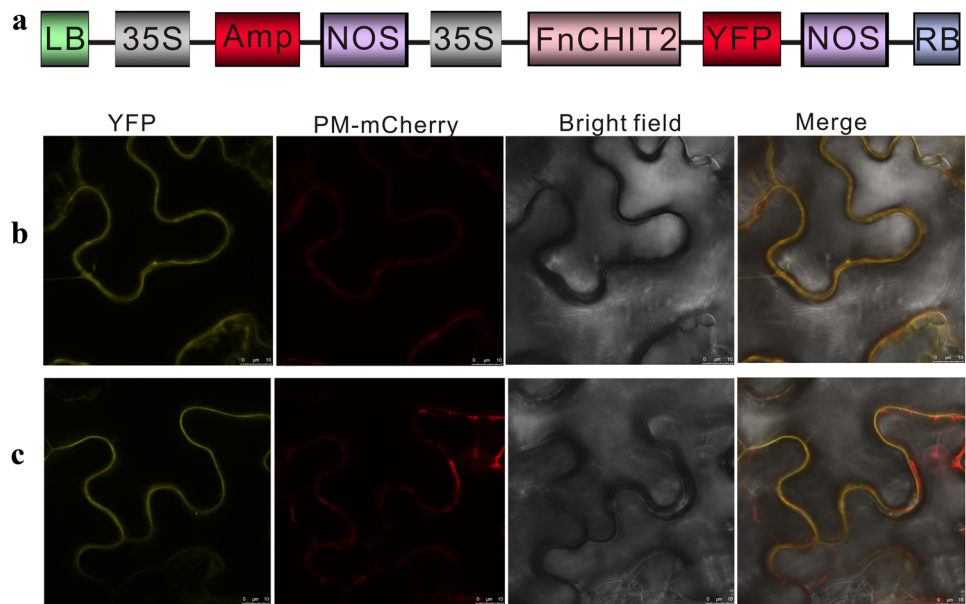
T2-26 lines. Initially, *AtPDF1.2* expression was down regulated at 12 h in three transgenic lines and then up-regulated compared to wild type plants.

### Constitutive expression of FnCHIT2 in *A. thaliana* enhances resistance to *Pst* DC3000

To further elucidate the biological function of the FnCHIT2 gene against bacterial disease infection, transgenic and wild type Col-0 *A. thaliana* plants were inoculated with *Pst* DC3000. Plants and leaves were examined after 3 dpi. After inoculation, the leaves of Col-0

plants displayed symptoms of chlorosis and necrosis, while the three transgenic lines have no or less symptoms compared with Col-0 plants (Fig. 5a). After trypan blue staining, wild type plants showed more cell death compared to three transgenic lines (Fig. 5d). Regarding NBT staining, three transgenic lines exhibited more  $O_2^-$  accumulation compared with Col-0 plants (Fig. 5c). Bacterial populations were quantified, and the quantities of bacteria in three transgenic plants were significantly ( $p < 0.05$ ) reduced compared with Col-0 plants (Fig. 5b). Compared with Col-0 plants, FnCHIT2 overexpression in three transgenic lines inhibited *Pst* DC3000 growth

**Fig. 2** Subcellular localization of FnCHIT2 protein. Transient expression of 35S::FnCHIT2-YFP and pm-rk CD3-1007 fusion protein in tobacco leaves, pm-rk CD3-1007 is a plasma membrane marker protein. Fluorescence signals were visualized using confocal laser scanning microscopy. **a** Structure of the CaMV35S promoter-FnCHIT2-YFP construct; *LB* left border, *RB* right border; *35S* CaMV35S promoter, *NOS* terminator, *Amp* ampicillin; **b** indicate injection of pm-rk CD3-1007 and free YFP vector. **c** Indicate injection of 35S::FnCHIT2-YFP and pm-rk CD3-1007. The 35S::FnCHIT2-YFP signals can be merged with fluorescent signals of the plasma membrane

