The protein elicitor Hrip1 enhances resistance to insects and early bolting and flowering in Arabidopsis thaliana

Xin-yue Miao1, Hong-pan Qu1, Ya-lei Han2, Cong-fen He1, De-wen Qiu3, Zhi-wei Cheng1,3*

1 Beijing Key Laboratory of Plants Resource Research and Development, School of Sciences, Beijing Technology and Business University, Beijing, China, 2 Aerospace Center Hospital, Cardiovascular Department, Beijing, China, 3 State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China

* zw_cheng1979@163.com

Abstract

The elicitor Hrip1 isolated from necrotrophic fungus Alternaria tenuissima, could induce systemic acquired resistance in tobacco to enhance resistance to tobacco mosaic virus. In the present study, we found that the transgenic lines of Hrip1-overexpression in wild type (WT) Arabidopsis thaliana were more resistant to Spodoptera exigua and were early bolting and flowering than the WT. A profiling of transcription assay using digital gene expression profiling was used for transgenic and WT Arabidopsis thaliana. Differentially expressed genes including 40 upregulated and three downregulated genes were identified. In transgenic lines of Hrip1-overexpression, three genes related to jasmonate (JA) biosynthesis were significantly upregulated, and the JA level was found to be higher than WT. Two GDSL family members (GLIP1 and GLIP4) and pathogen-related gene, which participated in pathogen defense action, were upregulated in the transgenic line of Hrip1-overexpression. Thus, Hrip1 is involved in affecting the flower bolting time and regulating endogenous JA biosynthesis and regulatory network to enhance resistance to insect.

Introduction

Plants are constantly adapting to environmental changes of abiotic and biotic stresses, because of their evolved ability to appropriately respond to changes in stressful conditions [1–4]. The innate immune systems of plants detect the invasion signals’ response to biotic stress and initiate the regulation of plant growth and defense [3]. Some effectors including plant phytohormones, pathogenesis-related protein (PR), and GDSL-type esterases/lipases play critical roles to mediate these regulatory signaling networks.

Phytohormones are produced in the plants and they control the behavior of growth and defense in plants. Jasmonates (JAs), a set of fatty acid-derived signaling molecules, are involved in many developmental processes, such as root growth, tuberization, tendril coiling, pollen development, and seed germination [5]. They are also involved in plant response to...
environmental stresses, such as water deficit, ozone exposure, pathogen infection, wounding, and pest attack [6–16]. The biosynthesis of JA in plants starts from α-linolenic acid (18:3) in chloroplast membranes [15, 17]. Subsequently, α-linolenic acid is catalyzed by a sequence of biosynthesis enzymes, including 13-lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC), to produce OPDA in plastid [15, 17–20]. OPDA was imported into peroxisome by PXA1, an ATP-binding cassette transporter, and catalyzed by a series of enzymes that included OPR3 (OPDA reductase), OPCL1 (OPC-8:0 CoA ligase), five acyl-CoA oxidases (ACX1–ACX5), and MFP (multifunctional protein) to yield (+)-7-iso-JA [15, 21, 22]. In cytosol, (+)-7-iso-JA is conjugated with the amino acid isoleucine (Ile) to synthesize JA-Ile, which is known as a bioactive form of JAs [23]. The JA receptor coronatine insensitive 1 (COI1) [24–26] recuits JA-ZIM-domain proteins (JAZs) [27, 28] for ubiquitination and degradation by 26S proteasome, when JA-Ile is recognized by COI1 in plants [27–29]. The transcription factors (such as MYC2) which are repressed by JAZs, are released to launch the expression of JA-response gene and initiate JA-regulated functions including JA-inhibitory root growth [30], plant fertility [30, 31], and resistance against pathogens and insects [32].

