Flagellin FLiC Enhances Resistance of Upland Cotton to *Verticillium dahliae*

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Abstract:

The mechanism by which flagellin induces an immune response in plants is still unclear. The purpose of this study is to reveal the effect and mechanism of flagellin in inducing plants to produce an immune response to increase the resistance to *Verticillium dahliae* (VD). The full-length flagellin gene C (FliC) was cloned from an endophytic bacteria (*Pseudomonas*) in the root of upland cotton cultivar Zhongmiansuo 41. The FliC protein purified in vitro has 47.50% and 32.42% biocontrol effects on resistant and susceptible cotton cultivars, respectively. FLiC can induce allergic reactions in tobacco leaf cells and immune responses in cotton plants. Smearing FLiC to cotton and performing RNA-seq analysis, it is significantly enriched in the activity of positive ion transporters such as potassium ions and calcium ions (Ca^{2+}), diterpenoid biosynthesis, phenylpropane biosynthesis and other disease-resistant metabolic pathways. FLiC inhibits the expression of calcium antiporter activity gene (*GhCAA*) to accelerate intracellular Ca^{2+} influx and stimulate the increase of intracellular hydrogen peroxide (H_2O_2) and nitric oxide (NO) content. The coordinated regulation of Ca^{2+}, H_2O_2 and NO enhances disease resistance. The resistance of transgenic *FLiC* gene Arabidopsis to VD was significantly improved. The *FLiC* gene can be used as an anti-VD gene and as a regulator to improve resistance to VD.

Key words: Flagellin, immune response, *Verticillium dahliae*, Ca^{2+}, RNA-seq

Abbreviations: 2-DDG: 2-deoxy-D-glucose; AOPP: α -aminooxyacetic acid- β -phenylpropionic acid; Ca^{2+}: calcium ions; H_2O_2: hydrogen peroxide; NO: nitric oxide; *GhCAA*: calcium antiporter activity gene; PTI: pattern triggered immunity; ETI: effector

triggered immunity; ROS: reactive oxygen species; SA: Salicylic acid；JA: Jasmonic acid；IPTG: isopropyl-β-D thiogalactopyranoside; PMSF: phenylmethanesulphonyl fluoride; PVP: polyvinyl pyrrolidone; CHI: chitinase; GLU: glucanase; PAL: Phenylalanine ammonia lyase; PPO: polyphenoloxidase; POD: Peroxidase; CAT: catalase; qRT-PCR: quantitative reverse transcriptase-PCR; DAB: 3,3'-diaminobenzidine; CAT: catalase; C-PTIO: carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-3-oxide-1-oxyl); L-NAME: nitro-L-arginine methyl ester; EGTA: ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid; VD: *Verticillium dahliae*; *PR1*: disease-related protein 1; *LOX*: lipoxygenase; *VSP*: vegetative storage protein; *GPX7*: glutathione peroxidase; *GSTU3*: glutathione sulfur transfer Enzyme gene; *FLiC*: full-length flagellin gene C.

Introduction：

Immune response is closely related to the disease resistance of plants. Plants mainly rely on two levels of defense pathways to resist infection by pathogens: pathogenic microorganisms pattern triggered immunity (PTI) (Nürnberger and Brunner, 2002) and pathogens secreted effector triggered immunity (ETI) (Thomma et al., 2011; Naveed et al., 2020). The defense response is realized by mutual recognition and interaction between the recognition receptors of plants and the elicitors secreted by pathogenic microorganisms. Through the transmission and transduction of a series of signals to activate the immune defense response in the plant, and finally make the plant obtain systemic disease resistance (Jennings et al., 2001; Bouizgarne et al., 2006; Jones et al., 2006; Kumar et al., 2020). The early defense reactions of plants mainly include the production of early disease resistance signals such as allergic reactions, reactive oxygen species (ROS) outbreaks, and NO accumulation. These stimulus signals are converted from extracellular to intracellular signals and amplified by a cascade reaction to induce downstream defense reactions. These reactions always occur first around the infected tissue and gradually spread to the surrounding uninfected tissues. In the end, the immune system of the entire plant is activated to defend against the infection of various pathogens (Dixon et al.,1994; Ebel et al.,1998; Yano et al.,1998; Durrant et al., 2004; Buxdorf et al., 2013; Holmes et al., 2021).

Flagellin can induce immune responses in rice, algae and kelp but the mechanism is unclear (Takai et al., 2008; Wang, 2012; Wang et al., 2013). The flagellin Flg22 cloned from *Pseudomonas syringae* is a 22-amino acid peptide conserved at the N-terminus, which acts as an active site for elicitor to induce immune responses in higher plants (Felix et al., 1999). After being induced by flagellin, plants will produce a series of defense responses, including ethylene (ETH) production, callose deposition, ROS burst, defense gene expression and growth inhibition (Asai et al., 2002; Zipfel et al., 2004). Flg22 is mainly based on salicylic acid (SA) signal transduction pathway, and also related to jasmonic acid (JA) or ethylene signal transduction pathway (Gómez-Gómez et al., 2000; Yuan et al., 2020). Activated SA and JA signaling pathways have an effect on the ROS burst and callose deposition triggered by Flg22 (Yi et al., 2014). The effect and mechanism of exogenous protein and Flg22 in inducing the immune response of upland cotton have not been studied.

Verticillium wilt of cotton is mainly a soil-borne vascular disease caused by VD. It seriously affects cotton yield and fiber quality and lacks effective control measures. The effect and mechanism of exogenous protein inducing immune response in cotton to increase resistance to VD has not been reported. In this study, a full-length flagellin gene *FliC* was cloned from cotton endophytic bacteria (*Pseudomonas*). The purpose of this study was to study the effect and mechanism of this protein in inducing cotton immune response and improving resistance to VD.

Materials and Methods

Microbial strains and cotton cultivar

VD were generously provided by the Institute of Plant Protection, Jiansu Academy of Agriculture Sciences. Hygromycin B-resistant GFP-labelled VD was provided by Hu (2012) and maintained on potato dextrose agar (PDA) at 25°C. For the inoculation assay, conidia from 10-day-old PDA plates inoculated with the V1070 were washed once with sterile water and diluted to a concentration of 10^7 conidia mL^{-1}. The expression vector Pgex-4T-2 and Escherichia coli expression competent cell E.coli BL21 (DE3) was purchased from Beijing Kinco Xinye Biotechnology Co., Ltd. The tested tobacco variety was *Nicotiana*

tabacum cv. Xanthi NN, which was cultivated in a greenhouse at 25℃ to the 7-8 leaf stage. The tested cotton varieties were the VD-susceptible variety Jimian 11 and the disease-resistant cotton variety Zhongzhimian 2.

Construction of FliC gene expression vector

Using Pgex-4T-2 as an expression vector and designing a pair of specific primers based on the *FliC* gene sequence:

FliC-BamHI-F: 5'- CGCGGATCCATGGCCTTGACCGTCAACAC-3'

FliC-EcoRI-R: 5'- CCGGAATTCTTAGCGCAGCAGGCTCAGAAC-3'

PCR reaction system: *FliC*-BamHI-F: 2.0 μL; *FliC*-EcoRI-R: 2.0 μL; Gold Mix (green): 45.0 μL; Plasmid: 1.0 μL with a total volume of 50.0 μL. PCR amplification conditions: pre-denaturation at 98°C for 2 min; denaturation at 98°C for 10 s, annealing at 62°C for 30 s, extension at 72°C for 10 s, 35 cycles; extension at 72°C for 2 min. Finally, perform agarose gel electrophoresis detection (1.5% agarose, 120V, 20 min). Use TAKARA gel recovery kit to recover the amplified products. The recovered product and Pgex-4T-2 were digested at 37°C for 5 hours. Use T4 ligase to ligate overnight at 16°C. PCR detection conditions of bacterial solution: pre-denaturation at 98°C for 2 min; denaturation at 98°C for 10 s, annealing at 55°C for 30 s, extension at 72°C for 20 s, 35 cycles; extension at 72°C for 1 min. The recombinant expression plasmid *FliC*- Pgex-4T-2 verified by PCR amplification, restriction enzyme digestion and sequencing was transformed into competent cells of E.coli BL21(DE3) for prokaryotic expression.

Induced expression of recombinant protein FLiC

Pick a single colony of the positive strain, inoculate 5 mL in a fresh LB liquid medium containing 50 mg/L ampicillin, and culture with shaking at 37°C until the OD_{600} is 0.6-0.8. Add isopropyl-β-D thiogalactopyranoside (IPTG) with a final concentration of 0.5 mM, and continue shaking culture at 150 rpm for 5 hours at 28°C. Polyacrylamide gel electrophoresis (SDS-PAGE) detection, with non-induced bacterial liquid and BL21 (DE3) bacterial liquid as controls.

FLiC protein allergic reaction test

Using 5-6 true leaf tobacco seedlings as experimental materials, 50 μL (100 μg/mL) of purified protein was injected into the mesophyll from the back of the leaf, GST as controls, and each treatment was repeated 3 times(Felix et al.,1999; Gómez-Gómez et al., 2000)。

Detection of disease resistance of FLiC to cotton cultivars

Four-leaf stage seedlings of uniformly growing disease-resistant cotton varieties (Zhongzhimian 2) and susceptible varieties (Jimian 11) were selected, and FliC at a concentration of 50 μg/mL was uniformly smeared to treat cotton seedling leaves. Water is used as a control. After treatment for 2 days, inoculate the pathogenic spore suspension (2 $\times 10^7$ cfu/mL). The method of inoculation of the pathogen is as follows: remove the cotton seedlings, soak the roots in the pathogen spore suspension for 20 minutes, inoculate 15 strains of each cotton cultivar with sterile water as the control. 15 days after inoculation, the incidence of cotton was counted. At the same time, observe the infection of disease bacteria in cotton roots, stems and leaves during this period under a microscope. The condition was investigated 30 days after inoculation, and all experiments were repeated 3 times.

Lignin detection

According to Pomar's method, Wiesner reagent is used to detect the content of lignin (Pomar et al., 2002). Each treatment is repeated three times.

callose detection

Refer to Millet's method for corpus callosum detection (Millet et al., 2010). The quantification of callose is calculated by Image J software. Each treatment was repeated three times.

Biomass detection of VD

Cut two cotyledons from cotton of different treatment groups in a sterile environment,

weigh and add 2 mL of sterile water for grinding. After grinding into a homogenate, it is diluted according to the method of gradient dilution. 100 μL of diluents of different concentrations were spread on the red bengal resistant medium containing 50 μg/mL hygromycin and 50 μg/mL streptomycin, and then placed in a 28℃ constant temperature incubator and cultured upside down for 2 days. Count the number of colonies in each petri dish and calculate the content of VD per gram of leaf. All experiments were repeated three times（Wang, 2014）。

Chitinase(CHI) and glucanase(GLU) detection

Enzyme extracts from cotyledon were prepared in 0.2 M boric acid-borax buffer, pH 7.6 with 0.1% (v/v) β-mercaptoethanol, 0.57 mM phenylmethanesulphonyl fluoride (PMSF) and 1% (w/v) polyvinyl pyrrolidone (PVP) and 50 mM potassium acetate buffer (pH 5.0) for CHI and GLU, respectively. The homogenate was centrifuged at 12,000×g for 20 min at 4℃ and the supernatant served as enzyme source. CHI activity was measured according to the protocol described by Emani et al (2003) using 4-methylumbelliferyl-β-D-N, N″-triacetylchitotrioside [4-MU-β-(GlucNAc)3] (Sigma, St. Louis, MO, USA) as the substrate. Enzyme extract were further diluted (16-fold) with 0.1 M citrate buffer, pH 3.0 prior to the enzyme assay. One hundred microlitres of diluted protein extract were mixed with 25 μL of substrate (250 μM) and incubated at 30℃ for 1 h. The reaction was terminated with 1 mL of 0.2 M sodium carbonate and fluorescence was measured using a DyNA Quant™ 200 fluorometer (Hoefer). The endochitinase activity is presented as pmole 4-MU/h/mg protein. Each assay was carried out in three replicates.

The GLU assay was performed using the method of Abeles and Forrence (1970). Laminarin was used as the substrate and dinitrosalicylic reagent was used to measure the reducing sugars produced in the enzymatic reaction. 0.5 mL enzyme extract was routinely added to 0.5 mL of 2% (w/v) laminarin in water and incubated at 50℃ for 1 or 2 h. The laminarin was dissolved by heating the 2% solution briefly in a boiling water bath before use. The reaction was stopped by adding 3 mL of the dinitrosalicylic reagent and heating the tubes for 5 min at 100℃. The tubes were then cooled to 25℃, the contents were diluted

1:10 with water, and the optical density was read at 500 nm. The enzyme activity is expressed as glucose equivalents, mg/h/mg protein. Each assay was carried out in three replicates.

Phenylalanine ammonia lyase (PAL) and polyphenoloxidase (PPO) detection

Enzyme extracts from cotyledon were prepared in 0.1 M sodium borate buffer, pH 7.0 containing 0.1 ginsoluble PVP and 0.1M sodium phosphate buffer (pH 6.5) for PAL assays and PPO, respectively. The homogenate was centrifuged at 12,000×g for 20 min at 4℃ and the supernatant served as enzyme source. Samples containing 0.4 mL of enzyme extract were incubated with 0.5 mL of 0.1 M borate buffer, pH 8.8, and 0.5 mL of 12 mM L-phenylalanine in the same buffer for 30 min at 30℃. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm as described by Dickerson et al (1984) and was expressed as nmoles of cinnamic acid unit min/mg protein.

The method for determining the activity of PPO is slightly modified with reference to the method of Ali et al (2006). Add 1 mL of 0.05 mol/L phosphate buffer (pH 5.5) to the 0.2g leaves uniformly ground with liquid nitrogen, shake and mix, and centrifuge at 4° C, 12000rpm for 15min. Take 0.5 mL of the supernatant enzyme solution and add 1.0 mL of 0.1 M catechol solution and 1.5 mL of 0.05 mol/L pH 5.5 phosphate buffer, the total volume is 3 mL. After mixing uniformly, measure the absorbance at 398 nm every 2 minutes, and use 0.05 mol/L (pH 5.5) phosphate buffer as a control. Take $\Delta A398/\Delta t$ =0.01/min to express an enzyme activity unit (U). Each assay was carried out in three replicates

Peroxidase (POD) and catalase (CAT) detection

Enzyme extracts from cotyledon were prepared in 50 mM Tris-HCl buffer, pH 7.0. The extract was centrifuged at 12 000 g for 20 min at 4℃. The supernatant was passed through a Sephadex G-25 column and fractions containing enzyme were pooled and used as enzyme source for the assay of catalase and POD by Sudhakar et al (2001). The determination of peroxidase activity is slightly modified according to the method of Dong et al (2003). Add 0.2g of the leaves uniformly ground with liquid nitrogen to 1mL of

0.1M pH 5.5 phosphate buffer, shake and mix well, and centrifuge at 4° C at 12000rpm for 15min. Take 0.1mL of the supernatant enzyme solution and add 2.9mL of 0.05mol/L phosphate buffer, 1mL of 0.05mol/L guaiacol and 1mL of 2% H_2O_2. Measure the absorbance value at 470 nm immediately after mixing, and the change of A470 by 0.01 per minute is 1 peroxidase activity unit (u). Each assay was carried out in three replicates.

CAT activity was measured using the method of Plazek and Zur (2003) with a modification. CAT activity was assayed in reaction mixture (3 cm^3 final volume) composed of 50 mM Tris-HCl buffer pH 7.0 to which 30% (w/v) H_2O_2 was added to reach an absorbance value in the range 0.520 – 0.550 (λ =240 nm). The reaction was started after adding 200 µL of crude extracts to the reaction mixture. CAT activity was measured as a decrease in absorbance at 240 nm as a consequence of H_2O_2 consumption. Activity of the enzyme was expressed as mmol H_2O_2 decomposed per minute per milligram of protein. Each assay was carried out in three replicates.

RNA extraction and gene expression analysis by quantitative reverse transcriptase-PCR (qRT-PCR)

Leaves were collected and transcript analysis was conducted at different time points as indicated in the figures. Analyses were performed by quantitative qRT-PCR. RNA extraction and gene expression analysis were performed as previously described by Ren et al (2013) with slight modifications. Total RNA was extracted using an RNA Isolation Kit (Omega Bio-Tek) according to the manufacturer's instructions. First-strand complementary DNA was synthesized from total RNA using a PrimeScriptTM RT reagent kit with gDNA Eraser (Takara, China). Then, 5 µL of 1:10 diluted cDNA samples was used as the qRT-PCR template with 0.5 µM gene-specific primers and 10 µL SYBR Premix Ex Taq II (Takara, China) in a total volume of 20 µL. Experiments were performed in a Realplex2 Systems (Eppendorf, Germany) with the following thermal cycling profile: 95℃ for 10 min, followed by 40 cycles of 95℃ for 30 s, 55℃ for 30 s, and 72℃ for 30 s. Each real-time assay was tested in a dissociation protocol to ensure that each amplicon was a single product. Sequences of cotton defense gene primers were used for RT-qPCR (Supplemental Table S1). Relative gene expression of genes was calculated by the threshold cycle $2^{-\Delta\Delta CT}$

method by Livak and Schmittgen (2001) from three biological replicates and three technical replicates. For each gene, the mean fold-change±SD in transcript accumulation within treated leaves relative to the control (set at 1.0) was calculated from three biological replicates.