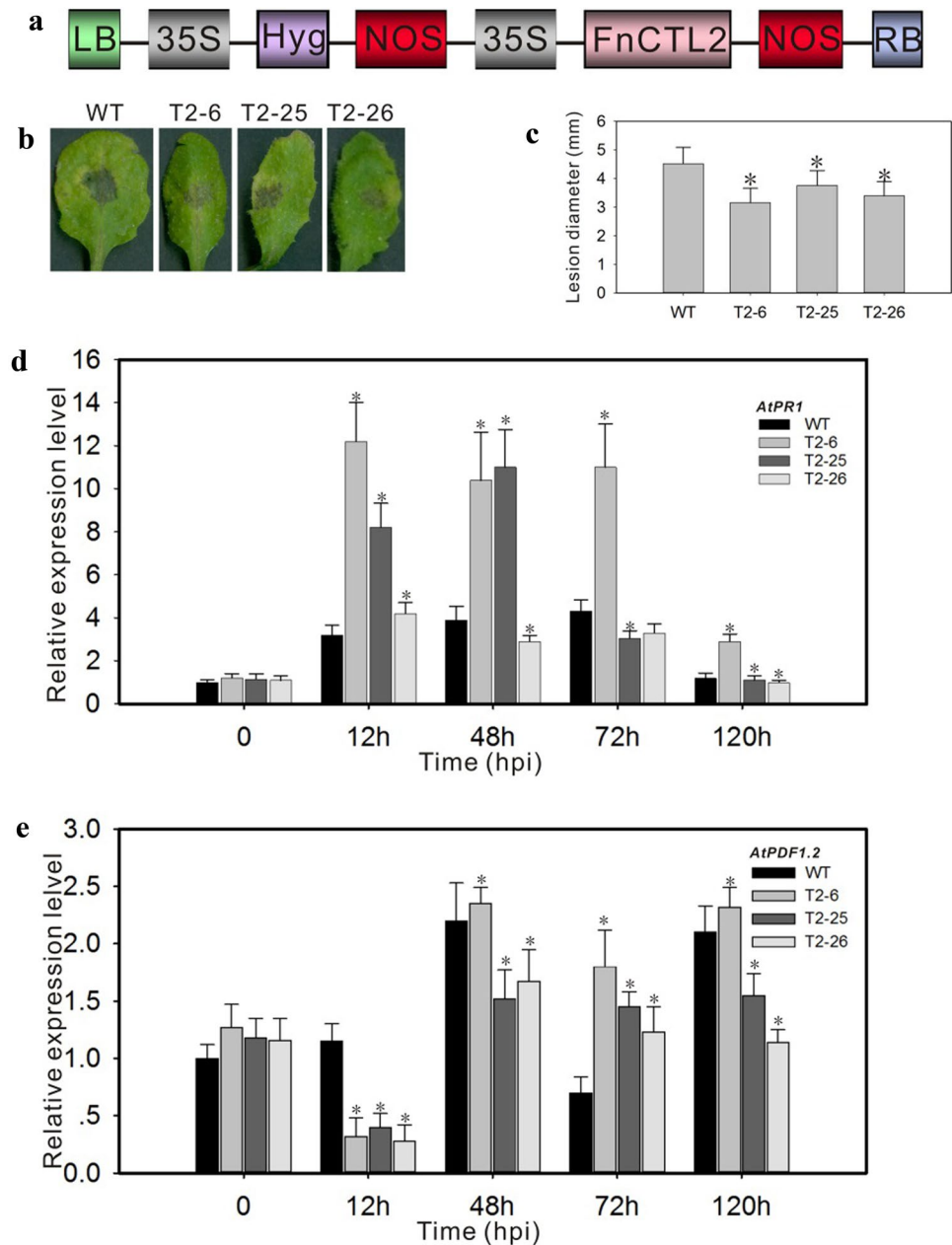


**Fig. 3** Generation of recombinant construct pET32A-FnCHIT2-1, expression of recombinant protein in *E. coli* (Rosetta, DE3), Western blot analysis of recombinant of pET32A-FnCHIT2-1 protein with His antibodies, and SDS-PAGE analysis of pET32A-FnCHIT2-1 protein expression induced with different concentrations of IPTG and temperature. **a** Diagram of FnCHIT2-1 prokaryotic expression vector; **b** Recombinant pET32A-FnCHIT2-1 vector in *E. coli*. M, protein molecular weight marker (Low); line 1, pET32A empty sample; line 2, recombinant protein expression of pET32A-FnCHIT2-1 un-induced with IPTG; lines 3–7, recombinant protein expression of pET32A-FnCHIT2-1 induced with IPTG (0.5 mmol). **c** Western blot of recombinant pET32A-FnCHIT2-1 protein. Protein molecular weight marker (Low); line 1, lysate of recombinant bacterial strain induced by IPTG for 4 h; line 2, positive control

and activity in the leaves. Further, qRT-PCR analysis was performed to investigate the expression of *AtPRI* and *AtPDF1.2* in transgenic and wild plants. According to qRT-PCR results, the transgenic lines showed significantly increased *AtPRI* gene expression after inoculation compared with wild type plants at 6, 12, 48, and 72 hpi

(Fig. 5e). The *AtPDF1.2* gene exhibited significantly increased expression at 48 or 72 hpi, and these levels subsequently decreased (Fig. 5f).