The plants have two modes of immunity: pathogen-associated molecular pattern-triggered immunity (PTI) and effector-triggered immunity (ETI). The pathogen-associated molecular patterns derived from the components of the structures microorganisms or pathogens, which are recognized by pattern recognition receptors of plants, induce the further activation of PTI [33]. The secret effector protein of microbial pathogens, which are distinguished by resistance (R) protein, initiates the activation of the ETI [34]. To date, many PR proteins that directly interact with identified pathogen effector proteins have been widely recognized [35]. The PR proteins are absent or present at low concentrations in healthy plants, but they are induced and accumulated at protein level during pathological infection (such as fungi, bacteria, viruses, insects, and herbivores) and related situations including the application of phytohormones and wounding [36–39]. Furthermore, PR proteins are low-molecular-weight proteins (5–75 kDa), which are thermostable, resistant to proteases, and able to dissolve at low pH (<3) [36, 39]. Presently, PR proteins are divided into 17 families that are based on protein sequence features, enzymatic activities, and other biological functions [36].

Some PR proteins (PR-6, PR-12, PR-13, and PR-14) with a molecular size below 10 kDa are defined as PR peptides or antimicrobial peptides, which are cysteine-rich molecules and play an important role in host resistance against microbial pathogens and pests in plants [36, 39, 40]. PR-6 peptides belong to a subclass of serine proteinase inhibitors (PIs), which are similar to "tomato/potato inhibitor I" [36, 41]. PIs are usually able to bind proteinases and control proteinase activity; they play a range of roles in defense, including weakening the ability of an attacker to (i) obtain lytic enzymes to resist fungi [42], (ii) disturb viral replication cycles [43], and (iii) act against the digestive protease used by nematodes and insects [44–46]. Numerous articles have reported that transgenic plants with heterologous PI gene overexpression enhance the resistance of plants to insect attack [47]. *Arabidopsis* has six PI genes encoding the PR-6-type protein, and the calculated isoelectric point (pI) of the predictive protein ranges from 4.6 to 11.3 [36]. A PR-6 protein (At2g38870) is overexpressed in *Arabidopsis*, and transgenic plants have the ability to enhanced resistance to *Botrytis cinerea* [48].

GDSL-type esterases/lipases, a type of lipid hydrolysis enzyme, are common in bacteria and plants including rice, maize, and *Arabidopsis*; they have multifunctional properties [49]. In *Arabidopsis*, 105 GDSL-type esterase/lipase (AtGELP) genes have been identified; these genes were divided into four clades depending on their functions related to morphological development, abiotic stress response, secondary metabolism, and pathogen defense [49]. In clade IIIa...
of AtGELP, several proteins exhibit functions related to biotic responses, such as AtGELP97 (GLIP1), ATGELP20 (GLIP2), AtGELP63 (ESM), and BrSil [49]. GLIP1 [50] regulates plant immunity via the ethylene signaling pathway, which upregulates the expression of ETHYLENE RESPONSE FACTOR1 (ERF1) and represses the expression of ETHYLENE INSENSITIVE 3 (EIN3) [51, 52]. Furthermore, the overexpression of GLIP1 in Arabidopsis increases the expression of SALICYLIC ACID INDUCTION-DEFICIENT2 (SID2), a salicylic acid biosynthesis gene [51]. The recombinant GLIP2 protein exhibits lipase and antimicrobial activities, resulting in resistance to Erwinia carotovora (Pectobacterium carotovora). The Arabidopsis T-DNA insertion mutant of glip2 is more susceptible to E. carotovora and manifests enhanced auxin response, which indicates that GLIP2 negatively regulates auxin signals to participate in pathogen defense in plants [53].

Protein elicitors, such as Harpin protein, flagellin, elicitin, activator, and glycoprotein [54, 55], are involved in both biotic and abiotic stress responses and trigger systemic acquired resistance (SAR) in plants infected by pathogens [56–58]. The Harpin protein has been isolated from Erwinia amylovora, and it is reported to trigger pathogen resistance in plants [59]. Transgenic tobacco plants with Harpin overexpressing exhibited phenotype resistance to pathogen infection [60, 61], and the expression of some genes is induced via the defense-related signaling pathway (generating nitric oxide [NO] and JA signaling pathway) [62]. MoHrip1 was purified from the extraction of Magnaporthe oryzae, which triggers the tobacco defense response, induces the expression of PR genes, and enhances systemic resistance to M. oryzae in rice seedlings [63]. The protein elicitor PevD1 isolated from Verticillium dahliae can enhance resistance to pathogen infection in plants, metabolite deposition, and cell wall modification [64, 65]. Transgenic lines with PevD1 overexpressing are highly resistant to B. cinerea and Pseudomonas syringae pv. Tomato DC3000 [66].