Supplemental Table S1 Primer sets used for quantitative real-time PCR

Primer	Sequence	Ref	Gene
CHI	ACCAAGCTACTCGCAAGAGG′ CGGAAGCGCAGTAAGATGA	CD485880	Pathogen induced class1 chitinase
GLU	CATTGATATGACCTTGATCG GTGAGATATCCCTTGGATTG	CD486342	Pathogen induced glucanase
HMG	GATTTGAAGTTGTATTTGGAG GAAATCAGTTTGAAGGAAA	CD486522	Pathogen induced HMG CoA reductase
LOX	AGTCGTCGGTTCATGCCTGAGAAA ATTCCCAGGAGTGTCTGCAGTTGA	Han, 2014	Pathogen induced lipoxygenase
POD4	TTTGCTGCTGCCATGGTGAAGATG CAATCAGTTGACCACCCTGCAGTT	AF155124	Pathogen induced peroxidase
PR1	TGCCCAAGACTCACAACAAG GGCCTTCTCATTAACCCACA	Han, 2014	Putative PR1
ERF5	GCTCAAGCCGGTTTAAATATGGGT TTCACCACATGTACAAGGTCCCAC	Han, 2014	Bacterial-induced ERF5 gene
CDN1	GCCAACTTGTGGTTATGCCATGCT TGCTGAGCAATCGTCTTCTCTCCT	Han, 2014	Terpenoid aldehydes and cadalene derivitives
PR10	ATGATTGAAGGTCGGCCTTTAGGG CAGCTGCCACAAACTGGTTCTCAT	Han, 2014	Putative PR10
MAPK2	TTACAATCTTATTCCACACACGC TCCCTATTTATAGAAAACCTCCC	DQ132852.1	Mitogen-activated protein kinase
MAPK6	GGACACTGAGATGGCGGAGC ACCAATGGGCATAATAGGAG	JF727638.1	Mitogen-activated protein kinase
MAPK7	CAGAGCAAACTATGCCGTATG TTACAATCTTATTCCACACACGC	FJ966888.1	Mitogen-activated protein kinase
MAPK16	GTGTTGTTTGTTCAGCGTATG CGTAGGAGCCTGAGAAGTTTA	FJ966889.1	Mitogen-activated protein kinase

WRKY2	TTCTTCCCATAATACCATCC CTCGCATAAAACTGTTAGCATC	DQ864758.1	Transcription factor
WRKY3	GGGACGAAAGTTGTCAAAGGGAA TCGTGTTGCTGTTTCGGTTG	FJ966887.1	Transcription factor
WRKY4	ATTGATAACAACTTACGAGGGC TTGAGGAGCAAGGAAGGATT	JQ081265.1	Transcription factor
WRKY5	GCAAAGGGAACCGAGATT ATCGGGTAGGGATGCTTG	JQ081266.1	Transcription factor
WRKY6	AAGCCAATCAAGGGTTCTCC GTTCTTCCAAGCACCTCTCT	JQ081267.1	Transcription factor
18S rRNA	CCATAAACGATGCCGACCAG AGCCTTGCGACCATACTCCC	HQ658359.1	18S ribosomal RNA gene

Measurement of cytosolic Ca^{2+} concentration

To measure cytosolic calcium concentration, epidermal strips of four-week-old cotton leaves were loaded with the Ca^{2+}-sensitive fluorescent dye 1-[2-amino-5-(2, 7-dichloro-6-hydroxy-3-oxo-9-xanthenyl) phenoxy]-2-(2-amino-5-methylphenoxy) ethane-N, N, N’, N’-tetraacetic acid, pentaacetoxymethyl ester (Fluo-3/AM) (Molecular Probes, Eugene, OR, USA), which was observed with the laser-scanning confocal microscopy (LSCM) according to the method described by Chen et al (2004). The cotton epidermal strips were incubated in 10 μM Fluo-3 AM loading buffer (10 mM MES-Tris, pH 6.1) at 4℃ for 2 h in darkness. After incubation at 4℃ for 2 h in the dark, the epidermal strips were washed twice with anisotonic solution and incubated at 25℃ for 1 h in the dark. The epidermal peels loaded with Fluo-3/AM were exposed to FLiC (100 μg/mL) and distilled water (control) for 12 min. Fluorescent probes were excited with a 488 nm laser, and the emission fluorescence was filtered by a 515 nm filter to eliminate the autofluorescence of the epidermal strips. Images were recorded every 20 s. Images were analyzed using Leica IMAGE software. All of the experiments were repeated three times, and the Ca^{2+} fluxes of 6 horizons of epidermal cells were measured in each treatment at each time. Pictures were taken by scanning the field of view three times each 20 s; then, the fluorescence intensities

of these pictures were measured by fluorescence microscopy after establishing a stable baseline.

H_2O_2 detection and quantification

Detection of H_2O_2 was also performed based on the method described by Kumar et al (2009) with slight modifications. The leaves were harvested and placed in a solution containing 1 mg mL^{-1} 3, 3’-diaminobenzidine (DAB) (pH 3.8) for 8 h under light at 25℃. The leaves were boiled in ethanol (96%, v/v) for 10 min and then stored in 96% ethanol. H_2O_2 production was visualized as a reddish-brown coloration of the leave. The concentration of H_2O_2 in plant leaves was measured by monitoring the A_{415} of the titanium-peroxide complex following the method described by Brennan and Frenkel (1977). Absorbance values were calibrated to a standard curve generated with known concentrations of H_2O_2. The total H_2O_2 content was recalculated for 1 g of fresh weight of plant leaves.

NO detection and quantification

The levels of NO in cotton leaves were determined with the Griess reagent kit (Beyotime Institute Biotech) described by Li et al (2010) with slight modifications. The optical density at 550 nm of the reaction product was measured with a UV mini-1240 spectrophotometer (Shimadzu, Japan). The production of NO (mol g^{-1} of fresh weight) was calculated with a formula supplied in the kit. There were three replicates in each treatment. Nitric oxide in cotton epidermal cell was visualized using the specific NO probes 3-Amino, 4-aminomethyl-2’,7’-difluorescein diacetate 4, 5-diaminofluorescein diacetate (DAF-FM DA, Sigma-Aldrich) (Sun et al., 2012) using the method described by Bright et al(2006) . FLiC or inhibitors as indicated the figures or figures legends, and then incubated in MES-KCl buffer (10 mM MES, 5 mM KCl, 50 μM $CaCl_2$, pH 6.15) and DAF-FM DA at a final concentration of 10 M for 30 min in the dark at 25℃, followed by washing twice in the same MES-KCl buffer for 15 min each. All images were visualized using CLSM (excitation 488 nm, emission 515 nm). Images acquired were analyzed using Leica IMAGE software. All treatment s were repeated three times. The data are presented as the average

fluorescence intensity.

Transcriptome Differential Gene Significance Screening Conditions

Define the genes with $|\log2FC| \geqslant 1$ and P-value $\leqslant 0.05$, and screen them as significantly differentially expressed genes. The significant enrichment conditions of metabolic pathways are used to calculate the P-value by the hypergeometric distribution method (the standard for significant enrichment is P-value <0.05).

Primer design for qRT-PCR verification of transcriptome results

According to the results of RNA-seq sequencing, 12 genes were randomly selected to design gene-specific primers by NCBI and verified by qRT-PCR (Supplemental Table S2). The relevant primer designs are as follows:

Supplemental Table S2 Specific primer sequences of qRT-PCR related genes

Gene name	Primer sequence（ 5′→3′）	
GH_D07G1376	F:GTTGGTCACGACCGAGGAG;	R:AGCCCTCTTGAAATTAGCCCC
GH_D09G0121	F:GGTCTTTATTTATTGTGACAGGTGG;	R:GCTGGTGGAATGGAAAACCG
GH_A11G1348	F:ACAGGCAGCATACTAGGTGG;	R:TCCTCTAGCTCTTGGCCCTT
GH_A02G0915	F: GCAGTCGACATCACAAAACCTC;	R:GGCCTCCACTACGGACTCTT
GH_A08G0063	F:ACCTATACCTGGCGAGTTTTG;	R:AATCCACCCAACCACAACACA
GH_D08G0067	F: ACCTATACCTGGCGAGTTTTG;	R:AATCCACCCAACCACAACACA
GH_A09G1367	F: CCGCTGGAGGTAGGAAAAGG;	R:CGATTTCTTCGCCCGGTTTC
GH_D03G1291	F: AACACTGATGCAGAAGGTAGG;	R:TGTTGCCAGGTCAAGATGTCTA
GH_A13G0836	F: CCATCTTCTCCCTCTTGGCA;	R:AAGCTGGGTTACAAGGCAAG
GH_D01G0069	F: GTGTCTGGCTTTGTCCTGGTA;	R:CTTCGATCGATGCAAACGACA
GH_D10G0737	F:ACTCCTTGTTACCAGCCGAAA;	R:CTCTGTGAAGACAAGCTGCC
GH_A10G0542	F:AGTTCGTCCAAGCGAAGGAG;	R:TCTTCAACCCCCGCTTTACC
ubiquitin	F: GAGTCTTCGGACACCATTG;	R:CTTGACCTTCTTCTTCTTGTGC

Statistical analysis

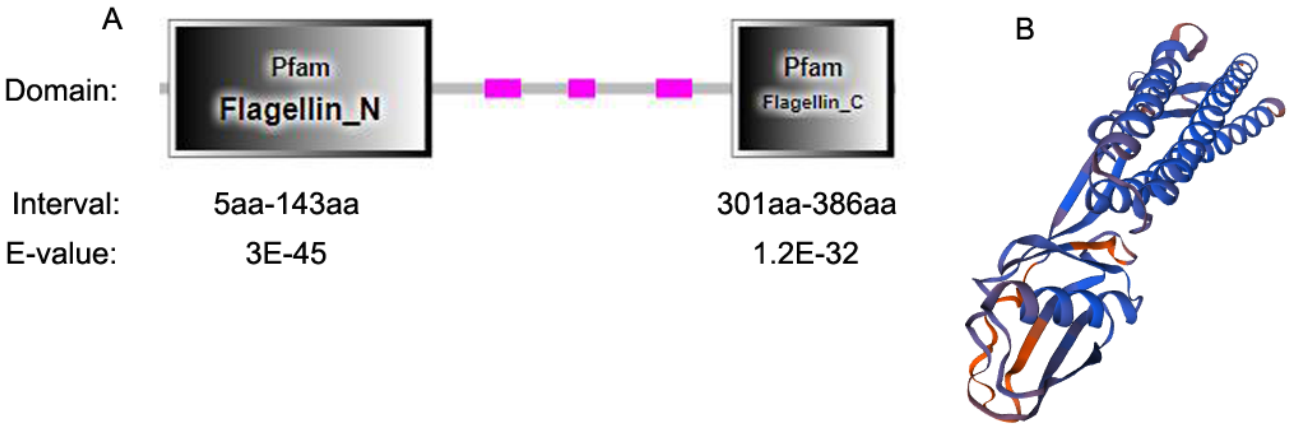
All experiments and data provided in this paper were repeated at least three times. The resulting data were subjected to an analysis of variance using GraphPad Prism software version 4.0 and SPSS 19.0 software (SPSS Inc.). Student's unpaired t test and Student –

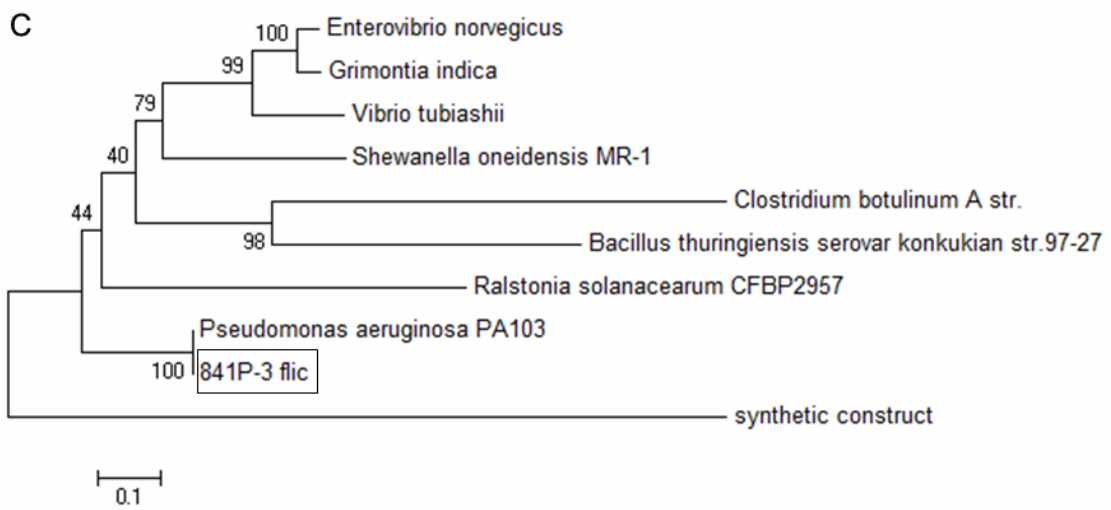
Newman – Keuls (S – N – K) test ($P < 0.05$) were used to determine the significance of the differences observed between the samples.

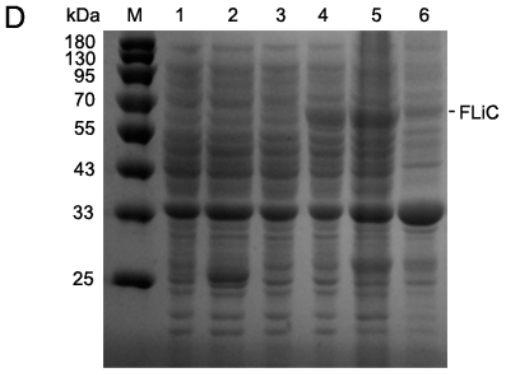
Results

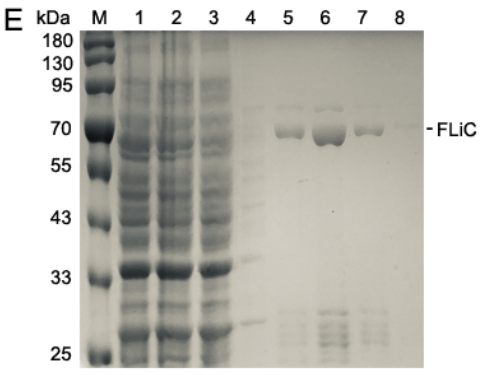
The structure and allergic reaction of flagellin FLiC

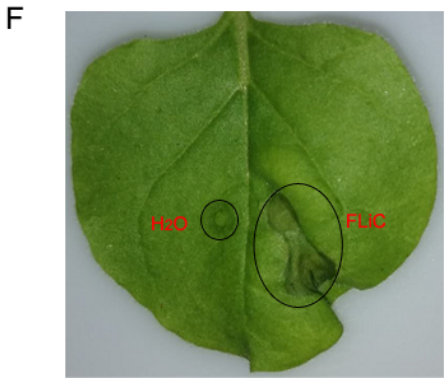
FLiC protein is flagellin and has two functional domains (Figure 1A). It has three-dimensional structure (Fig. 1B). The homology with *Pseudomonas aeruginosa* PA103 is 100% (Figure 1C). The recombinant plasmid was transformed into *E. coli* BL21 (DE3) strain, and induced by Isopropyl- β -D-thiogalactopyranoside (IPTG) (0.5 mM) at 28℃ for 5 h. The purified FLiC protein was 66 kDa (Figure 1, D and E). FLiC protein induces hypersensitive reaction and reactive oxygen species in tobacco leaves (Figure 1, F and G).











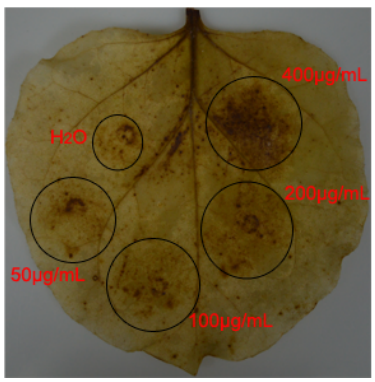


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Figure 1 The structure and allergic reaction of flagellin FLiC

A, two functional domains of FLiC protein; B, three-dimensional structure diagram of FLiC protein; C, evolutionary tree of FLiC protein; D: Expression of FLiC recombinant protein in *E. coli* at 28℃; M: protein marker; 1: control containing empty vector; 2: empty vector with the addition of inducer (0.5 mM IPTG); 3: Whole bacteria added with inducer (0 mM IPTG); 4: Whole bacteria added with inducer (0.5 mM IPTG); 5: The supernatant part after the bacterial cell is broken; 6: The part that settles after the bacterial cell is broken. E: purified FLiC protein; M：protein marker； 1：cell lysate； 2: flow through；3: wash1; 4: wash2; 5: elution1; 6：elution2；7：elution3; 8: elution4. F: hypersensitive reaction(HR) of FLiC on tobacco; G: reactive oxygen species (ROS) generated after treatment with different FLiC protein concentration,

FLiC induces resistance of cotton to VD

After smearing Jimian 11 and Zhongzhimian 2 with FLiC protein, the amount of VD in roots, stems and leaves was significantly lower than that in the control group, indicating that FLiC can induce resistance to the infection of VD (Figure 2). The incidence of resistant varieties was lower than that of susceptible varieties. After 30 days of FLiC treatment, the disease index of resistant varieties was lower than that of susceptible varieties. The relative biocontrol effects of FLiC on resistant and susceptible cotton varieties were 47.50% and 32.42% respectively (Table 1). Therefore, FLiC can not only induce systemic disease resistance in cotton, but the induction effect of resistant varieties is stronger than that of susceptible varieties.