**Fig. 4** Overexpression of *FnCHIT2* in *A. thaliana* conferred enhanced disease response to *C. higginsianum* inoculation. **a** Structure of the CaMV35S promoter-*FnCHIT2* construct; LB, left border; RB, right border; 35S, CaMV35S-promoter; NOS, terminator; Hyg, hygromycin. **b** Disease symptoms in Col-0 and transgenic *A. thaliana* leaves three days post-inoculation. **c** The average lesion diameter on leaves at 72 hpi. **d** qRT-PCR analysis of *AtPR1* transcripts after inoculation. **e** qRT-PCR analysis of *AtPDF1.2* transcripts after inoculation. Four-week-old Col-0 and transgenic *A. thaliana* leaves were sprayed with a spore suspension of *C. higginsianum* ( $1 \times 10^6$  spores / mL) and harvested at 0, 12, 48, 72 and 120 hpi. Data are the mean  $\pm$  SE of three replications. Asterisks indicate significant difference between wild type and transgenic lines (\*  $p < 0.05$ , \*\*  $p < 0.01$ , Student's *t* test). Scale bars = 0.5 mm



## Discussion

*Colletotrichum* is a genus of major plant pathogens causing anthracnose disease in many plants worldwide (Silva et al. 2017). This ascomycete genus is comprised of a highly diverse group of pathogenic fungi that can infect a wide range of commercially important crops (Gan et al. 2013). Several species of *Colletotrichum* spp. causes strawberry anthracnose in China. *C. gloeosporioides* has been defined as the major causal agent (Zhang et al. 2016). *C. gloeosporioides* is both intracellular hemibiotrophic and intramural necrotrophic (Gan et al. 2013; Xie et al. 2010; O'Connell et al. 2000; Kim et al. 2004; Moraes et al. 2013).

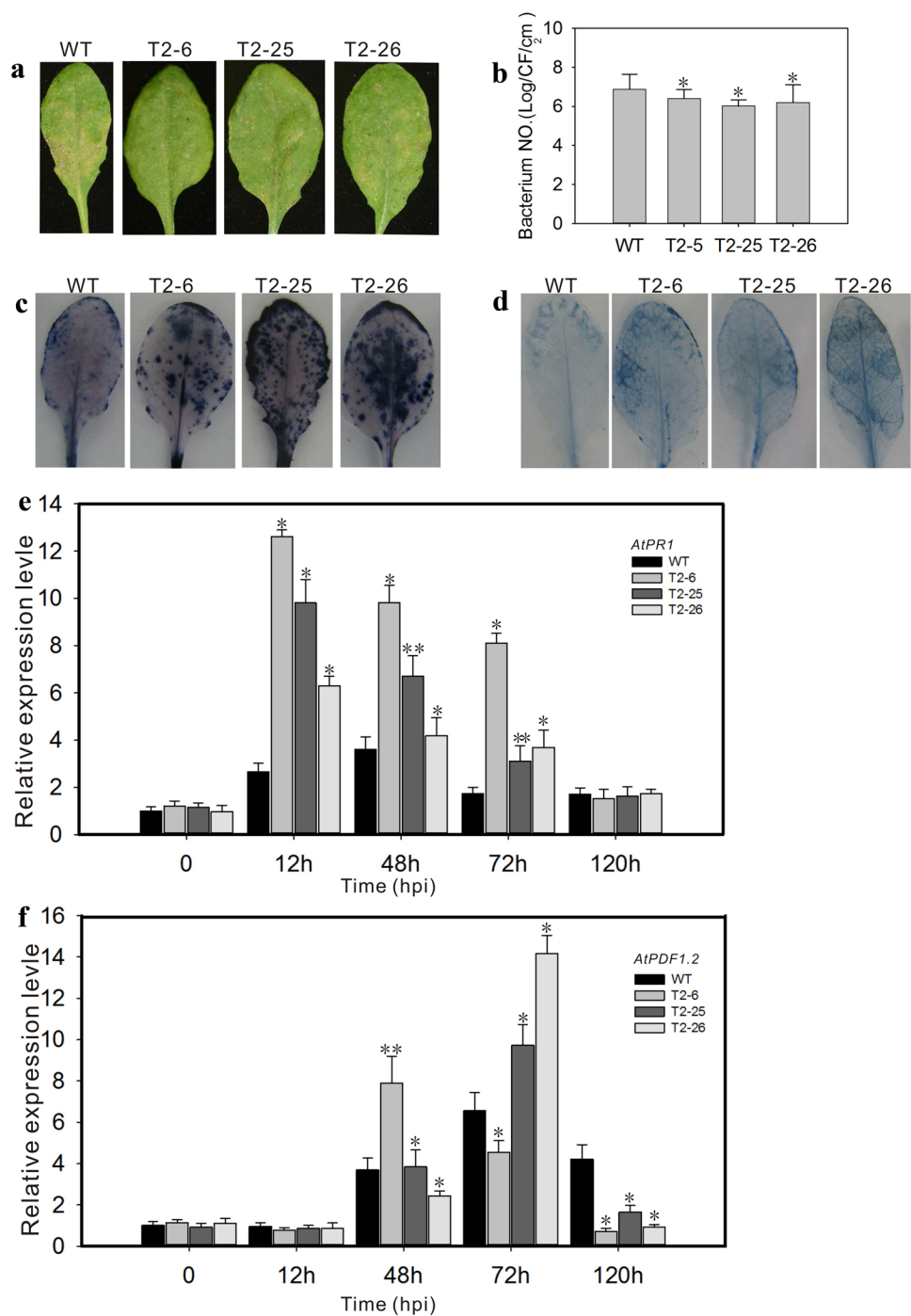
*C. higginsianum* causes anthracnose disease in many wild and cultivated crucifers, including *A. thaliana* (Narusaka et al. 2004; O'Connell et al. 2000; Takahara et al. 2009). This fungus also has a hemibiotrophic lifestyle (Plaumann et al. 2018). The *Arabidopsis*-*C. higginsianum* interaction is a convenient model for system analysis of fungal pathogenicity and plant resistance (Takahara et al. 2009). In this study, we isolated the *FnCHIT2* gene from *F. nilgerrensis* after *C. gloeosporioides* infection, overexpressed this gene in *Arabidopsis* and investigated its biological function after inoculation with *C. higginsianum*.