Hrip1 is a novel elicitor that was purified from the necrotrophic fungus Alternaria tenuissima [67]. The protein Hrip1 comprises 163 amino acid residues, which are encoded by a 495 bp open reading frame (GenBank accession number HQ713431). In our previous work, the results indicated that Hrip1 triggers the hypersensitive response, generates necrotic lesions, and induces SAR in tobacco leaves that were inoculated with mosaic virus [67]. Furthermore, the transgenic lines of Hrip1 in Arabidopsis were more resistant to stresses and exerted a significant effect on plant height, silique length, and plant dry weight under the conditions of salt and drought compared with the WT [68]. In this study, Hrip1 was transferred into the Arabidopsis genome by Agrobacterium tumefaciens. We investigated the bolting time and pathogen response phenotypes in transgenic plants distinguished from the WT. We also used high-throughput RNA-seq digital gene expression profiling (DGE) to explore the significant differential expression of genes in transgenic lines compared with WT. Our results furnished reliable information to facilitate our understanding of functions and mechanisms, including how Hrip1 regulated pathogen resistance and development in plants.

Materials and methods

Plant growth environment

Seeds including WT Arabidopsis thaliana (Col-0) and transgenic plants (35:Hrip1) were surface-sterilized with 10% bleach plus 0.1% tween-20 for 15 min, washed using sterile water for more than five times, and placed on growth medium (Murashige and Skoog, MS). The plates were transferred in a growth chamber (Percival AR800, USA) for 7 days until the seeds were germinated contained two euphylla. All plantlets grown in nutrient soil were grown in the growth chamber with 16 hrs light/8 hrs dark cycle at 22˚C and 60% relative humidity.
Vector construction and plant transformation

A truncated Hrip1 gene without a signal peptide was amplified with its special primer and cloned into the Nco I and BstE II sites of the pCAMBIA1301 vector. This recombinant vector was transformed into A. tumefaciens strain LBA4404. The floral dip method was employed to complete the Agrobacterium-mediated transformation of Col-0 [69]. The T1 seeds were collected from transformed Col-0 (T0 generation) and then generated in screening medium supplemented with 25 μg/mL hygromycin. Hygromycin-resistance T1 seedlings were transplanted into soil to harvest the T2 seeds. The T2 seeds were screened using hygromycin and selected in accordance with Mendel’s law. Six independent T3 transgenic lines were homozygous and used in our experiments.

Real-time PCR

Total RNA was isolated using TRIZol reagent (Invitrogen, Carlsbad, California, CA, USA) and reverse-transcribed into cDNA using the kit (TransScript All-in-One First-Strand cDNA Synthesis SuperMix, TransGen Biotech) according to the manufacturer’s protocol. Quantitative RT-PCR (qRT-PCR) was performed using Power SYBR Green Master Mix (Invitrogen) and an iQ5 real-time PCR instrument (Bio-Rad Laboratories). Reaction system and amplification protocol referred to the manufacturer’s introductions. ACTIN2 and ACTIN7 were used as normalizing controls. All primers were designed by online-tools Universal ProbeLibrary Assay Design Center (Roche, https://lifescience.roche.com/en_cn/brands/universal-probe-library.html#assay-design-center) for qRT-PCR. The result of each qRT-PCR reaction was repeated at least three times.

Western blot analysis

The leaves were collected and ground into powder in liquid nitrogen after the plants were grown in a growth chamber for 10 days. The powder was mixed well with extraction buffer (20 mM Tris-HCl pH 8.0, 20 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF], and 1×ProteinSafe Protease Inhibitor Cocktail [100×] [TransGen Biotech]) and incubated for 30 min under ice block. The mixer was centrifuged at 20,000 g for 30 min at 4˚C, and the supernatant was retained and used as crude protein. The boiled protein samples were electrophoresed using 15% SDS-PAGE and transferred onto PVDF membranes by the wetting transfer method. Immunoblotting was conducted using Anti-Hrip1 rabbit polyclonal antibodies (prepared by our lab) [67] or Anti-Actin Mouse Monoclonal Antibodies (TransGen Biotech, CAT: HC201) and ProteinFind Goat Anti-Rabbit IgG (H+L) with HRP conjugate (TransGen Biotech, CAT: HS101) or ProteinFind Goat Anti-Mouse IgG (H+L) with HRP conjugate (TransGen Biotech, CAT: HS201). The specific protein signals after immunoblotting were detected using photographic film under routine operation. These results were repeated more than three times.