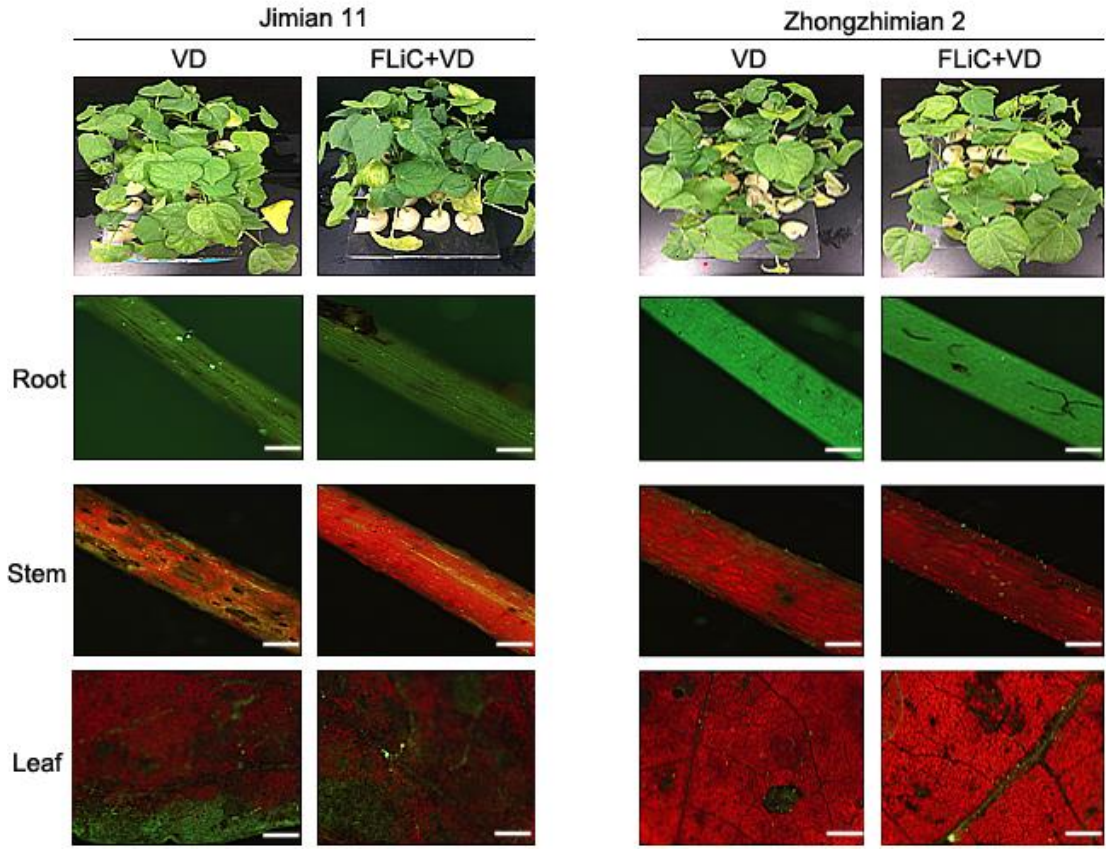


Figure 2 The effect of FLiC on the infection of VD in Jimian 11 and Zhongzhimian 2

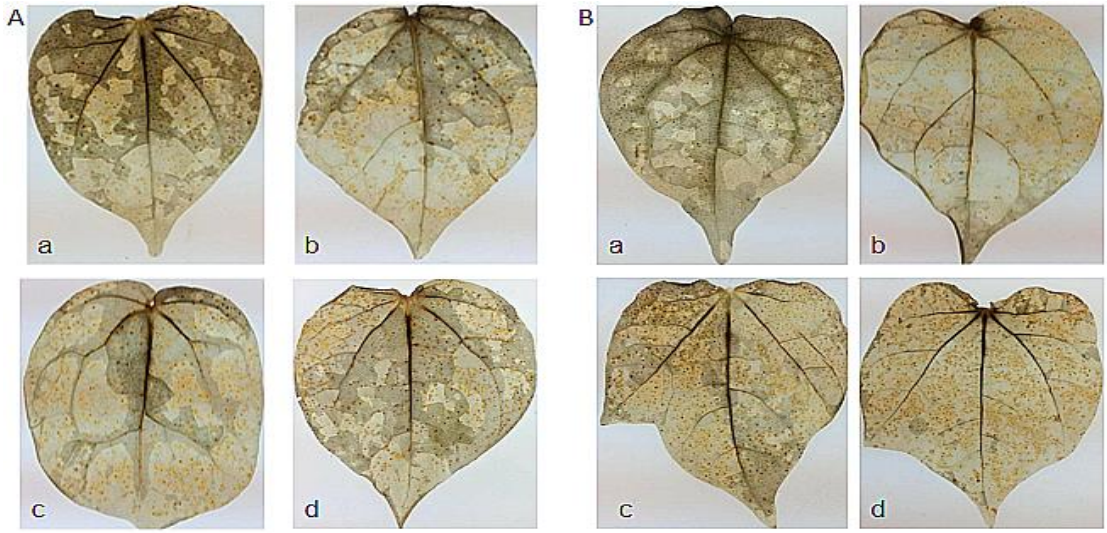
Table 1 FLiC induces disease resistance of different cotton varieties (30 d)

Treatment	Disease index(DI)	Relative control effect（%）
Jimian 11 （CK）	68.41±0.61a	
Jimian 11（FLiC）	46.23±1.12c	32.42±1.39b
Zhongzhimian 2（CK）	55.28±0.61b	
Zhongzhimian 2（FLiC）	29.00±0.83d	47.50±2.09a

Note: Different letters indicate significant at the 0.05 level.

FLiC induces H_2O_2 accumulation in cotton

After FLiC and VD treated cotton leaves for 24 hours, a large amount of H_2O_2 could be clearly detected. Pretreatment of FLiC protein before VD treatment can induce more H_2O_2 deposition. However, the accumulation of H_2O_2 in Jimian 11 was less than that of Zhongzhimian 2. Therefore, FLiC can induce the outbreak of H_2O_2 in the leaves to induce the defense response of cotton, and the immune response of resistant varieties is stronger than that of susceptible varieties (Supplemental Figure S1).

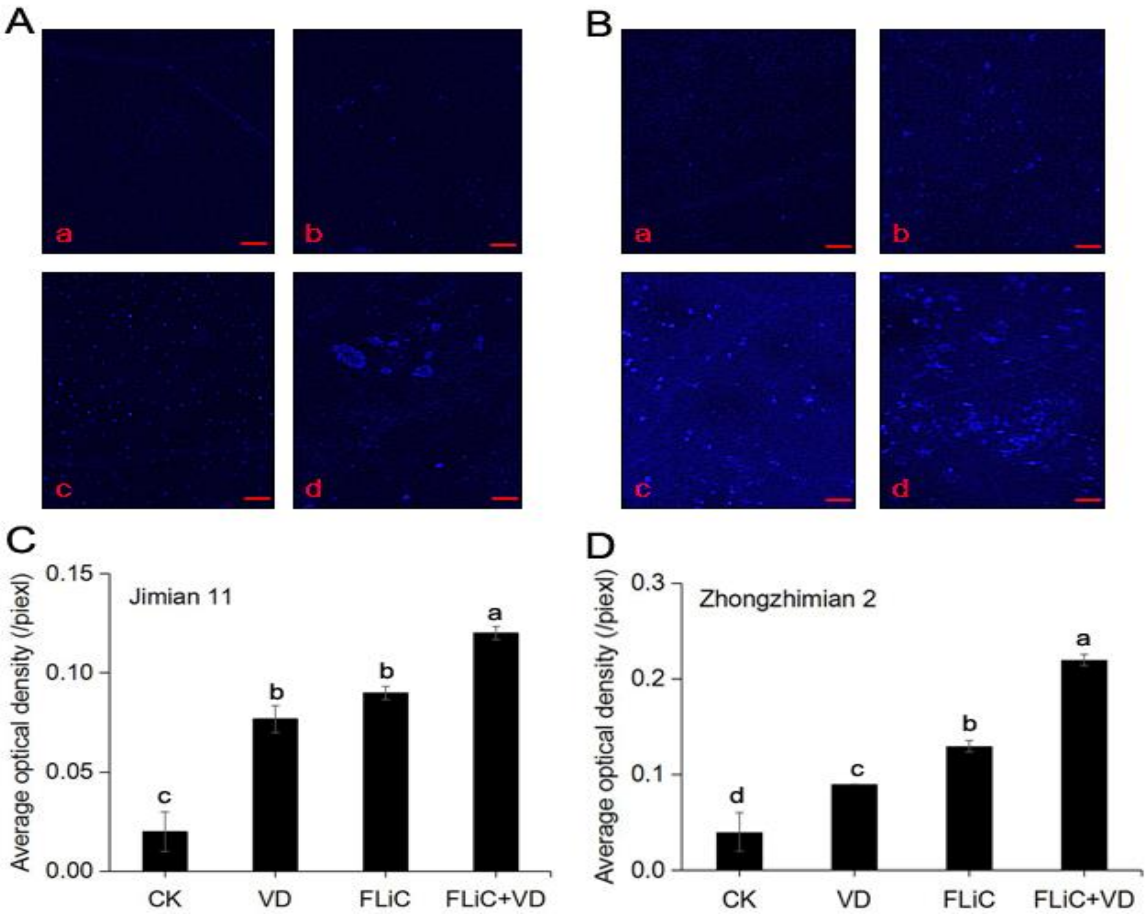


Supplemental Figure S1 FLiC induced the deposition of H_2O_2 in Jimian 11(A) and Zhongzhimian 2(B) leaves

a: Control; b: VD treatment; c: FLiC treatment; d: FLiC+VD treatment

FLiC induces the accumulation of callose in cotton

After FLiC treatment, both Jimian 11 and Zhongzhimian 2 can detect the obvious blue fluorescent substance (Supplemental Figure S2). VD and FLiC treatments had little difference in the corpus callosin content of Jimian 11, but significant difference in Zhongzhimian 2. Pretreatment of FLiC before VD inoculation, resistant and susceptible varieties produced a large amount of callus, and the induced resistance of resistant varieties was higher than that of susceptible varieties.Therefore, FLiC can induce cotton leaves to produce callose to enhance plant disease resistance.

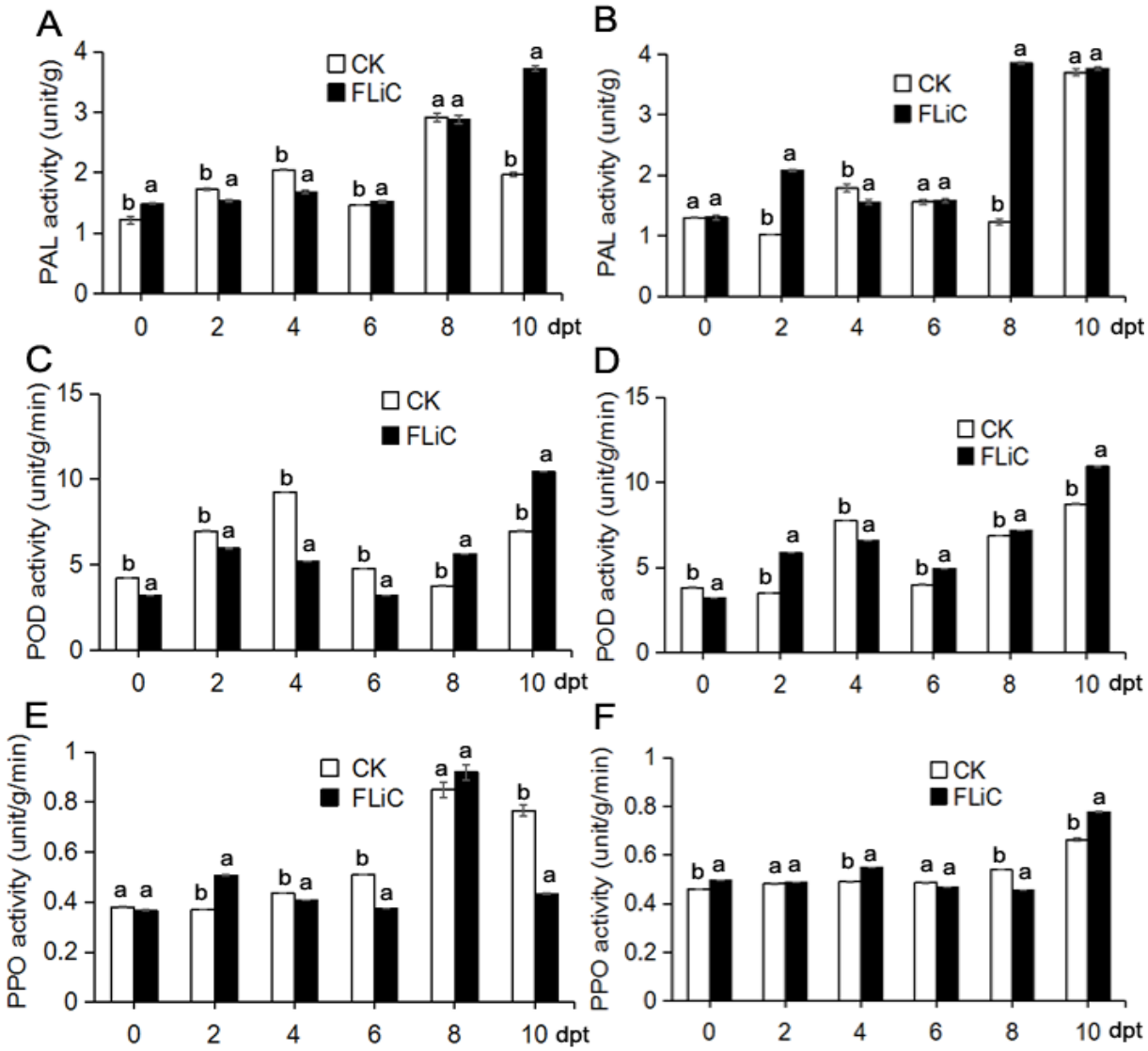


Supplemental Figure S2 FLiC-induced callose deposition in Jimian 11(A) and Zhongzhimian 2(B) leaves; a: Control; b: VD treatment; c: FLiC treatment; d: FLiC+VD treatment; C: FLiC protein induces the average optical density of Jimian 11 to accumulate callose; D: FLiC protein induces the average optical density of Zhongzhimian 2 to accumulate callose; different letters indicate significant at the 0.05 level.

FLiC-induced resistance depends on changes in related enzyme activities

After FLiC treatment of Jimian 11 and Zhongzhimian 2 cotton seedlings, the activities of the three defense-related enzymes in the cotton were increased to varying degrees. In Jimian 11, the PAL activity reached the maximum at 10 d, while the resistant variety reached the maximum at 8 d. The PAL activity in the resistant variety had a more obvious response than the susceptible variety. The POD activity of susceptible varieties increased significantly at 10 d, while the POD activity of resistant varieties peaked at 2 d, then

decreased to the control level after 6 d of inoculation, and peaked again after 10 d of inoculation. The PPO activity of the susceptible varieties increased at 2 d, and there was no significant difference from the control afterwards. The resistant varieties were higher than the control at 4 d, and the PPO activity peaked at 10 d (Supplemental Figure S3). Therefore, FLiC can induce different degrees of changes in the activities of three enzymes in cotton to enhance disease resistance and more obvious disease-resistant varieties.



Supplemental Figure S3 FLiC induces the activity detection of defense-related enzymes in cotton Jimian 11 (A, C, E) and Zhongzhimian 2 (B, D, F); different letters indicate significant at the 0.05 level.

FLiC induces the deposition of callose and lignin

After applying FLiC to the leaves for 2 days, there were obvious callose deposits in

the leaves and the lignin content increased in the stems of Zhongzhimian 2 (Figure 3A). This result is consistent with the result that FLiC induces callose deposits in resistant and susceptible cotton. After pretreatment of FLiC before inoculation with VD, the deposition of callus and lignin is more obvious. When 2-DDG and AOPP were pretreated, the amount of callose and lignin deposition decreased, the amount of spores in the roots increased significantly, and the FLiC induced resistance was weakened (Figure 3, C and D). Therefore, FLiC can induce the deposition of lignin in stem cells and callose of leaves to resist the infection of VD.

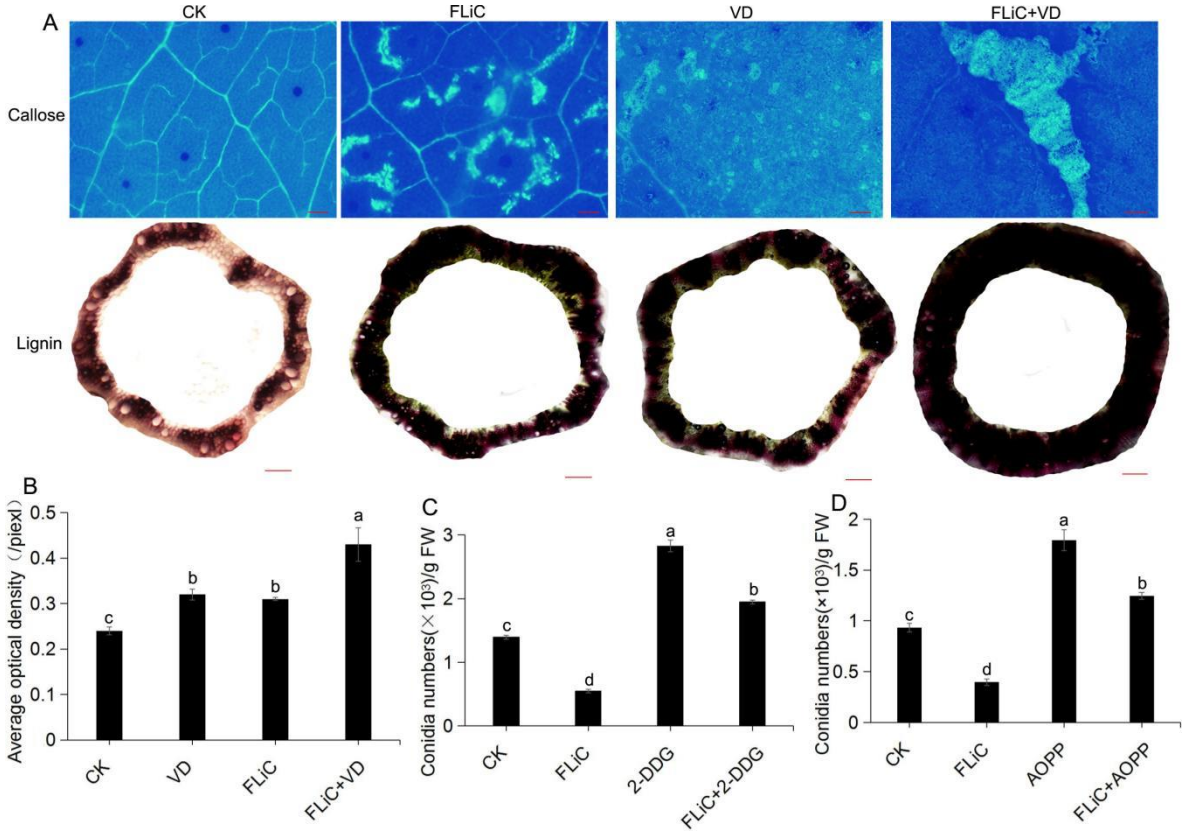
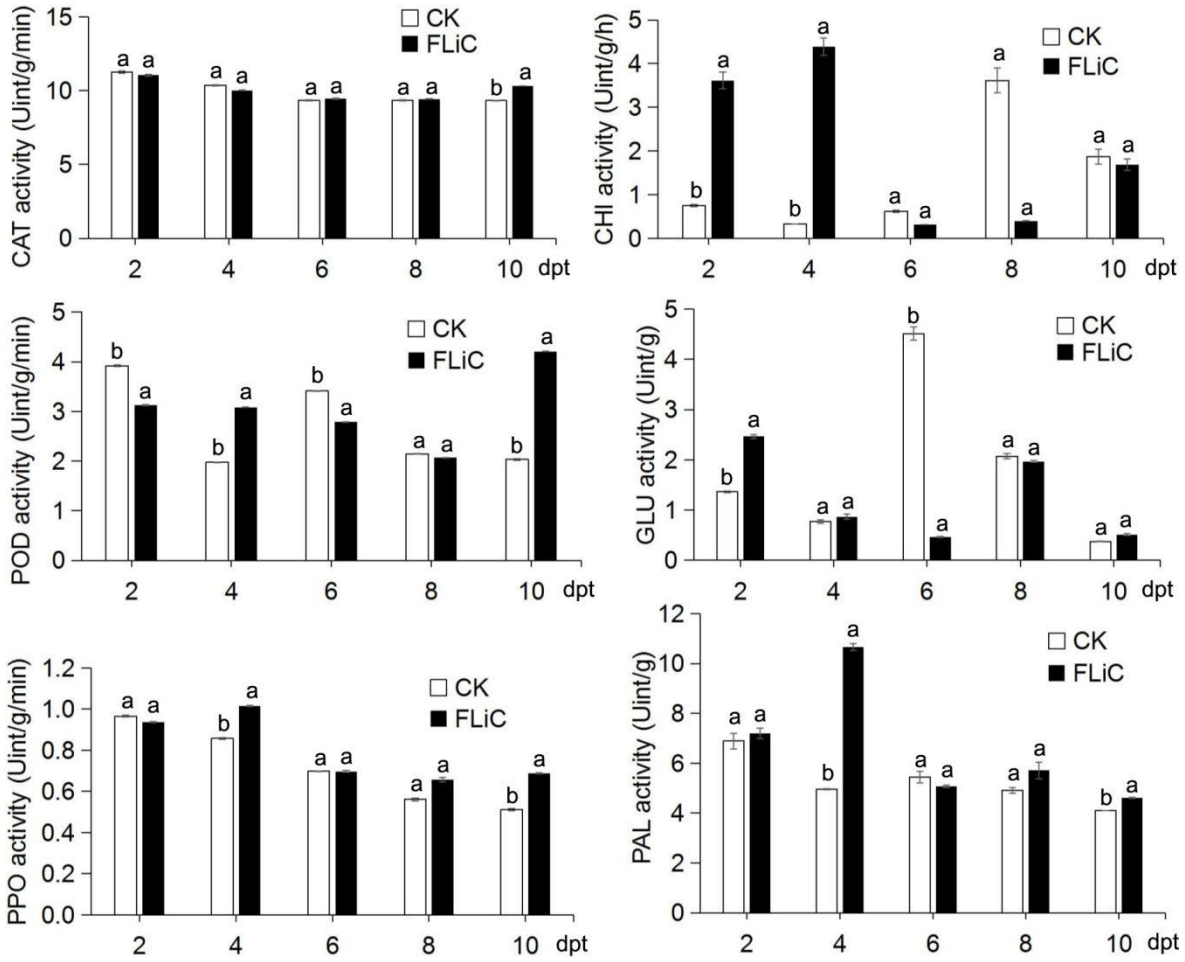