Chitinases are pathogenesis-related proteins that play important roles in host resistance to various pathogens and



**Fig. 5** Effect of *FnCHIT2* over-expression in *A. thaliana* on *Pst* DC3000 inoculation. Transgenic lines (T2-5, T2-25, and T2-26) and wild type *A. thaliana* were inoculated with *Pst* DC3000.

**a** Images of leaves at 3 days post-inoculation. **b** Bacterial population assays counted at 3 dpi. **c** Nitro blue tetrazolium (NBT) staining of  $O_2^-$  at 3 dpi. **d** Trypan blue staining of leaves at 3 dpi. **e** Relative expression levels of *AtPR1* examined using qRT-PCR in transgenic lines (T2-5, T2-25, and T2-26) and wild type *A. thaliana*. The leaves were collected at 0, 6, 12, 24, 72, and 120 hpi. **f** Relative *AtPDF1.2* expression levels examined with qRT-PCR in transgenic lines (T2-5, T2-25, and T2-26) and wild type *A. thaliana*. The leaves were collected at 0, 6, 12, 24, 72 and 120 hpi. Data represent mean  $\pm$  SD values from three independent experiments. Asterisks indicate significant differences between Col-0 and transgenic lines as determined by Student's *t*-test (\*  $p < 0.05$ , \*\*  $p < 0.01$ ). Scale bars = 0.5 mm



abiotic stress responses (Xu et al. 2016). Chitinase gene expression is strongly induced by infection with fungi, bacteria, and viruses. For example, 33 chitinase genes were identified in *Brassica rapa*, among them 14 genes were induced by *P. brassicae* infection (Chen et al. 2018). Eight chitinase genes were significantly induced by *V. dahliae* and quickly reached peak levels at different time points (Plauermann et al. 2018). In this study, we also found that *FnCHIT2* from *F. nilgerrensis* was strongly induced after inoculation

with *C. gloeosporioides*. According to previous studies, overexpression of chitinase genes in transgenic *A. thaliana* enhanced resistance to fungal infection (Hong et al. 2006) and inhibited fungal growth (Leah et al. 1991; Cao et al. 2009; Shah et al. 2010; Mercado et al. 2015; Durechova et al. 2019). According to our results, *FnCHIT2* gene over-expression in *A. thaliana* enhanced resistance against *C. higginsianum* infection. Over-expression of the chitinase gene has been previously reported to impart enhanced