Detection of H2O2 assay

Detection of H2O2 assay was performed as previously described [70]. Seeds including WT Arabidopsis thaliana (Col-0) and transgenic plants (35:Hrip1) were surface-sterilized, and placed on MS medium to germination and growth. The leaves were detached from Arabidopsis which were grown MS medium for 7 days to 14 days (the days started from seed germination), was infiltrated with a solution of 1 mg/L diaminobenzidine dissolved in water. Leaves were placed on 3MM filter paper, then fixed with a solution of ethanol/lactic acid:glycerol (3:1:1, v/v/v), washed with 75, 50, and 25% ethanol, equilibrated with water, and photographed [70].
Insect defense assay

Insect defense assay was performed as previously described [71]. The second-instar larvae of *S. exigua* were purchased from Henan Jiyuan Baiyun Industry Co., Ltd.. The larvae were transferred in plastic Petri dishes (150 mm) containing 1.5% phytogel and fed with 30 rosette leaves (these leaves were replaced with fresh leaves every 2 days from 2-week-old plants growing on nutrient soil) from WT or *Hrip1* transgenic plants. The larvae were fed for 6 days in the plastic Petri dish [71]. The larvae were weighed at 6 days after feeding using ten independent replicates. Student's *t*-test was employed to determine statistically significant differences compared with the WT (*p* < 0.05; **p** < 0.01). These results were repeated at least three times.

Bolting assay of transgenic lines

The seedlings of each genotype were germinated on MS medium for 1 week and then transferred into nutrient soil. After WT and transgenic plants were grown for 10 days under a growth chamber, the bolting phenotype was observed and recorded daily. Flowering time was measured by scoring the number of rosette leaves at the time of bolting. The data measured from 30 independent lines were analyzed with Student’s *t*-test, and asterisks indicate statistically significant differences compared with WT (*p* < 0.05; **p** < 0.01). All the plants were grown in the same growth chamber under the same conditions (16 hrs light/8 hrs dark cycle at 22 °C with 60% relative humidity). These results were repeated more than three times.

RNA-sequence with DGE

The *Arabidopsis* leaves (before bolting of plants grown for two-week-old on nutrient soil) were ground into a powder under liquid nitrogen, and total RNA was isolated from powders of plants using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the protocol provided by the manufacturer. The quality of total RNA was monitored on 1% agarose gels and checked by NanoPhotometer Spectrophotometer (IMPLEN, CA, USA). The quantity of total RNA of each samples was measured using Qubit RNA Assay Kit in a Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was evaluated by RNA Nano 6000 Assay Kit in a Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

Approximately 3 μg of RNA from each sample was used to construct DGE libraries, which were made by NEBNext Ultra RNA Library Prep kit for Illumina (NEB) following the manufacturer’s recommendations. The preferential cDNA fragment (library fragments) whose length was 150–200 bp was collected using an AMPure XP system (Beckman Coulter, Beverly, CA, USA). The library fragments were amplified by PCR with Phusion High-Fidelity DNA polymerase, universal PCR primers, and an index (X) primer. The PCR products were purified (AMPure XP system), and library quality was appraised using an Agilent Bioanalyzer 2100 system. The index-coded samples were clustered on a cBot Cluster Generation System using a TruSeq PE Cluster Kit v3-cBot-HS (Illumina) in accordance with the manufacturer’s instructions. The sequence of each library fragments was read by an Illumina HiSeq 2000 platform.