Figure 3 FLiC induces the deposition of lignin in cotton leaves and stems;(A) callose scale = 1000 μm; lignin scale = 2000 μm; (B), The statistics of the average optical density of callose; (C), the statistics of the bacterial mass after the corpus callose is cleared with 2-DDG; (D), the statistics of the bacterial mass after the corpus lignin is cleared with AOPP; Different letters indicate significant at the 0.05 level.

FLiC-induced resistance depends on changes in defensive enzyme activity

In order to further verify that FLiC can induce changes in enzyme activity in cotton to enhance the defense response. We determined the changes in the activities of six enzymes in the body of the disease-resistant variety Zhongzhimian 2 treated with FLiC alone. After FLiC smeared the leaves, the activity of defense-related enzymes changed to varying degrees (Supplemental Figure S4). CAT was significantly higher than the control on the 10th day. PPO, POD, and PAL all increased significantly on the 4th day compared with the control, and on the 8th day after that they were comparable to the control, and on the 10th day they all reached a significant level compared with the control. This result is basically consistent with the changes in the activities of defense enzymes induced by FLiC in resistant and susceptible cotton varieties. CHI reached a significant level compared with the control on the 2nd and 4th day, and then began to drop to the control level. GLU increased significantly on 2nd day compared with the control, and then began to decrease. This result shows that after FLiC treatment, the activity of defense-related enzymes changes differently under different induction time to improve disease resistance.

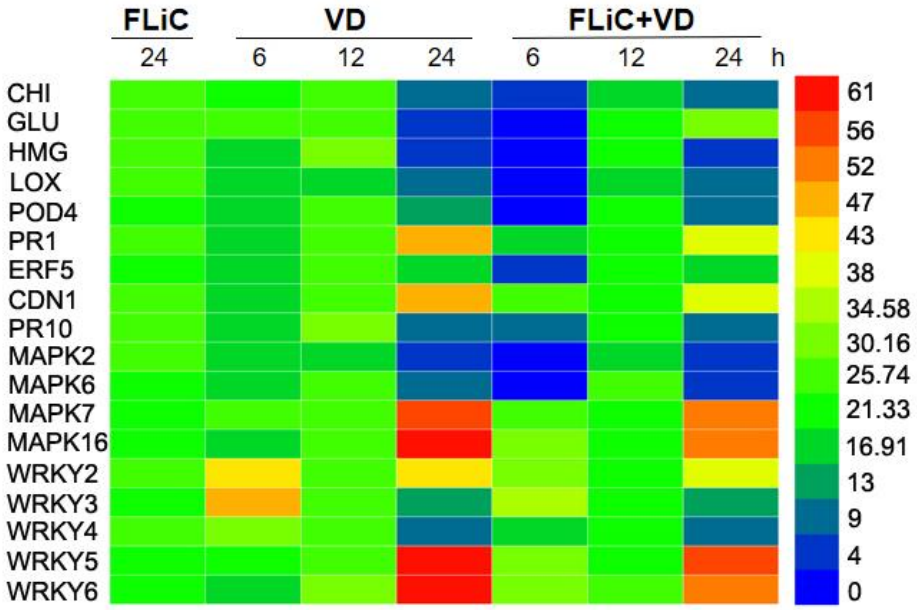


Supplemental Figure S4 FLiC induces changes in defense-related enzyme activities, the same letter indicates that there is no significant difference at the 0.05 level.

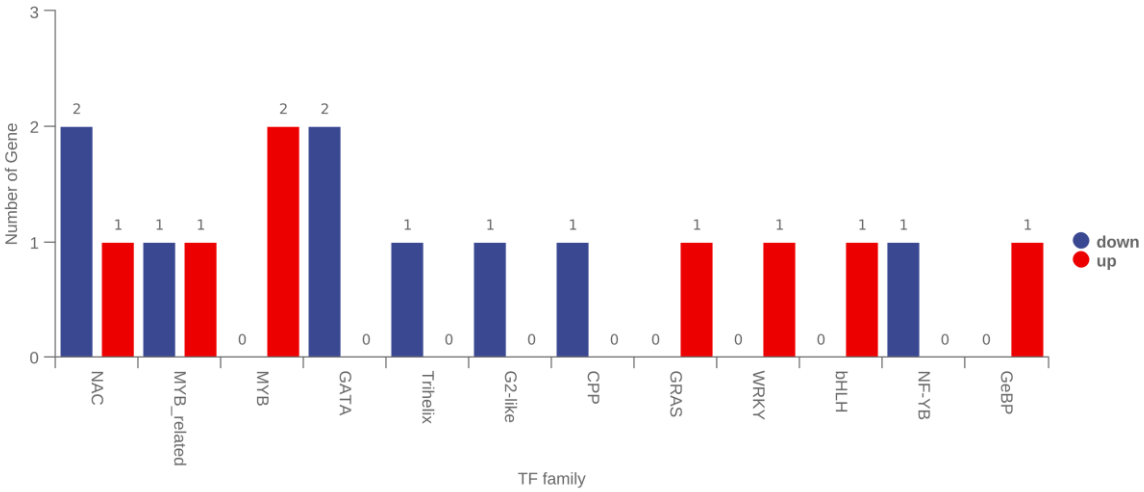
FLiC induces the expression of disease resistance-related genes

After the cotton seedlings were smeared with flagellin FLiC for 24 h, defense genes such as *PR1*, *CHI*, *GLU*, *POD4*, *ERF5*, *PR10*, *LOX* and *CDN1* were induced to varying degrees (Supplemental Figure S5). FLiC pretreated before inoculation with VD was the treatment group, and only VD was inoculated as the control group. After 6 hours of inoculation, the expression levels of *MAPK2*, *MAPK6* and *MAPK7* genes in the seedling roots of the treatment group were lower than those of the control, and the expression levels of the *MAPK16* gene were higher than those of the control. After 12 h and 24 h of inoculation, the expression levels of *MAPK2*, *MAPK6*, *MAPK7* and *MAPK16* genes were not significantly different from those of the control. It indicates that *MAPK16* gene may be

involved in the early immune response induced by FLiC. After 6 hours of inoculation with VD, the expression levels of *WRKY5* and *WRKY6* genes were higher than the control except for *WRKY2*, *WRKY3* and *WRKY*4. At 12 h and 24 h after inoculation, *WRKY2*, *WRKY3*, *WRKY4*, *WRKY5* and *WRKY6* were not significantly different from the control. These two transcription factors, *WRKY5* and *WRKY6*, may be involved in the early immune response induced by FLiC. This is consistent with the up-regulated expression of the differentially expressed transcription factor WRKY in the transcriptome results (Supplemental Figure S5).



Supplemental Figure S5 Flagellin FLiC induced defence related genes expression



Supplemental Figure S6 Distribution of differentially expressed transcription factors in the transcriptome

Metabolic pathway enriched by differentially expressed genes in the transcriptome

The transcriptome results showed that 87 genes were up-regulated and 90 genes were down-regulated. The significantly enriched differential genes were classified and clustered as follows (Figure 4, A and B). Differentially expressed genes were significantly enriched in related disease-resistant metabolic pathways such as potassium ion, calcium antiporter activity, diterpenoid biosynthesis and phenylpropane biosynthesis (Figure 4, C-F). Significantly enriched to two down-regulated calcium antiporter activity regulatory genes, namely GH-A08G0063 and GH-D08G0067. Therefore, calcium antiporter activity regulatory genes may negatively regulate plant disease resistance.

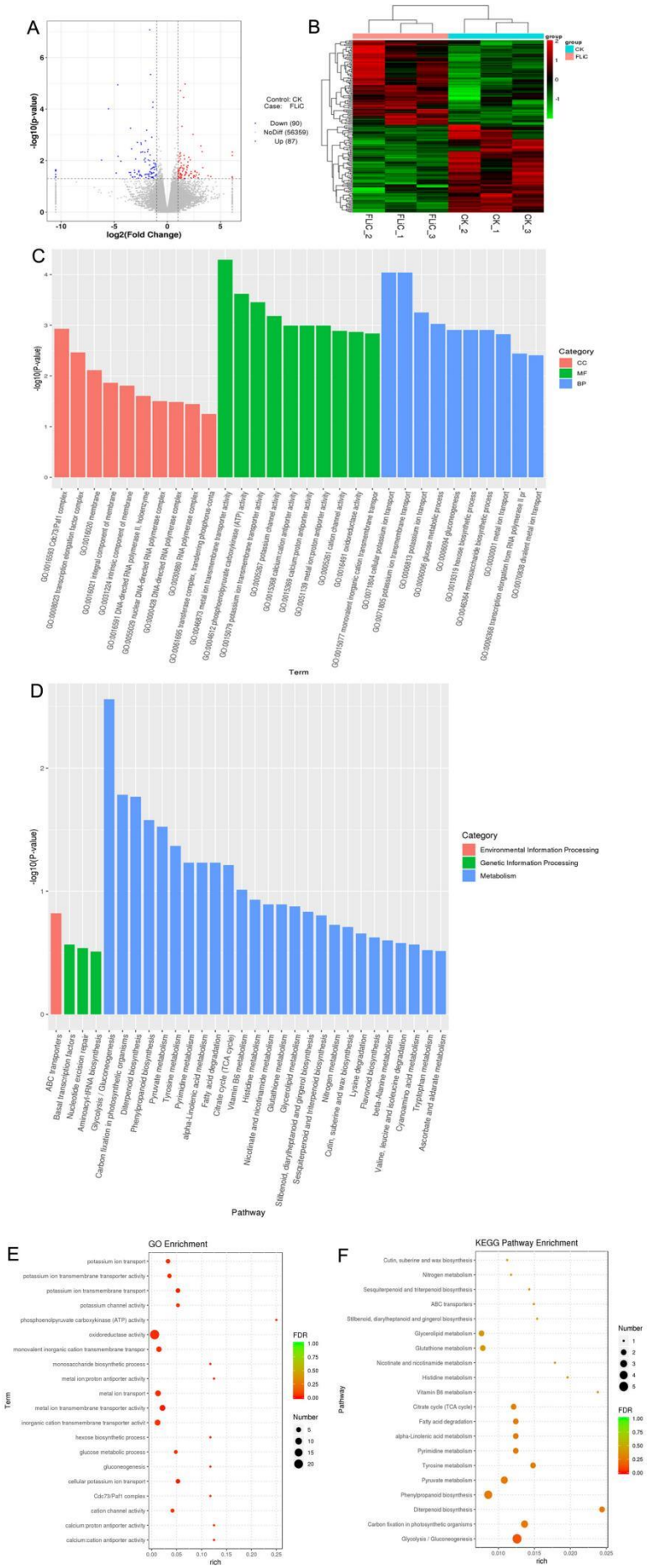


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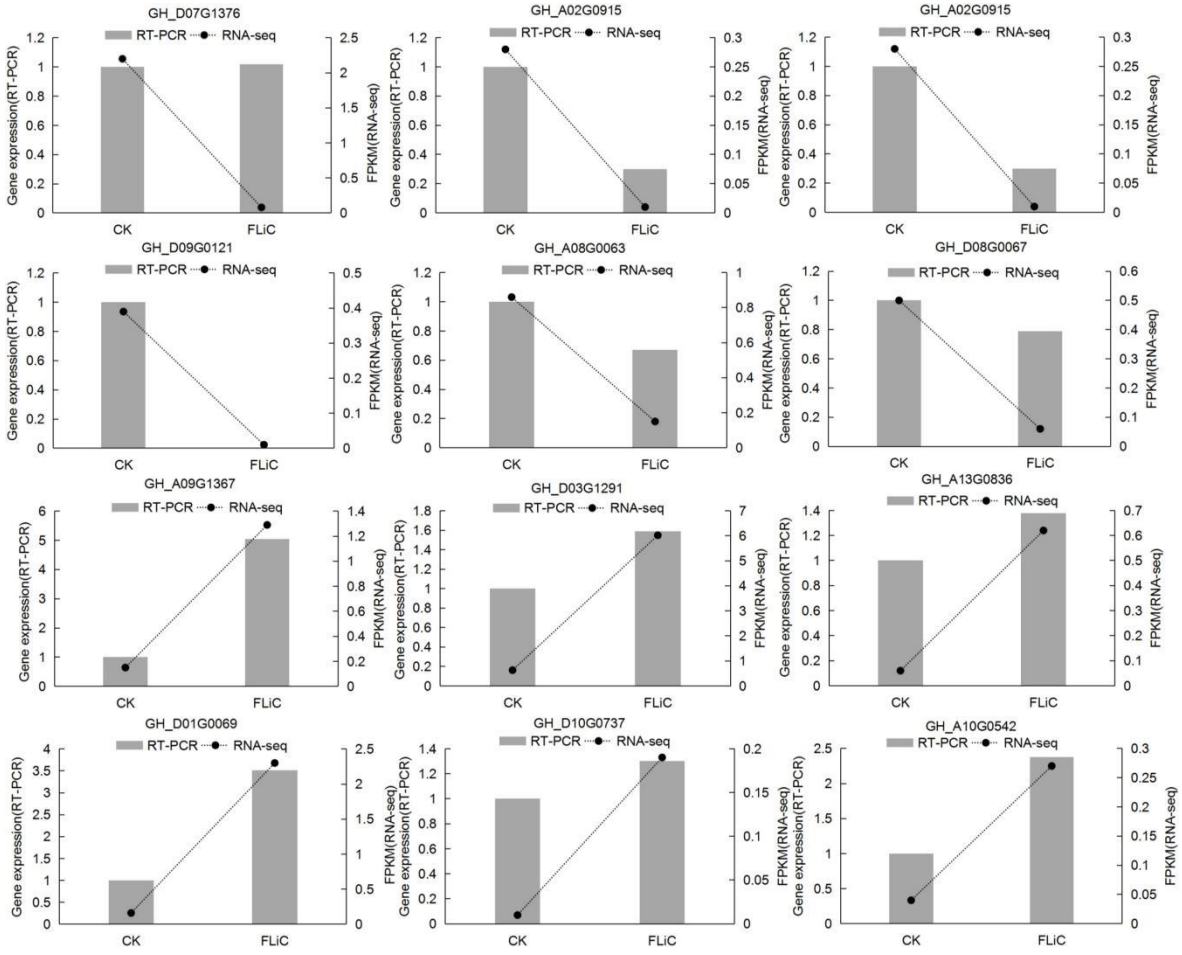
Figure 4 Pathways to which differentially expressed genes in the transcriptome are significantly enriched; A, differential gene volcano map; B, differential gene cluster analysis; C, GO-enriched metabolic pathway; D, KEGG-enriched metabolic pathway; E , The bubble chart of the first 20 metabolic pathways enriched by GO; F, the bubble chart of the first 20 metabolic pathways enriched by KEGG. Note: C, the abscissa is the term of Go level 2, and the ordinate is the -log10 (p-value) enriched for each term. D, the abscissa is the name of the pathway, and the ordinate is the -log10 (p-value) enriched for each pathway.

RT-PCR verification of transcriptome results

Twelve groups were randomly selected from the RNA-seq sequencing results to verify the transcriptome results by RT-PCR. The results showed that 11 of the 12 groups had the same expression trend as the transcriptome, with an accuracy rate of 91.67%. It shows that the transcriptome results are credible (Supplemental Figure S7).