disease resistance via two mechanisms: degradation of chitin in hyphae, which retards fungal growth, and release of pathogen-borne elicitors that induce defence reactions in plants (Prasad et al. 2013). Chitinases confer plant resistance against microbial attack by partially digesting isolated cell walls of several pathogenic fungi, such as ascomycetes, basidiomycetes, and deuteromycetes (Zhu et al. 1991; González et al. 2015). Furthermore, expression of the PR1 gene (SA-signalling pathway) in strawberry was up-regulated after *C. gloeosporioides* infection (Wang et al. 2017). In *A. thaliana*, the SA pathway was involved in resistance against *C. higginsianum* (Liu et al. 2017). According to our results, *AtPR1* expression was strongly induced after inoculation with *C. higginsianum* in transgenic *A. thaliana* at 12 hpi. This finding suggests that SA-dependent pathways regulated defence response activation. Overexpression of chitinase genes in plants results in the release of pathogen-borne elicitors, which induce defence reactions in plants. In tobacco, overexpression of the chitinase gene conferred enhanced disease resistance to *Trichoderma harzianum* and enhanced levels of PR1 transcripts in transgenic lines compared with basal levels, suggesting the involvement of chitinase in the signal transduction of defence pathways (Voll et al. 2012). Therefore, we hypothesized that infection with *C. higginsianum* and overexpression of the *FnCHIT2* gene in transgenic plants results in the release of pathogen-borne elicitors and induces defence responses via SA-dependent pathways. We also examined the expression pattern of the *AtPDF1.2* gene (JA-signalling pathway marker gene) after infection with *C. higginsianum*. We found that *AtPDF1.2* transcription levels in transgenic lines were reduced at 12 hpi and increased at 72 hpi compared with wild type plants. The JA-dependent signalling pathway was also activated after inoculation with *C. higginsianum* in transgenic plants. According to these results, SA and JA defence signalling pathways were activated in transgenic *A. thaliana* during *C. higginsianum* infection, suggesting the positive role of *FnCHIT2* against *Colletotrichum* spp. infection. Our results are consistent with the previous findings that both SA and JA pathways are involved in plants resistance against *C. higginsianum* (Dana et al. 2006). Zhang et al. (2018) and Amil-Ruiz et al. (2016) reported that both SA and JA defence pathways were activated during *Colletotrichum* spp. infection in strawberry. We inferred that overexpression of *FnCHIT2* in transgenic *A. thaliana* lead to increased chitinase expression and played an antifungal role by directly hydrolysing *C. higginsianum* chitin and subsequently activating SA and JA signalling pathways.

We also evaluated bacterial disease resistance in transgenic *A. thaliana*. We infected transgenic and wild type *A. thaliana* lines with *Pst* DC3000 and observed that transgenic plants exhibited enhanced resistance to *Pst* DC3000 and *C. higginsianum*. It was previously reported that *CaChi2*

overexpression in *A. thaliana* enhanced resistance to *Pst* DC3000 (Hong et al. 2006). In our study, transgenic lines showed reduced disease symptoms, more programmed cell death and increased  $O_2^-$  levels compared with wild type *A. thaliana* after inoculation. Reactive oxygen species (ROS) production is thought to be directly toxic to pathogens (Lambeth 2004), and restrict pathogens by triggering stomatal closure (Zhang et al. 2020a, b). In the SA-mediated signalling pathway, enhanced plant resistance against biotrophic and hemibiotrophic pathogens might be due to increased PR1 gene expression. We observed increased *AtPR1* transcript levels in transgenic lines compared with wild type *A. thaliana* at 12–72 hpi. In transgenic lines, *AtPDF1.2* exhibited increased expression after inoculation with *Pst* DC3000. These results suggest that overexpression of *FnCHIT2* improved resistance to *Pst* DC3000 via activation of both SA and JA-mediated pathways and confirm that *FnCHIT2* has a positive role in resistance against *Pst* DC3000 infection.

In the present study, we demonstrated that the *FnCHIT2* gene from *F. nilgerrensis* was strongly induced following the inoculation of *C. gloeosporioides* and *FnCHIT2* overexpression in *A. thaliana* conferred enhanced resistance to *C. higginsianum* and *Pst* DC3000. In the future, detailed studies are needed to evaluate disease resistance mechanisms against *C. gloeosporioides* infection in transgenic strawberries.

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