Analysis of DGE sequence results

Raw reads processed by removing reads containing adapters, reads containing poly-N, low quality reads, and cleaned reads were prepared for further analysis. Index of the *Arabidopsis* genome established by Bowtie v2.0.6 and single-end reads were matched to the reference genome using TopHat v2.0.9. The Reads Per Kilobase of exon model per million (RPKM) of each gene was calculated by HTSeq v0.6.1, which was based on the length of the gene and the reads count mapped to this gene [72]. The DESeq R package was used to distinguish the
differential expression between the transgenic lines and WT [73]. *P*-values were adjusted using the Benjamini and Hochberg Method to assess the false results of significantly differential expression (adjusted *P*<0.01) [74]. The GOseq R package was used to analyze gene ontology (GO) enrichment of differentially expressed genes (DEGs) [75]. The DEGs were corrected at *P* < 0.05 to build GO terms. KOBAS software was used to test the statistical enrichment of differential expression in the KEGG pathway [76]. The raw data of RNA-seq were uploaded in the Sequence Read Archive database at the National Center for Biotechnology Information under access number.

**Extraction and determination of JA**

To measure the JA content of each genotype, a previous method was employed with slight modification [77]. In brief, 0.5 g of two-week-old leaf tissue (growing on nutrient soil) of each genotype was ground into powder using liquid nitrogen. The powders were added with internal standards (2H5-JA 95 pmol), extracted with methanol, and incubated at ~20°C overnight. The mixture was centrifuged at 4°C for 15 min at 20,000 g. The supernatant was maintained, dried under nitrogen gas, and dissolved in 1 mL of ammonia solution (5%). The Oasis MAX SPE column was used to purify the crude extract. The purified eluent through the column was dried with nitrogen gas and finally dissolved in 200 μL of water/methanol (20:80, v/v) for UPLC-MS/MS analysis (Waters, Milford, MA, USA). The parameters of UPLC-MS/MS referred to a previously described method [77, 78]. These results were repeated more than three times.

**Results**

**Construction of Hrip1 overexpressing transgenic plants**

The gene sequence of *Hrip1* without a signal peptide was cloned and fused into the *Nco* I and *BstE* II sites of the pCAMBIA1301 vector with the CaMV35s promoter and transferred into *Col-0*. More than 20 positive transgenic seedlings of (T0) were screened as hygromycin-resistant. Six of the positive transgenic seeds (T1) were hygromycin-resistant and showed phenotypic ratios that corresponded to the Mendelian ratio of 3:1. All homozygote seeds of the T2 and T3 were selected and confirmed using hygromycin resistance. We checked the expression level of *Hrip1* in the independent T3 homozygote seedlings (35S:*Hrip1-1* to 35S:*Hrip1-6*) with qRT-PCR. The results showed that *Hrip1* was successfully overexpressed in *Col-0* (Fig 1A).

Subsequently, we detected the Hrip1 protein expression level in three individual transgenic lines (35S:*Hrip1-1*, 35S:*Hrip1-2*, and 35S:*Hrip1-3*) using the specific antibody of Hrip1 (Anti-Hrip1). As shown in Fig 1B, the protein Hrip1 was highly expressed in *Col-0*, and the expression pattern was in accordance with the results of the *Hrip1* RNA expression pattern in qRT-PCR. To eliminate the phenotypic characteristic that was caused by transplant, three independent *Hrip1*-overexpression lines (35S:*Hrip1-1*, 35S:*Hrip1-2*, and 35S:*Hrip1-3*) with High, middle and low expression levels were selected for further analysis.

**Hrip1 accelerated bolting in Arabidopsis under long day treatment**

The lines were transferred to soil to grow after all genotype seeds were germinated on MS medium for 7 days under long day conditions (16 hrs light/8 hrs night). The timing of floral induction was determined by counting the number of rosette leaves at the time of bolting (S2 Fig). The *Hrip1* transgenic plants initiated bolting at approximately 18 days after they were transplanted on nutrition soil, which was ahead of the flower bolting time compared with WT.
lines (approximately 24 days after growing on nutrient soil) (Fig 2A and 2B). The Hrip1 transgenic plants were ahead by about 6 days to bolting compared with control WT.

FLOWERING LOCUS T (FT) is a mobile protein translated in leaves, and it interacts with the bZIP transcription factor FD in shoot apical meristem (SAM), and resulted in the activation of the floral meristem genes [79–81]. To explain why the bolting time of the Hrip1 transgenic line was shorter than that of WT, the expression level of FT was examined via qRT-PCR using the special primers listed in S5 Table. The results showed that the expression level of FT was significantly upregulated in the leaves of the Hrip1 transgenic lines compared with that in control WT prior to bolting in plants (Fig 2C).