Supplemental Figure S7 Verification of FLiC transcriptome results

The relationship between Ca^{2+}, NO and H_2O_2

1. FLiC induces an increase in intracellular Ca^{2+}

According to the results of the transcriptome, the calcium antiporter activity was significantly enriched. In order to verify that the FLiC protein induced resistance, the increase in calcium ion influx caused the downstream immune response. The cotton epidermal cells were loaded with Ca^{2+} sensitive fluorescent probe Fluo-3/aM, and then FLiC protein at a concentration of 100 μg/mL was processed and observed with LSCM. The results show that FLiC treatment can cause a significant increase in the fluorescence intensity of epidermal cells (Figure 5). Pretreatment of Ca^{2+} chelating agent EGTA and Ca^{2+} channel blocker $LaCl_3$ significantly reduced the fluorescence intensity induced by FLiC. Therefore, FLiC can induce the increase of Ca^{2+} concentration in cotton epidermal cells. This is consistent with the results of calcium-related metabolic pathways that are significantly enriched in the transcriptome.

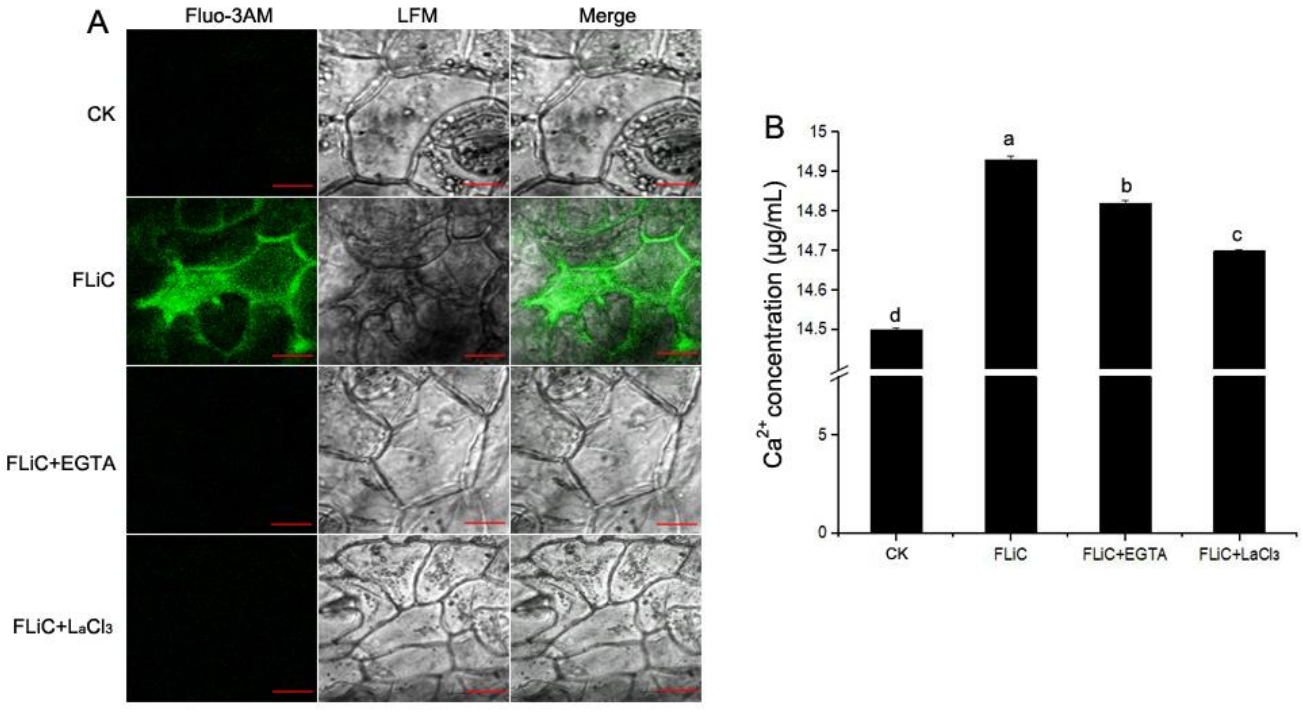
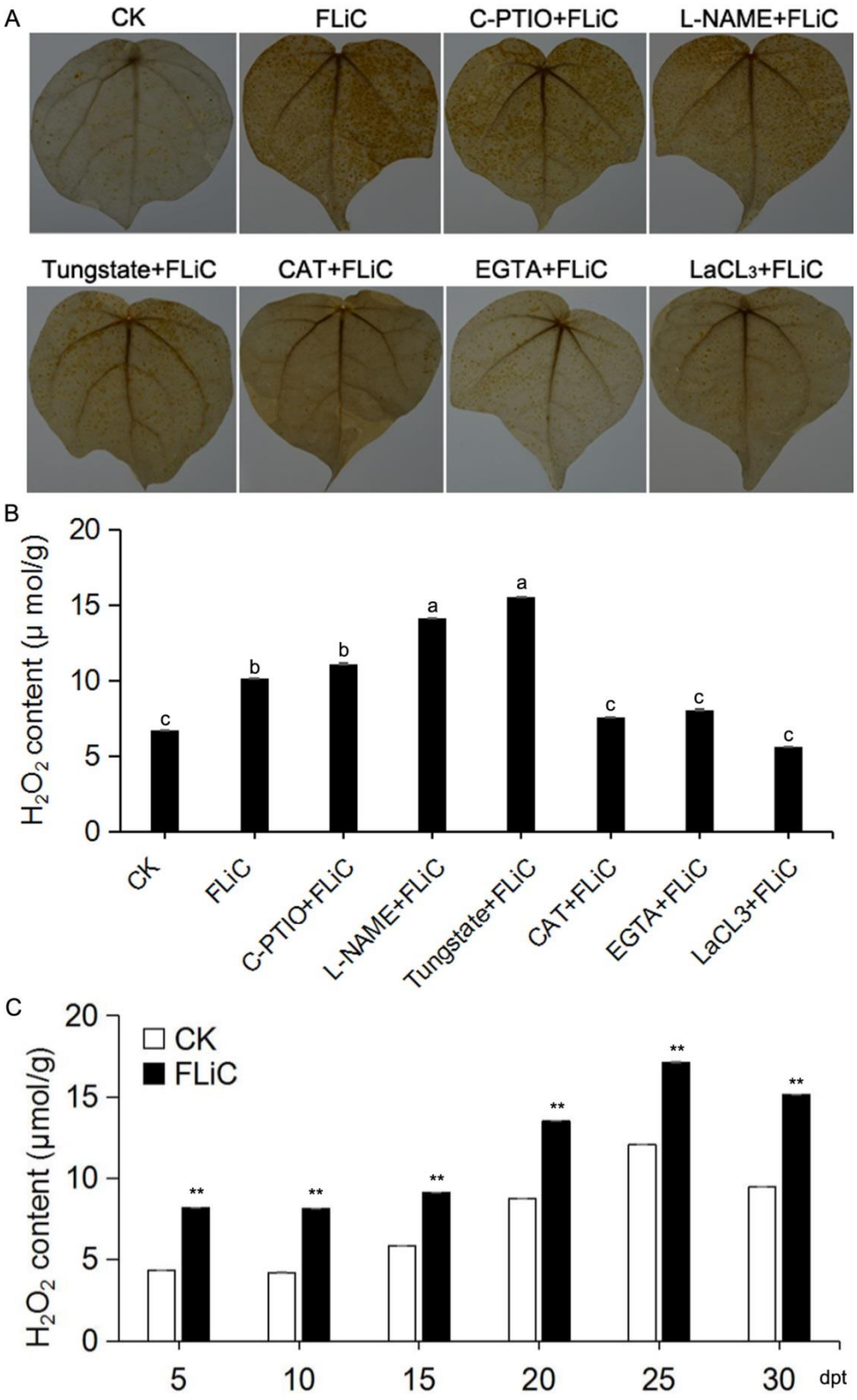


Figure 5 FLiC induces Ca^{2+} production in cotton epidermal cells; different letters indicate significant differences at the 0.05 level; bar= 50 μm

2. Different pretreatments affect the H_2O_2 burst induced by FLiC

To determine the relationship between ROS, Ca^{2+} and NO. Two days after the FLiC smearing treatment, there was an obvious burst of ROS in the leaves (Supplemental Figure S8A). In order to study the influence of FLiC treatment on the relationship among Ca^{2+}, NO and H_2O_2, after pretreatment of CAT, EGTA and $LaCl_3$, the H_2O_2 content in the leaves was significantly reduced to the control level. After pretreatment of C-PTIO, L-NAME and Tungstate, the H_2O_2 content in the leaves was higher than that induced by FLiC alone (Supplemental Figure S8B). The above results indicate that Ca^{2+} are located upstream of H_2O_2 and affect the synthesis of H_2O_2, while NO may be located beside or downstream of H_2O_2. After FLiC treatment for 5-30 days, the H_2O_2 in the leaves of the seedlings continued to be significantly higher than that of the control. Until the 30th day, the H_2O_2 content began to decrease, but it was still higher than the control (Supplemental Figure S8C). Therefore, FLiC protein can induce cotton to produce an immune response and last longer.



Supplemental Figure S8 The effect of NO scavenger C-PTIO, NO synthesis inhibitor L-NAME and sodium tungstate, H_2O_2 scavenger CAT, Ca^{2+} chelating agent EGTA and Ca^{2+} channel inhibitor $LaCl_3$ on FLiC-induced H_2O_2 production. The brown substance represents the H_2O_2 in the leaves. Different letters

indicate significant differences at the 0.05 level.**, means that the difference is extremely significant at the 0.01 level.

3. FLiC induces NO production in cotton cells

There was strong fluorescence around the epidermal cells and stomata of cotton treated with FLiC, indicating that FLiC can induce the production of NO by cotton epidermal cells (Figure 6A). When the concentration of FLiC was 400 μg/mL and the treatment for 1 h, the fluorescence intensity was the strongest (Figure 6B), indicating that the production of NO induced by FLiC depends on the concentration of FLiC and the treatment time. After pretreatment of NO scavenger C-PTIO and NOS pathway inhibitor L-NAME, the fluorescence intensity decreased significantly (Figure 6A), indicating that C-PTIO and L-NAME have an inhibitory effect on FLiC-induced NO production in cotton epidermal cells. Nitrate reductase(NR) pathway inhibitor tungstate has only a small inhibitory effect on FLiC-induced NO production in cotton epidermal cells. It indicates that FLiC induces the production of NO by cotton epidermal cells, which may be mainly synthesized through the nitric oxide synthase (NOS) pathway. After pretreatment with CAT, EGTA and $LaCl_3$, the NO content was significantly reduced. It shows that Ca^{2+} and H_2O_2 are involved in the production of NO. Preliminary experiments have shown that pretreatment of C-PTIO and L-NAME, FLiC can still induce more H_2O_2 production. It shows that NO has an inhibitory effect on FLiC-induced H_2O_2. After pretreatment of EGTA and $LaCl_3$, the content of H_2O_2 is significantly reduced (Figure S8, A and B). Therefore, in the immune response induced by FLiC, Ca^{2+} located upstream of H_2O_2 and participate in the production of H_2O_2. H_2O_2 will stimulate the production of NO, and excessive NO will inhibit the production of H_2O_2.

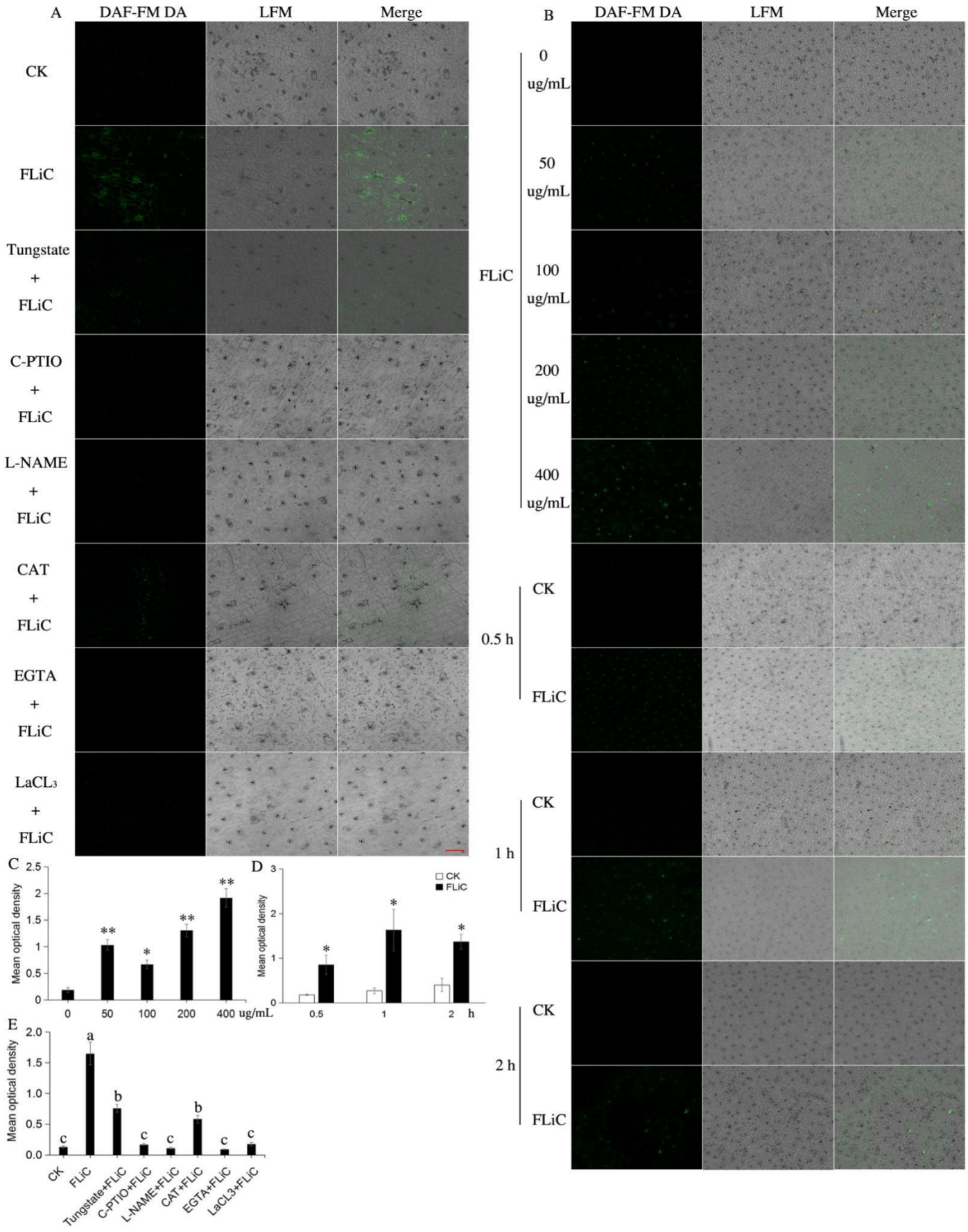


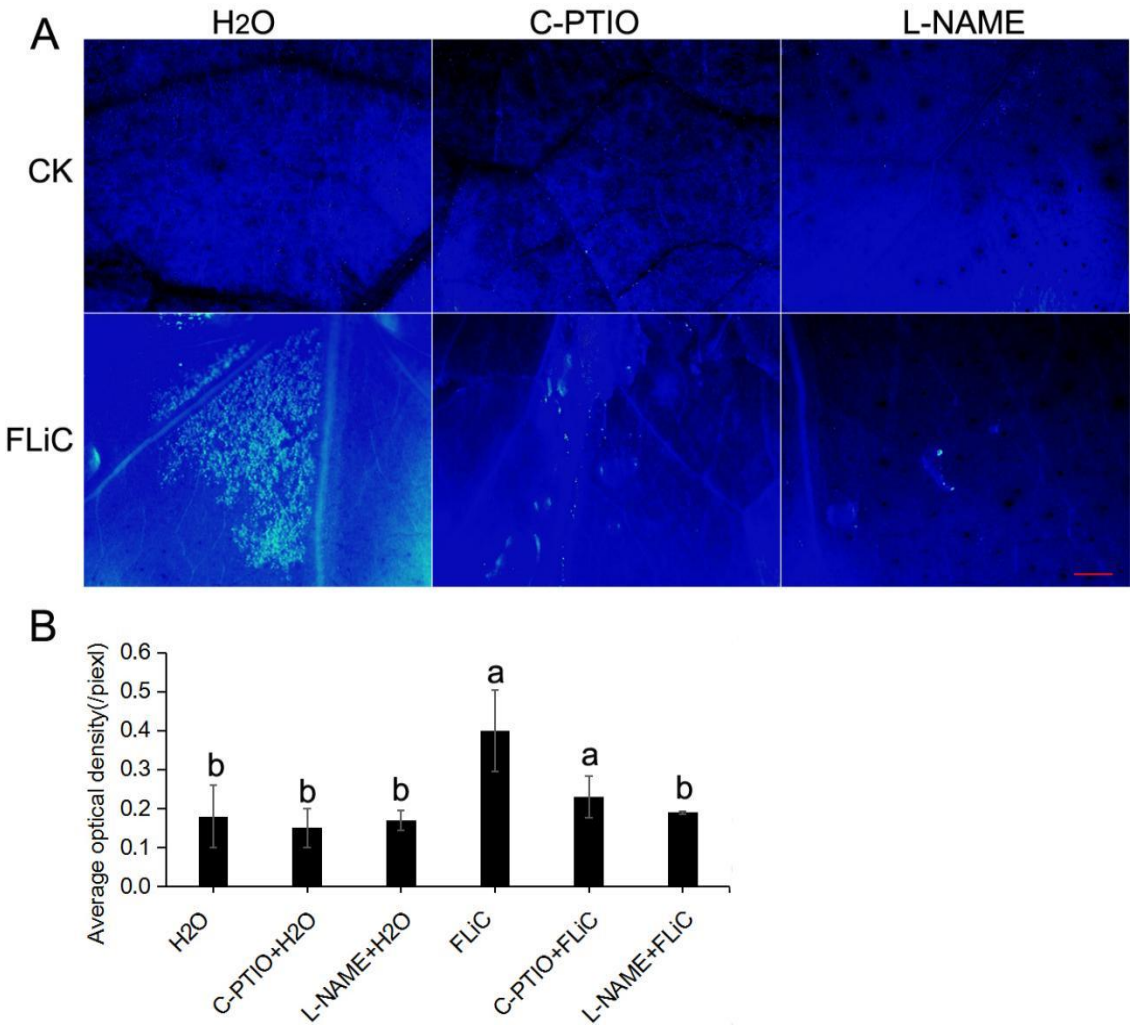
Figure 6 NO production in response to FLiC treatment in cotton leaves

A. Fluorescence in cotton epidermal cells during different pretreatments (200 μM C-PTIO, 200 μM L-NAME, 100 μM tungstate, 100 unit/mL CAT, 5 mM EGTA and 200 μM $LaCl_3$); B. Fluorescence produced in cotton epidermal cells with different protein concentration and different treatment time; C. The average fluorescence density of NO in cotton epidermal cells treated with different protein concentrations; D. The average fluorescence density of NO in cotton epidermal cells after different treatment time; E. The average fluorescence density of NO in cotton epidermal cells after different pretreatments; *: The difference is significant at the 0.05 level; **: the difference is extremely significant at the 0.01 level; different letters indicate that the difference between the treatments is significant at the 0.05 level; Bars = 20 μm.

NO is involved in FLiC-induced resistance

1. NO is involved in the deposition of callose induced by FLiC

FLiC protein can induce callose in cotton leaves (Supplemental Figure S9), and after pre-treatment of C-PTIO and L-NAME, FLiC-induced callose is significantly reduced (Supplemental Figure S9). Therefore, the increase of callose induced by FLiC is dependent on NO.

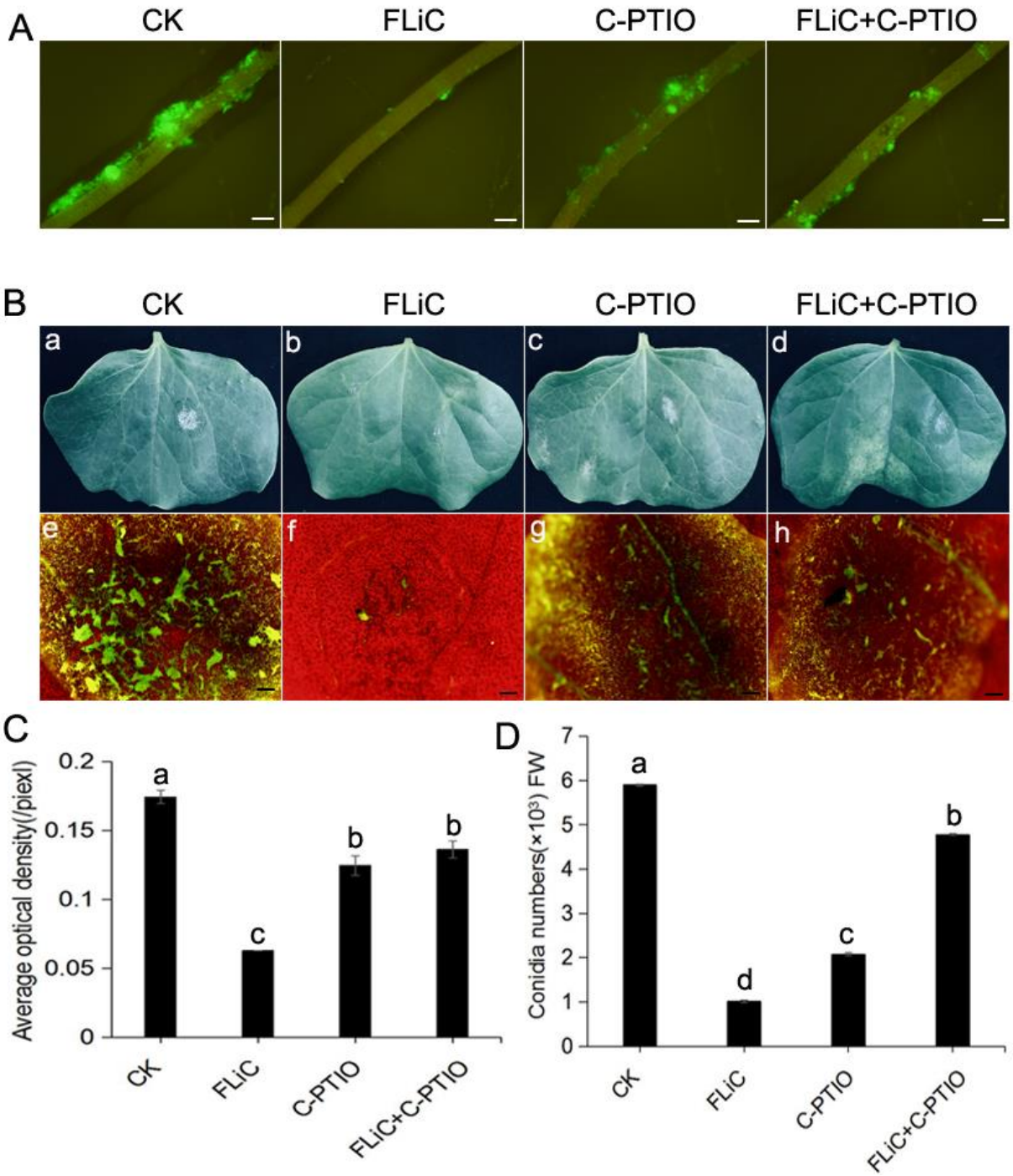


Supplemental Figure S9 NO is involved in the deposition of callose induced by FLiC; different letters indicate significant at the 0.05 level; bars=500 μm

2. NO reduces the damage of VD

After 48 hours of inoculation with VD, the number of pathogens colonizing the roots of cotton seedlings pretreated with FLiC was significantly less than that of the control (Supplemental Figure S10A). After pretreatment of C-PTIO, the number of pathogen colonization increased, and the difference reached a significant level compared with the control (Supplemental Figure S10D). Five days after the leaf was inoculated with spores of VD, lesions were observed on the leaf surface (Supplemental Figure S10B). The disease degree of the leaves of the seedlings treated with FLiC was significantly less than that of

the control. The area of leaf damage of pretreatment with C-PTIO, FLiC treatment and control seedlings all increased. This result further indicates that the NO induced by FLiC participates in the resistance of cotton to VD.



Supplemental Figure S10 NO is involved in FLiC-induced disease resistance

A. Colonization of fluorescently labeled VD on cotton roots. B. The infection of leaves inoculated with VD under different treatments. a, b, c and d: leaf parts inoculated with VD. e, f, g and h: observe the infection of VD on cotton cotyledons under a fluorescence microscope. C. The average

fluorescence density value of cotton roots. D. The number of spores on cotton roots. Different letters indicate significant differences at the 0.05 level. Bars=500 μm.

Calcium antiporter activity (GhCAA) increases in anti-disease substances after silence

In order to verify that the calcium antiporter activity regulatory gene negatively regulates the disease resistance of cotton. After the positive control true leaves appeared albino, the silencing efficiency of GhCAA was determined to reach 62% (Figure 7, A and B). After the *GhCAA* gene was silenced, the intracellular Ca^{2+} and NO content in the epidermis of cotton leaves after applying FLiC were significantly higher than that of WT and TRV2::00 (Figure 7, C-G); The accumulation of ROS, callose and lignin was significantly higher than that of WT and TRV2::00 (Figure 7E). Therefore, FLiC negatively regulates the disease resistance of cotton by inhibiting the expression of *GhCAA* gene.

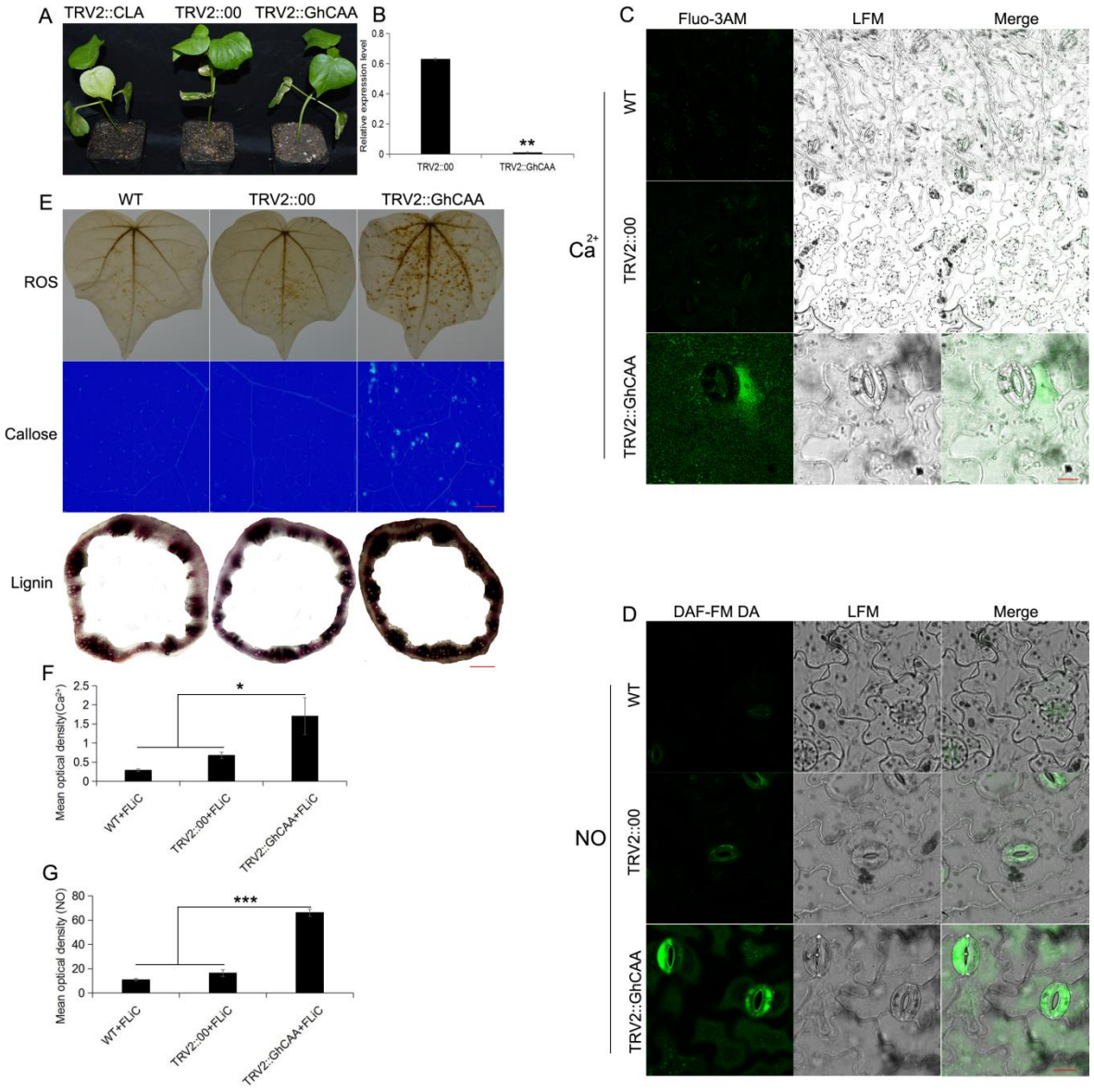
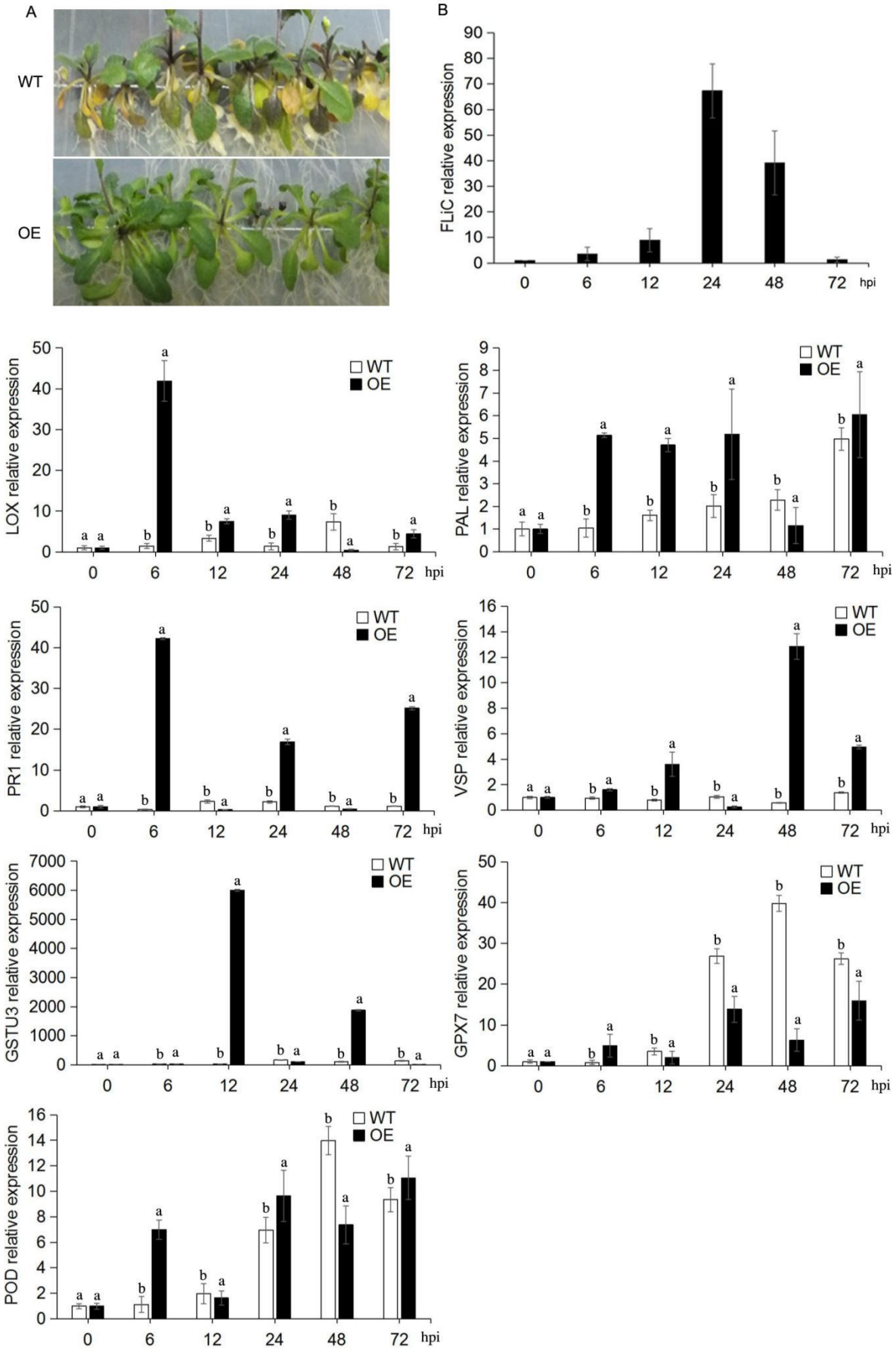


Figure 7 Anti-disease substances increase after the silencing of the calcium antiporter activity gene *GhCAA*; *: The difference is significant at the 0.05 level; ***: the difference is extremely significant at the 0.001 level; C and D,bars= 50 μm; E , bars= 2000 μm.

Transgenic Arabidopsis with *FLiC* gene enhances resistance to VD and increases the expression of disease-resistant genes

The *FLiC* gene-transformed Arabidopsis has enhanced disease resistance to VD (Supplemental Figure S11A). The expression of *FLiC* gene increased significantly at 24 h after inoculation (Supplemental Figure S11B). Therefore, transgenic *FLiC* gene

Arabidopsis can be highly expressed in vivo to improve plant disease resistance to VD. In order to verify that *FLiC* gene-transformed Arabidopsis has enhanced resistance to VD, we tested lipoxygenase (*LOX*), phenylalanine ammonia lyase (*PAL*), disease-related protein (*PR1*) and vegetative storage protein (*VSP*). The expression of the four disease resistance genes increased significantly. Therefore, *FLiC* gene-transformed Arabidopsis can effectively increase the expression of disease resistance genes related to the SA and JA signal pathways and enhance the plant's disease resistance. The expression level of glutathione peroxidase gene (*GPX7*) showed no increasing trend or even lower than the control; peroxidase (*POD*) was expressed more obviously when exposed to VD than the control; glutathione sulfur transferase gene (*GSTU3*) expression increased significantly (Supplemental Figure S11). Therefore, *FLiC* gene-transformed Arabidopsis can effectively regulate the up-regulated expression of ROS and NO signal-related genes in plant roots and enhance disease resistance.



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Supplemental Figure S11 A, *FLiC* gene-transformed Arabidopsis has enhanced disease resistance. B, *FLiC* expression in transgenic *Arabidopsis thaliana* increased after inoculation; WT: wild type; OE: over expression; *PAL*: phenylalanine ammonia lyase; *PR1*: disease-related protein; *LOX*: lipoxygenase; *VSP*: vegetative storage protein; *GPX7*: glutathione peroxidase; *GSTU3*: glutathione sulfur transfer Enzyme gene; *POD*: peroxidase; Different letters indicate significant differences at the 0.05 level.