**Hrip1 overexpression in Arabidopsis enhanced the resistance to S. exigua**

Hrip1 protein infiltrated in tobacco leaves and induced SAR against tobacco mosaic virus (TMV) [67], and stimulated oxidative burst and the H$_2$O$_2$ accumulation at early time of application [70]. As show in S1 Fig, the H$_2$O$_2$ level in leaves of Hrip1-overexpression transgenic plants and WT was not significantly different. Furthermore, Hrip1 can elevate the expression level of LOX2, which is a JA synthesis-related gene, after Hrip1 is infiltrated in tobacco leaves;
therefore, we speculated that Hrip1 may be resistant to pests [67, 82]. The second instar larvae of S. exigua were fed with rosette leaves (after the plants grew for approximately two weeks on nutrient soil) for 6 days, and the leaves were replaced every 2 days. After growth for 6 days, the weight of larvae fed with leaves of 35S::Hrip1-1, 35S::Hrip1-2, and 35S::Hrip1-3 transgenic lines significantly decreased by 27.30%, 37.39%, and 39.76% compared with the weight of larvae fed with leaves of WT (Fig 3A and 3B). Taken together, Hrip1 could enhance resistance to S. exigua in Arabidopsis.

Analysis of RNA-sequencing results

Three biological replicates of WT (C1, C2, and C3) and transgenic plants 35S::Hrip1-2 (H1, H2, and H3) were used to perform RNA sequencing, which was completed with an Illumina HiSeq 2000 platform (Novogene Bioinformatics Technology Co., Ltd, Beijing, China). To assess the quality of the sequencing data, we used the error rate and base contents as reference (S2 Table). The cleaned sequences of samples were mapped to the Arabidopsis reference genome analyzed with software TopHat v2.0.9 [83, 84], and the results of special reads matched to the genome are showed in S3 Table. To evaluate the expression level of each gene on the basis of RPKM [72], we set the RPKM > 1 as threshold for significant gene expression. More than half of the matched genes were significantly expressed (S3 Table). We calculated the average RPKM value from the three biological replicates of each gene and then compared the difference in gene expression levels between WT and transgenic plants 35S::Hrip1-2 (Fig 4A and 4B). To explore the gene expression difference between the transgenic lines 35S::Hrip1-2 (treat, AtRNA_H) and WT (control, AtRNA_C), the average read count data were used for analysis. The fold change of DEGs was determined based on the ratio of read count value of one gene between AtRNA_H and AtRNA_C. We set \( p_{\text{adj}} < 0.01 \) as the analysis condition to statistically decide the DEGs AtRNA_H and AtRNA_C. Subsequently, we found 40 genes that were upregulated and three genes that were significantly downregulated in AtRNA_H (Fig 4C).

Six DEGs between AtRNA_H and AtRNA_C were used for further analysis. Flowering locus (FT), a mobile protein in the leaf that determines the flower bolting time, showed 2.9-fold changes in transgenic line 35S::Hrip1-2 (S4 Table). Some genes were involved in JA biosynthesis, such as defective anther dehiscence 1 (DADI), LOX2, and AOS, and these genes were upregulated 1.9-, 2.6- and 2.3-fold, respectively (S4 Table). DEGs results revealed that
GLIP1, clade IIIa of GDSL-type esterases/lipases (*AtGELP*), which participates in the activation of biotic responses, exhibited 2.2-fold changes. PR6, a low-molecular-weight peptide grouped into PR protein, was only expressed in the transgenic line of *Hrip1*-overexpression (S4 Table). To confirm the DEGs results, we collected leaves from the transgenic lines (35S: *Hrip1*-1, 35S: *Hrip1*-2, and 35S: *Hrip1*-3) and WT of *Arabidopsis* plants that were not bolting (approximately two weeks after growing on nutrient soil). All of the interesting genes were confirmed with qRT-PCR with the specific primers listed in S5 Table. Changes in the expression level of genes of interest are shown in Fig 2C, and Fig 5. These findings were in agreement with the results of DEGs.