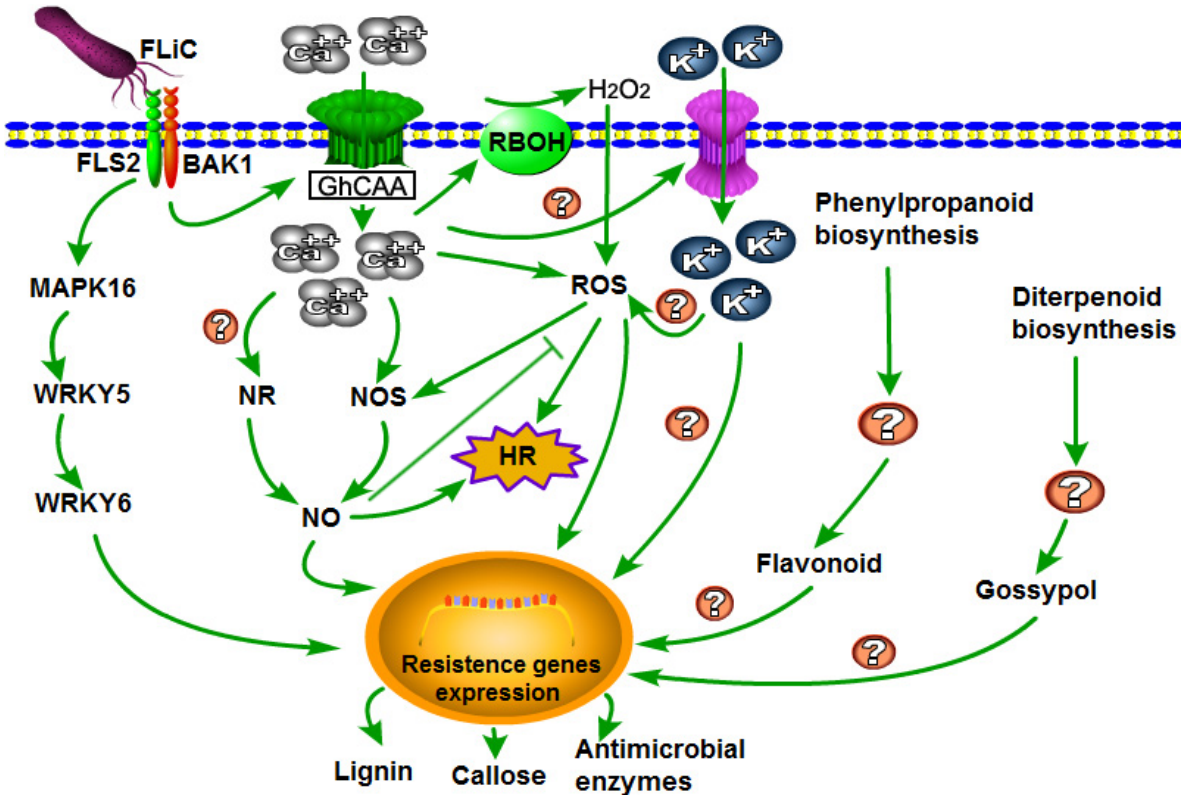

Figure 8 Reasoning of FLiC-induced resistance signal pathway

Discussion:

The metabolic pathways of FLiC protein and Flg22 protein to induce immune response are different

Rice is not sensitive to Flg22 but can recognize full-length flagellin (Takai, et al., 2007). However, the mechanism by which full-length flagellin regulates the immune response of plants is unclear. The effect and mechanism of FLiC full-length protein on plant immune response have not been reported yet. Flg22 can induce immune response effects in different plants, but there is no systematic metabolic pathway study in upland cotton. We cloned a new type of flagellin gene *FLiC*, which contains the amino acid sequence of Flg22. It can induce cotton to produce an immune response, such as the *MAPK* cascade reaction and the up-regulation of key genes in disease resistance pathways such as SA, JA, and ETH (Supplemental Figure S5). After the protein is treated with cotton, the differentially expressed genes can be significantly enriched in potassium ions, calcium ions, diterpenoid synthesis, phenylpropane biosynthesis, lignin biosynthesis, nitrogen metabolism and other disease-resistant metabolic pathways through transcriptome analysis. Therefore, the disease resistance induced by FLiC protein in cotton is related to the activation of these pathways. We found that after silencing *GhCAA* on the membrane, the intracellular Ca^{2+} content increased and induced NO, H_2O_2 and disease resistance related substances to increase. It is clear that Ca^{2+}, NO and H_2O_2 are coordinately regulated to enhance the resistance of cotton to VD (Figure 8). A 32 kDa flagellin Flg22 extracted from *Pseudomonas syringae* can be used as an elicitor to induce defense responses in tomato cells (Felix et al., 1999). Flg22 can induce a strong defense response in Arabidopsis, including ROS bursts, callose deposition, ethylene production and the expression of defense-related genes (Gómez-Gómez et al., 2000). Peroxidase-dependent oxidation burst plays an important role in the basic resistance of *Arabidopsis thaliana* mediated by Flg22 recognition (Daudi et al., 2012; Liu et al., 2015)。Nonexpresser of PR genes (*NPR1*) play a role in the SA-induced initiation event, which enhances the oxidative burst triggered by Flg22. This is related to the enhancement of callose deposition induced by Flg22 (Yi and

Kwon, 2014). Treatment of non-host plant tobacco with Flg22 will cause a strong defense response, indicating that Flg22 is a PAMP that can act on a variety of plants and has a broad spectrum of resistance (Nicaise, 2009). Therefore, our research not only found a new full-length flagellin gene, but also used upland cotton as research material to explore a new way to induce an immune response in plants and provide a new protein for cotton to resist VD.

Ca^{2+}, NO and H_2O_2 synergistically enhance cotton disease resistance

The relationship among Ca^{2+}, NO and H_2O_2 in plants to regulate plant disease resistance is still unclear. The interaction between the two to regulate the immune response has been reported. NO not only participates in the regulation of plant growth and development, but also participates in the signal transmission of plants in response to various biotic and abiotic stresses (Yan et al., 2007; Sang et al., 2008; Martínez-Medina et al., 2019). Ca^{2+} can activate the NO signal, and can also sense the NO signal. Ca^{2+} participates in the production of NO in tobacco and grapes induced by the elicitor, and the NO produced can in turn cause the increase of intracellular Ca^{2+} concentration (Lamotte et al., 2004; Vandelle et al., 2006; Besson-Bard et al., 2008). These indicate that there is an interaction between Ca^{2+} and NO signals. it was found that an increase in Ca^{2+} concentrations in mesophyll cells was necessary for the cells to produce ROS after pathogen infection(Qiao et al., 2015). The synergistic effect of NO and H_2O_2 triggers the death of hypersensitive cells. The absence of any one of this system cannot induce cell death (Tada et al., 2004)。Transient changes in the content of NO and H_2O_2 can activate a series of physiological responses in plants, and they can interact to regulate the same or related signal pathways to enhance a certain response. H_2O_2 can induce the synthesis and accumulation of NO, and H_2O_2 regulates the NO content by affecting the activity of NO synthase (Zhang et al., 2007)。Similarly, NO can regulate H_2O_2 levels. H_2O_2 is involved in mediating NO-induced resistance of tomato to *Rhizopus Nigricans*(Fan et al., 2008). $Ca^{2+}$and H_2O_2 are involved in upstream of NO production to induce the HR cell death (Qiao et al., 2015). However, it is not clear whether the content of NO negatively adjusts

the content of H_2O_2 . This study found that FLiC binds to membrane receptors and negatively regulates calcium antiporter activity to increase the intracellular influx of Ca^{2+} and induce the production of H_2O_2 and NO; H_2O_2 acts as a signal molecule to induce the production of NO, and NO inhibits the synthesis of H_2O_2; H_2O_2 and NO can induce the production of defense responses. It shows that Ca^{2+}、NO and H_2O_2 are synergistically regulating the resistance of cotton to VD.

GhCAA negatively regulates immune response

No calcium antiporter-related regulatory genes have been found in cotton to negatively regulate calcium ion levels and participate in cotton disease resistance. Bacterial flagellin is the most in-depth study of PAMP (Wang, 2012). After Flg22 processes *Arabidopsis thaliana*, the Ca^{2+} channel and its activation mechanism of stomatal closure in the process of immune signal transduction indicate the specificity of the Ca^{2+} influx mechanism in response to different stresses (Thor et al., 2020). HopZ-Activated Resistance 1 (ZAR1) resistant body acts as a calcium permeable cation channel to trigger plant immunity and cell death (Bi et al., 2021). After Flg22 treatment of *Arabidopsis thaliana*, the tonoplast targeting pump aca4/11 with double gene knockout showed higher basal Ca^{2+} levels and higher amplitude Ca^{2+} signals than wild-type plants (Richard et al., 2020)。It shows that calcium transporter can negatively regulate calcium ion influx. The calcium transporter AtANN1 in *Arabidopsis thaliana* positively regulates the freezing tolerance of *Arabidopsis thaliana* by affecting the influx of calcium signals mediated by low temperature（Liu et al., 2021). Calcium transporter-related regulatory genes have positive regulation and negative regulation of intracellular calcium ion levels to participate in plant defense responses.Through transcriptome analysis, we found for the first time that calcium antiporter activity related regulatory genes negatively regulate calcium levels to enhance cotton resistance to VD.

FLiC-transformed Arabidopsis has enhanced resistance to VD

In order to verify whether the *FLiC* gene-transformed Arabidopsis can improve the resistance to VD. Transformation of flagellin gene can improve rice resistance to bacterial streaks(Wang et al., 2014). The flagellin gene of *Bacillus subtilis* was transferred into rice to increase the resistance to rice blast and the genetically modified rice leaves produced allergic reaction spots (Wang et al., 2015). After the *FLiC* gene Arabidopsis was inoculated with VD, the relative expression levels of SA and JA defense signal-related genes *LOX*, *PAL*, *PR1*, and *VSP* all increased significantly, and the expression levels of *LOX* and *PR1* both reached 40 times. The expression of genes *GSTU3*, *GPX7* and *POD* related to ROS and NO signal pathway changed to varying degrees. Therefore, transgenic Arabidopsis with *FLiC* gene can induce the expression of key genes in SA, JA, ROS and NO signaling pathways to improve plant disease resistance.

Acknowledgments: This work was financially supported by the National Key R & D Program of China (2016YFD0102105) and the Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX20_0582).

Conflict of interest: The authors declare that they have no conflict of interest.

Author Contributions Heng Zhou: responsible for the concepts, design, definitions of intellectual content, literature search, data analysis and manuscript preparation. Yijing Xie: provided assistance for data acquisition and data analysis. Yi Wang: carried out the literature search and data acquisition. Heqin Zhu and Canming Tang: performed manuscript editing and manuscript review. All authors have read and approved the content of the manuscript.

References

Abeles FB, Forrence LE (1970) Temporal and hormonal control of beta-1, 3-glucanase in Phaseolus vulgaris L. Plant Physiol **45**: 395–400

Ali MB, Singh N, Shohael AM, et al. (2006) Phenolics metabolism and lignin synthesis in root suspension cultures of Panax ginseng in response to copper stress[J]. Plant Science **171**: 147-154.

Asai T, Tena G, Plotnikova J, et al. (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature **415**: 977-983

Besson-Bard A, Courtois C, Gauthier A, Dahan J, Dobrowolska G, Jeandroz S, Pugin A, Wendehenne D（2008）Nitric oxide in plants: production and cross-talk with Ca^{2+} signaling. Mol Plant **1**:218-228

Bi G, Su M, Li N, Liang Y, Dang S, Xu J, Hu M, Wang J, Zou M, Deng Y, Li Q, Huang S, Li J, Chai J, He K, Chen YH, Zhou JM (2021) The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. Cell **184**:3528-3541

Bouizgarne B, El-Maarouf-Bouteau H, Frankart C, et al. (2006) Early physiological responses of *Arabidopsis thaliana* cells to fusaric acid: toxic and signalling effects. The New Phytologist **169**: 209-218

Brennan T, Frenkel C (1977) Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiol **59**: 411–416

Bright J, Desikan R, Haneoek J T, Weir1 S, Neill S J (2006) ABA induced NO generation and stomatal elosure in Arabilopsisare dependent on H_2O_2 sythesis .The Plant J **45**:113-122

Buxdorf K, Rahat I, Gafni A, Levy M (2013) The epiphytic fungus Pseudozyma aphidis induces jasmonic acid- and salicylic acid/nonexpressor of PR1-independent local and systemic resistance. Plant Physiol **161**:2014-2022

Chen YL, Huang R, Xiao YM, Lü P, Chen J, Wang XC (2004) Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and H_2O_2. Plant Physiol **136**: 4096–4103

Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP (2012) The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. Plant Cell **24**:275-87

Dickerson DP, Pascholati SF, Hagerman AE, Butler LG, Nicholson RL (1984) Phenylalanine ammonialyase and hydroxy cinnamate: CoA ligase in maize mesocotyls inoculated with Helminthosporium maydis or Helminthosporium carbonum. Physiol Plant Pathol **25**: 111–123

Dixon RA, Harrison MJ, and Lamb CJ (1994) Early events in the activation of plant defense responses. Annual Review of Phytopathology **32**: 479-501

Dong H, Li W, Zhang D, et al. (2003) Differential expression of induced resistance by an aqueous extract of killed Penicillium chrysogenum against Verticillium wilt of cotton[J]. Crop Protection **22**: 129-134.

Durrant WE, Dong X(2004) Systemic acquired resistance. Annual Review of Phytopathology **42**: 185-209

Ebel J, Mithöfer A (1998) Early events in the elicitation of plant defence. Planta **206**: 335-348

Emani C, Garcia JM, Lopata-Finch E, Pozo MJ, Uribe P, KimDJ, Sunilkumar G, Cook DR, Kenerley CM, Rathore KS (2003) Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from Trichoderm virens. Plant Biotechnol J **1**: 321–336

Fan B, Shen L, Liu KL, et al. (2008) Interaction between nitric oxidenand hydrogen peroxide in postharvest tomato resistance response to Rhizopus Nigricans. J Sci Food Agric **88**: 1238-1244

Felix G, DuranJ D, Volko S, et al. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal **18**: 265-276

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Molecular Cell **5**: 1003-1011

Han Q (2014) Screening and identification of cotton anti-VD biocontrol bacteria and research on disease resistance mechanism. Nanjing: Nanjing Agricultural University

Holmes EC, Chen YC, Mudgett MB, Sattely ES. (2021) Arabidopsis UGT76B1 glycosylates N-hydroxy-pipecolic acid and inactivates systemic acquired resistance in tomato. Plant Cell **33**:750-765

Hu HL (2012) Construction of transgenic VD with GFP gene and study on the process of infecting cotton. Nanjing Agricultural University

Jennings JC, Birkhold PC, Mock NM (2001) Induction of defense responses in tobacco by the protein Nep1 from Fusarium oxysporum. The Plant Science **161**: 891-899

Jones JD, Dangl JL (2006) The plant immune system. Nature **444**: 323-329

Kumar R, Barua P, Chakraborty N, Nandi AK (2020) Systemic acquired resistance specific proteome of *Arabidopsis thaliana*. Plant Cell Rep **39**:1549-1563

Kumar V, Parkhi V, Kenerley CM, et al. (2009) Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from Trichoderma virens in response to interaction with Rhizoctonia solani. Planta **230**: 277-291

Lamotte O， Gould K， Lecourieux D， Sequeira-Legrand A， Lebrun -Garcia A， Durner J， Pugin A & Wende-henne D (2004) Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein . Plant Physiol **135**: 516-529

Li B, Fu Y, Jiang D, Xie J, Cheng J, Li G, Hamid MI, Yi X (2010) Cyclic GMP as a second messenger in the nitric oxide-mediated conidiation of the mycoparasite Coniothyrium minitans. Appl Environ Microbiol **76**: 2830–2836

Liu P, Zhang H, Yu B, Xiong L, Xia Y (2015) Proteomic identification of early salicylate- and flg22-responsive redox-sensitive proteins in Arabidopsis. Sci Rep **27**:8625

Liu Q, Ding Y, Shi Y, Ma L, Wang Y, Song C, Wilkins KA, Davies JM, Knight H, Knight MR, Gong Z, Guo Y, Yang S (2021) The calcium transporter ANNEXIN1 mediates cold-induced calcium

signaling and freezing tolerance in plants. EMBO J **40**:e104559

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods **25**: 402–408

Martínez-Medina A, Pescador L, Terrón-Camero LC, Pozo MJ, Romero-Puertas MC (2019) Nitric oxide in plant-fungal interactions. J Exp Bot **70**:4489-4503

Millet Y A, Danna C H, Clay N K, et al. (2010) Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. Plant Cell **22**: 973-990

Naveed ZA, Wei X, Chen J, Mubeen H, Ali GS (2020) The PTI to ETI Continuum in Phytophthora-Plant Interactions. Front Plant Sci **17**: 593965

Nicaise V, Roux M, Zipfel C (2009) Recent advances in PAMP-triggered immunity adainst bacteria: pattern recognition receptors watch over and raise the alarm. Plant Physiol **150**: 1638-1647

Nürnberger T, Brunner F (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Current Opinion in Plant Biology **5**: 318-324

Plazek A, Zur I (2003) Cold-induced plant resistance to necrotrophic pathogens and antioxidant enzyme activities and cell membrane permeability. Plant Sci **164**: 1019–1028

Pomar F, Merino F, Barceló A R (2002) O-4-Linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction. Protoplasma **220**: 17-28

Qiao M, Sun J, Liu N, Sun T, Liu G, Han S, Hou C, Wang D (2015) Changes of Nitric Oxide and Its Relationship with H_2O_2 and Ca^{2+} in Defense Interactions between Wheat and Puccinia Triticina. PLoS One **10**:e0132265

Ren A, Li MJ, Shi L, Mu DS, Jiang AL, Han Q, Zhao MW (2013) Profiling and quantifying differential gene transcription provide insights into ganoderic acid biosynthesis in Ganoderma lucidum in response to methyl jasmonate. PLoS One **8**: e65027

Richard Hilleary, Julio Paez-Valencia, Cullen Vens, Masatsugu Toyota, Simon Gilroy (2020) Tonoplast-localized Ca^{2+} pumps regulate Ca^{2+} signals during pattern-triggered immunity in *Arabidopsis thaliana*. Proceedings of the National Academy of Sciences **117**:18849-18857

Sang J, Jiang M, Lin F, et al. (2008) Nitric oxide reduces hydrogen peroxide accumulation involved in water stress-induced subcellular antioxidant defense in maize plants. J Integr Plant Biol **50**: 231-243

Sudhakar C, Lakshmi A, Giridarakumar S (2001) Changes in the antioxidant enzyme efficacy in two high yielding. Plant Sci **161**: 613– 619

Sun A, Nie S, Xing D (2012) Nitric oxide-mediated maintenance of redox homeostasis contributes to NPR1-dependent plant innate immunity triggered by Lipopolysaccharides. Plant Physiol **160**:1081–1096

TadaY, Mori T, Shinogi T, Yao N, Takahashi S, Betsuyaku S. Sakamoto M, Park P. Nakayashiki H, Tosa Y, and Mayama S (2004) Nitrie Oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defense response of oat. Mol Plant Microbe Interaet **17**: 245-253

Takai R, Isogai A, Takayama S, et al. (2008) Analysis of flagellin perception mediated by Flg22 receptor OsFLS2 in rice. Molecular Plant-Microbe Interactions **21**: 1635-1642

Takai R., Kaneda T, Isogai A, Takayama S, Che FS (2007) A new method of defense response analysis using a transient expression system in rice protoplasts. Biosci Biotechnol Biochem **71**: 590-593

Thomma BP, Nurnberger T, Joosten MH (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell **23**: 4-15

Thor K, Jiang S, Michard E, et al. (2020) The calcium-permeable channel OSCA1.3 regulates plant stomatal immunity.Nature **585**:569-573

Vandelle E， Poinssot B， Wendehenne D， Bentejac M & Pugin A (2006) Integrated signalling network involving calcium, nitric oxide, and active oxygen species but not mitogen - activated protein

kinases in BcPG1-elicited grapevine defenses. Mol Plant Microbe Interact **19**: 429-440

Wang S S, Zhao F Y, Wei X J, et al. (2013) Preliminary study on Flg22-induced defense response in female gametophytes in Saccharina japonica (Phaeophyta). Journal of Applied Phycology **25**: 1215-1223

Wang XN (2014) Study on the infection of cotton leaves and monocots by VD labeled with green fluorescent protein. Nanjing Agricultural University

Wang XY, Chen ZL, Liu WZ, et al. (2015) Breeding of fla transgenic rice and analysis of disease resistance spectrum.Molecular Plant Breeding **13**:61-65

Wang XY, Chen ZY, Liu WZ, et al. (2014) Resistance to Bacterial Leaf Streak of Transgenic Rice with Flagellin Gene.Acta Botanica Northwest **34**:1534-1539

Wang (2012) A preliminary study on the defense response of kelp induced by Flg22. Ocean University of China

Yan J, Tsuichihara N, Etoh T, et al. (2007) Reactive oxygen species and nitric oxide are involved in ABA inhibition of stomatal opening. Plant Cell Environ **30**: 1320-1325

Yano A, Suzuki K, Uchimiya H, Shinshi H (1998) Induction of hypersensitive cell death by a fungal protein in cultures of tobacco cells. Mol Plant Microbe Interact **11**: 115-123

Yi SY, Kwon SY (2014) How does SA signaling link the Flg22 responses? Plant Signal Behav **9**:e972806

Yi SY, Shirasu K, Moon JS, Lee SG, Kwon SY (2014) The activated SA and JA signaling pathways have an influence on flg22-triggered oxidative burst and callose deposition. PLoS One **9**:e88951

Yuan P, Jewell JB, Behera S, Tanaka K, Poovaiah BW (2020) Distinct Molecular Pattern-Induced Calcium Signatures Lead to Different Downstream Transcriptional Regulations via AtSR1/CAMTA3. Int J Mol Sci **21**:8163

Zhang A, Jiang M, Zhang J, Ding H, Xu SH, Hu X (2007) Nitric oxide induced by hydrogen peroxide

mediates abscisic acid-induced activation of mitogen-aetivated protein kinase cascade involved in antioxidant defense in maize leaves. New Phytol **175**: 36-50

Zipfel C, Robatzek S, Nacarro L, et al. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature **428**: 764-767

Parsed Citations

Abeles FB, Forrence LE (1970) Temporal and hormonal control of beta-1, 3-glucanase in Phaseolus vulgaris L. Plant Physiol 45: 395–400

Google Scholar: Author Only Title Only Author and Title

Ali MB, Singh N, Shohael AM, et al. (2006) Phenolics metabolism and lignin synthesis in root suspension cultures of Panax ginseng in response to copper stress[J]. Plant Science 171: 147-154.

Google Scholar: Author Only Title Only Author and Title

Asai T, Tena G, Plotnikova J, et al. (2002) MAP kinase signaling cascade in Arabidopsis innate immunity. Nature 415: 977-983

Google Scholar: Author Only Title Only Author and Title

Besson-Bard A, Courtois C, Gauthier A, Dahan J, Dobrowolska G, Jeandroz S, Pugin A, Wendehenne D(2008) Nitric oxide in plants: production and cross-talk with Ca2+ signaling. Mol Plant 1:218-228

Bi G, Su M, Li N, Liang Y, Dang S, Xu J, Hu M, Wang J, Zou M, Deng Y, Li Q, Huang S, Li J, Chai J, He K, Chen YH, Zhou JM (2021) The ZAR1 resistosome is a calcium-permeable channel triggering plant immune signaling. Cell 184:3528-3541

Google Scholar: Author Only Title Only Author and Title

Bouizgarne B, El-Maarouf-Bouteau H, Frankart C, et al. (2006) Early physiological responses of Arabidopsis thaliana cells to fusaric acid: toxic and signalling effects. The New Phytologist 169: 209-218

Google Scholar: Author Only Title Only Author and Title

Brennan T, Frenkel C (1977) Involvement of hydrogen peroxide in the regulation of senescence in pear. Plant Physiol 59: 411–416

Google Scholar: Author Only Title Only Author and Title

Bright J, Desikan R, Hancock J T, Weir1 S, Neill S J (2006) ABA induced NO generation and stomatal closure in Arabilopsisare dependent on H2O2 sythesis .The Plant J 45:113-122

Google Scholar: Author Only Title Only Author and Title

Buxdorf K, Rahat I, Gafni A, Levy M (2013) The epiphytic fungus Pseudozyma aphidis induces jasmonic acid- and salicylic acid/nonexpressor of PR1-independent local and systemic resistance. Plant Physiol 161:2014-2022

Google Scholar: Author Only Title Only Author and Title

Chen YL, Huang R, Xiao YM, Lü P, Chen J, Wang XC (2004) Extracellular calmodulin-induced stomatal closure is mediated by heterotrimeric G protein and H2O2. Plant Physiol 136: 4096–4103

Google Scholar: Author Only Title Only Author and Title

Daudi A, Cheng Z, O'Brien JA, Mammarella N, Khan S, Ausubel FM, Bolwell GP (2012) The apoplastic oxidative burst peroxidase in Arabidopsis is a major component of pattern-triggered immunity. Plant Cell 24:275-87

Google Scholar: Author Only Title Only Author and Title

Dickerson DP, Pascholati SF, Hagerman AE, Butler LG, Nicholson RL (1984) Phenylalanine ammonialyase and hydroxy cinnamate: CoA ligase in maize mesocotyls inoculated with Helminthosporium maydis or Helminthosporium carbonum. Physiol Plant Pathol 25: 111–123

Google Scholar: Author Only Title Only Author and Title

Dixon RA, Harrison MJ, and Lamb CJ (1994) Early events in the activation of plant defense responses. Annual Review of Phytopathology 32: 479-501

Google Scholar: Author Only Title Only Author and Title

Dong H, Li W, Zhang D, et al. (2003) Differential expression of induced resistance by an aqueous extract of killed Penicillium chrysogenum against Verticillium wilt of cotton[J]. Crop Protection 22: 129-134.

Google Scholar: Author Only Title Only Author and Title

Durrant WE, Dong X(2004) Systemic acquired resistance. Annual Review of Phytopathology 42: 185-209

Google Scholar: Author Only Title Only Author and Title

Ebel J, Mithöfer A (1998) Early events in the elicitation of plant defence. Planta 206: 335-348

Google Scholar: Author Only Title Only Author and Title

Emani C, Garcia JM, Lopata-Finch E, Pozo MJ, Uribe P, KimDJ, Sunilkumar G, Cook DR, Kenerley CM, Rathore KS (2003) Enhanced fungal resistance in transgenic cotton expressing an endochitinase gene from Trichoderm virens. Plant Biotechnol J 1: 321–336

Google Scholar: Author Only Title Only Author and Title

Fan B, Shen L, Liu KL, et al. (2008) Interaction between nitric oxidenand hydrogen peroxide in postharvest tomato resistance response to Rhizopus Nigricans. J Sci Food Agric 88: 1238-1244

Google Scholar: Author Only Title Only Author and Title

Felix G, DuranJ D, Volko S, et al. (1999) Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant Journal 18: 265-276

Google Scholar: Author Only Title Only Author and Title

Gómez-Gómez L, Boller T (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in Arabidopsis. Molecular Cell 5: 1003-1011
Google Scholar: Author Only Title Only Author and Title

Han Q (2014) Screening and identification of cotton anti-VD biocontrol bacteria and research on disease resistance mechanism. Nanjing: Nanjing Agricultural University
Google Scholar: Author Only Title Only Author and Title

Holmes EC, Chen YC, Mudgett MB, Sattely ES. (2021) Arabidopsis UGT76B1 glycosylates N-hydroxy-pipecolic acid and inactivates systemic acquired resistance in tomato. Plant Cell 33:750-765
Google Scholar: Author Only Title Only Author and Title

Hu HL (2012) Construction of transgenic VD with GFP gene and study on the process of infecting cotton. Nanjing Agricultural University
Google Scholar: Author Only Title Only Author and Title

Jennings JC, Birkhold PC, Mock NM (2001) Induction of defense responses in tobacco by the protein Nep1 from Fusarium oxysporum. The Plant Science 161: 891-899
Google Scholar: Author Only Title Only Author and Title

Jones JD, Dangl JL (2006) The plant immune system. Nature 444: 323-329
Google Scholar: Author Only Title Only Author and Title

Kumar R, Barua P, Chakraborty N, Nandi AK (2020) Systemic acquired resistance specific proteome of Arabidopsis thaliana. Plant Cell Rep 39:1549-1563
Google Scholar: Author Only Title Only Author and Title

Kumar V, Parkhi V, Kenerley CM, et al. (2009) Defense-related gene expression and enzyme activities in transgenic cotton plants expressing an endochitinase gene from Trichoderma virens in response to interaction with Rhizoctonia solani. Planta 230: 277-291
Google Scholar: Author Only Title Only Author and Title

Lamotte O, Gould K, Lecourieux D, Sequeira-Legrand A, Lebrun -Garcia A, Durner J, Pugin A & Wende-henne D (2004) Analysis of nitric oxide signaling functions in tobacco cells challenged by the elicitor cryptogein . Plant Physiol 135: 516-529
Google Scholar: Author Only Title Only Author and Title

Li B, Fu Y, Jiang D, Xie J, Cheng J, Li G, Hamid MI, Yi X (2010) Cyclic GMP as a second messenger in the nitric oxide-mediated conidiation of the mycoparasite Coniothyrium minitans. Appl Environ Microbiol 76: 2830–2836
Google Scholar: Author Only Title Only Author and Title

Liu P, Zhang H, Yu B, Xiong L, Xia Y (2015) Proteomic identification of early salicylate- and flg22-responsive redox-sensitive proteins in Arabidopsis. Sci Rep 27:8625
Google Scholar: Author Only Title Only Author and Title

Liu Q, Ding Y, Shi Y, Ma L, Wang Y, Song C, Wilkins KA, Davies JM, Knight H, Knight MR, Gong Z, Guo Y, Yang S (2021) The calcium transporter ANNEXIN1 mediates cold-induced calcium signaling and freezing tolerance in plants. EMBO J 40:e104559
Google Scholar: Author Only Title Only Author and Title

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT Method. Methods 25: 402–408
Google Scholar: Author Only Title Only Author and Title

Martínez-Medina A, Pescador L, Terrón-Camero LC, Pozo MJ, Romero-Puertas MC (2019) Nitric oxide in plant-fungal interactions. J Exp Bot 70:4489-4503
Google Scholar: Author Only Title Only Author and Title

Millet Y A, Danna C H, Clay N K, et al. (2010) Innate immune responses activated in Arabidopsis roots by microbe-associated molecular patterns. Plant Cell 22: 973-990
Google Scholar: Author Only Title Only Author and Title

Naveed ZA, Wei X, Chen J, Mubeen H, Ali GS (2020) The PTI to ETI Continuum in Phytophthora-Plant Interactions. Front Plant Sci 17: 593965
Google Scholar: Author Only Title Only Author and Title

Nicaise V, Roux M, Zipfel C (2009) Recent advances in PAMP-triggered immunity adainst bacteria: pattern recognition receptors watch over and raise the alarm. Plant Physiol 150: 1638-1647
Google Scholar: Author Only Title Only Author and Title

Nürnberger T, Brunner F (2002) Innate immunity in plants and animals: emerging parallels between the recognition of general elicitors and pathogen-associated molecular patterns. Current Opinion in Plant Biology 5: 318-324
Google Scholar: Author Only Title Only Author and Title

Plazek A, Zur I (2003) Cold-induced plant resistance to necrotrophic pathogens and antioxidant enzyme activities and cell membrane permeability. Plant Sci 164: 1019–1028
Google Scholar: Author Only Title Only Author and Title

Pomar F, Merino F, Barceló A R (2002) O-4-Linked coniferyl and sinapyl aldehydes in lignifying cell walls are the main targets of the Wiesner (phloroglucinol-HCl) reaction. Protoplasma 220: 17-28
Google Scholar: Author Only Title Only Author and Title

Qiao M, Sun J, Liu N, Sun T, Liu G, Han S, Hou C, Wang D (2015) Changes of Nitric Oxide and Its Relationship with H2O2 and Ca2+ in Defense Interactions between Wheat and Puccinia Triticina. PLoS One 10:e0132265
Google Scholar: Author Only Title Only Author and Title

Ren A, Li MJ, Shi L, Mu DS, Jiang AL, Han Q, Zhao MW (2013) Profiling and quantifying differential gene transcription provide insights into ganoderic acid biosynthesis in Ganoderma lucidum in response to methyl jasmonate. PLoS One 8: e65027
Google Scholar: Author Only Title Only Author and Title

Richard Hilleary, Julio Paez-Valencia, Cullen Vens, Masatsugu Toyota, Simon Gilroy (2020) Tonoplast-localized Ca2+ pumps regulate Ca2+ signals during pattern-triggered immunity in Arabidopsis thaliana. Proceedings of the National Academy of Sciences 117:18849-18857
Google Scholar: Author Only Title Only Author and Title

Sang J, Jiang M, Lin F, et al. (2008) Nitric oxide reduces hydrogen peroxide accumulation involved in water stress-induced subcellular antioxidant defense in maize plants. J Integr Plant Biol 50: 231-243
Google Scholar: Author Only Title Only Author and Title

Sudhakar C, Lakshmi A, Giridarakumar S (2001) Changes in the antioxidant enzyme efficacy in two high yielding. Plant Sci 161: 613– 619
Google Scholar: Author Only Title Only Author and Title

Sun A, Nie S, Xing D (2012) Nitric oxide-mediated maintenance of redox homeostasis contributes to NPR1-dependent plant innate immunity triggered by Lipopolysaccharides. Plant Physiol 160:1081–1096
Google Scholar: Author Only Title Only Author and Title

TadaY, Mori T, Shinogi T, Yao N, Takahashi S, Betsuyaku S. Sakamoto M, Park P. Nakayashiki H, Tosa Y, and Mayama S (2004) Nitrie Oxide and reactive oxygen species do not elicit hypersensitive cell death but induce apoptosis in the adjacent cells during the defense response of oat. Mol Plant Microbe Interaet 17: 245-253
Google Scholar: Author Only Title Only Author and Title

Takai R, Isogai A, Takayama S, et al. (2008) Analysis of flagellin perception mediated by Flg22 receptor OsFLS2 in rice. Molecular Plant-Microbe Interactions 21: 1635-1642
Google Scholar: Author Only Title Only Author and Title

Takai R., Kaneda T, Isogai A, Takayama S, Che FS (2007) A new method of defense response analysis using a transient expression system in rice protoplasts. Biosci Biotechnol Biochem 71: 590-593
Google Scholar: Author Only Title Only Author and Title

Thomma BP, Nurnberger T, Joosten MH (2011) Of PAMPs and effectors: the blurred PTI-ETI dichotomy. Plant Cell 23: 4-15
Google Scholar: Author Only Title Only Author and Title

Thor K, Jiang S, Michard E, et al. (2020) The calcium-permeable channel OSCA1.3 regulates plant stomatal immunity.Nature 585:569-573
Google Scholar: Author Only Title Only Author and Title

Vandelle E, Poinssot B, Wendehenne D, Bentejac M & Pugin A (2006) Integrated signalling network involving calcium, nitric oxide, and active oxygen species but not mitogen - activated protein kinases in BcPG1-elicited grapevine defenses. Mol Plant Microbe Interact 19: 429-440
Google Scholar: Author Only Title Only Author and Title

Wang S S, Zhao F Y, Wei X J, et al. (2013) Preliminary study on Flg22-induced defense response in female gametophytes in Saccharina japonica (Phaeophyta). Journal of Applied Phycology 25: 1215-1223
Google Scholar: Author Only Title Only Author and Title

Wang XN (2014) Study on the infection of cotton leaves and monocots by VD labeled with green fluorescent protein. Nanjing Agricultural University
Google Scholar: Author Only Title Only Author and Title

Wang XY, Chen ZL, Liu WZ, et al. (2015) Breeding of fla transgenic rice and analysis of disease resistance spectrum.Molecular Plant Breeding 13:61-65
Google Scholar: Author Only Title Only Author and Title

Wang XY, Chen ZY, Liu WZ, et al. (2014) Resistance to Bacterial Leaf Streak of Transgenic Rice with Flagellin Gene.Acta Botanica Northwest 34:1534-1539
Google Scholar: Author Only Title Only Author and Title

Wang (2012) A preliminary study on the defense response of kelp induced by Flg22. Ocean University of China
Google Scholar: Author Only Title Only Author and Title

Yan J, Tsuichihara N, Etoh T, et al. (2007) Reactive oxygen species and nitric oxide are involved in ABA inhibition of stomatal opening. Plant Cell Environ 30: 1320-1325

Google Scholar: Author Only Title Only Author and Title

Yano A, Suzuki K, Uchimiya H, Shinshi H (1998) Induction of hypersensitive cell death by a fungal protein in cultures of tobacco cells. Mol Plant Microbe Interact 11: 115-123
Google Scholar: Author Only Title Only Author and Title

Yi SY, Kwon SY (2014) How does SA signaling link the Flg22 responses? Plant Signal Behav 9:e972806

Yi SY, Shirasu K, Moon JS, Lee SG, Kwon SY (2014) The activated SA and JA signaling pathways have an influence on flg22-triggered oxidative burst and callose deposition. PLoS One 9:e88951
Google Scholar: Author Only Title Only Author and Title

Yuan P, Jewell JB, Behera S, Tanaka K, Poovaiah BW (2020) Distinct Molecular Pattern-Induced Calcium Signatures Lead to Different Downstream Transcriptional Regulations via AtSR1/CAMTA3. Int J Mol Sci 21:8163
Google Scholar: Author Only Title Only Author and Title

Zhang A, Jiang M, Zhang J, Ding H, Xu SH, Hu X (2007) Nitric oxide induced by hydrogen peroxide mediates abscisic acid-induced activation of mitogen-aetivated protein kinase cascade involved in antioxidant defense in maize leaves. New Phytol 175: 36-50
Google Scholar: Author Only Title Only Author and Title

Zipfel C, Robatzek S, Nacarro L, et al. (2004) Bacterial disease resistance in Arabidopsis through flagellin perception. Nature 428: 764-767
Google Scholar: Author Only Title Only Author and Title