The 40 DEGs of upregulation were classified using GO enrichment to illustrate their potential functions, which were divided into 22 GO accessions with the most enrichment (P<corrected<0.05; S3 Fig). These categories were as follows: “response to stimulus” (GO: 0050896), “biological regulation” (GO: 0065007), “cellular process” (GO: 0009887) and “metabolic process” (GO: 0008152). The two GO terms of molecular accessions were “catalytic activity” (GO: 0003824) and “binding” (GO: 0005488). Most DEGs were from biological process activity. The FT was presented in the top of four significant GO terms of biological process, namely, “developmental process” (GO: 0032502), “multicellular organismal process” (GO: 0032501), “reproductive process” (GO: 0000003), and “reproductive process” (GO: 0022414). The JA biosynthesis genes (*DAD1*, *LOX2*, and *AOS*) were included in the GO terms “response to stimulus”, “regulation of biological process”, and “biological process”.

The DEGs were analyzed using KEGG enrichment to illustrate the signal transduction or metabolism network that these genes took part in. The DEGs were divided into 15 pathways. The JA biosynthesis genes *DAD1*, *LOX2*, and *AOS* were involved in the “α-linolenic acid metabolism” (ath00592) pathway, which was significantly enriched at P<corrected<0.01 (S4 Fig). The protein FT mentioned above was involved in the pathway named “circadian rhythm-plant”, identified at P<corrected=0.2146 (S4 Fig); this protein acted as a long-distance signal to induce flowering [85].

**JA content were slightly higher in transgenic lines than in WT**

Some genes involved in JA biosynthesis were upregulated in transgenic lines of *Hrip1*-overexpression, so we speculated that JA level must be enhanced in transgenic lines. To verify this
hypothesis, JA was isolated from leaves (approximately two weeks after plants grew on nutrient soil) and quantified via UPLC-MS/MS. In the transgenic line of Hrip1-overexpression, the contents of endogenous JA were examined to compared with WT Arabidopsis, and values of 184.70, 218.21, and 209.31 ng.g⁻¹ fresh weight were obtained (Fig 6). In the transgenic lines of Hrip1-overexpression, the expression level of genes involved in JA biosynthesis were enhanced and the JA level increased. Therefore, Hrip1 could trigger JA biosynthesis.

![Fig 5](https://doi.org/10.1371/journal.pone.0216082.g005)

Fig 5. Verification of differentially expressed DGE results using qRT–PCR. (A–E) The relative expression level of each gene significantly fold-changed in transgenic Hrip1-overexpressing Arabidopsis lines. Actin2 and Actin7 were used as internal controls in real-time PCR analysis. Each experiment was repeated more than thrice. Data are presented as the means ± SD of three independent experiments. Asterisks indicate significant differences between transgenic and WT Arabidopsis (Student’s t-test: **P < 0.01).

![Fig 6](https://doi.org/10.1371/journal.pone.0216082.g006)

Fig 6. Jasmonic acid contents of rosette leaves from wild-type (WT) and Hrip1-overexpressing transgenic lines. Jasmonic acid contents of 2-week-old rosette leaves from WT, 35S:Hrip1-1, 35S:Hrip1-2 and 35S:Hrip1-3 transgenic plants (growing on nutrient soil). Data are presented as the means ± SE of three biological replicates. Asterisks represent a significant difference between transgenic and WT Arabidopsis lines (Student’s t-test: *P < 0.05, **P < 0.01).
Discussion

The novel elicitor Hrip1 was purified from the necrotrophic fungus A. tenuissima using an ion exchange chromatography column [67]. In our previous work, Hrip1 was found to induce SAR in tobacco and enhance the resistance of plants to salt and drought [56, 67]. Here, we showed that Hrip1 promoted flower bolting and resistance to insects in Arabidopsis.

The previously article reported that the transgenic plants can enhance biotic and abiotic resistance when Hrip1 is overexpressed in Arabidopsis [56]. The Hrip1 transgenic lines of Arabidopsis displayed significantly higher changes in plant height, siliqule length, and plant dry weight, and the Hrip1 gene was induced by the rd29A promoter [56]. In this assay, we found that the transgenic plants of Hrip1-overexpression had other biological functions, including resistance to insects (Fig 3) and early bolting time in transgenic plants (Fig 2). These results were similarly observed in other elicitors isolated from different pathogens. The Harpin protein is an elicitor with many functions, including increased resistance to fungi and insects in many plants; it also regulates plant growth and flower bolting time by influencing the expression of plants, such as JA signaling-related genes and components of the ethylene (ET) signaling pathway [59–62, 86–90]. PevD1, an elicitor isolated from the cotton Verticillium wilt fungus V. dahliae, which can trigger resistance to pathogens in many plants by affecting the plant signaling pathway (including phytohormones JA, calcium ions, and WRKY) and regulating plant growth [64–66]. The enzymes of JA biosynthesis, PR-6 and GLIP1, were activated to enhance plant resistance to insects in Hrip1-overexpressing Arabidopsis.

The flower bolting time was determined by day length and mobile floral stimulus in leaves [35]. FT is a member of the phosphatidylethanolamine binding proteins, which is a key flowering promoter [80, 81, 91, 92] that is triggered by CONSTANTS (CO) in vascular tissues of leaves under long days [81, 93–96]. The FT protein moves from leaves to the SAM through the phloem to induce flowering in Arabidopsis [85, 97–99]. Furthermore, FT interacts with FD in SAM, resulting in the activation of SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1) and APETALA1 to initiate flower development [80, 81]. Our DEG results revealed that the transcription level of FT was significantly higher than that of Col-0 (S4 Table), which was confirmed by qRT-PCR (Fig 2C). Other elicitors, such as PevD1 and Harpin Hpa1, were reported to contribute to flower development among the plants [90, 100]. These results suggested that the expression of FT may be affected directly or indirectly in the transgenic lines of Hrip1-overexpression.

The phytohormone JA plays an important role to triggering plant responses against biotic infections, including insect and pathogen [17, 101–103]. Genes responsive to JA were rapidly triggered by JA biosynthesized by a series of well-organized enzymes, including DAD1, LOX, AOS, AOC, and OPDA [15, 17, 32]. In our assay, the results of the DEGs and qRT-PCR demonstrated that three genes of JA-biosynthesis, namely, DAD1, LOX-2, and AOS, were highly expressed in the transgenic lines of Hrip1-overexpression compared with WT (Fig 4 and S4 Table). The endogenous JA levels in transgenic lines of Hrip1-overexpression detected by LC-MS/MS were higher than those in the control Col-0 in Arabidopsis (Fig 6). These results proved that some genes involved in JA biosynthesis were activated when Hrip1 was overexpressed in Arabidopsis, which may laterally explain the transgenic resistance to larvae of S. exigua (Fig 3) and necrotrophic fungi [56, 67]. Similarly, the Harpin protein was reported to triggered the expression of TDF89H1 and TDF249H2 in Phalaenopsis orchids; these genes were similar to JAR1 and JAR4 of Arabidopsis [62]. In the results of DEGs, no significantly expressed gene related to the SA signaling pathway, such as PR1, was found. However, a pathogenesis-related peptide PR-6 was highly expressed in the transgenic lines of Hrip1-overexpression, which played a major role in defense action [42–44, 46].
In *Arabidopsis*, 105 GDSL-type esterase/lipase genes were divided into four clades based on function, namely, morphological development, abiotic stress response, secondary metabolism, and pathogen defense [49]. *GLIP1* belongs to clade IIIa of *AtGELP*; it participates in the plant’s immunity action via the ethylene signaling pathway and enhances the expression of *ERF1* and suppresses the expression of *EIN3* [51, 52]. Furthermore, *SID2*, a SA biosynthesis gene, is significantly expressed compared with WT, when *GLIP1* is overexpressed in *Arabidopsis*. In the transgenic lines of *Hrip1*-overexpression, *GLIP1* and *GLIP4* were differentially expressed compared with WT (S4 Table), and these results were confirmed by qRT-PCR (Fig 5). These results implied that *GLIP* could be activated when *Hrip1* was overexpressed in *Arabidopsis*, which may induce the immune system of the plant via the ethylene signaling pathway to lead to fungal resistance [56, 63].

**Additional information**

Sequence data are available in the Sequence Read Archive (SRA) database at the National Center for Biotechnology Information (NCBI) with access number PRJNA498541.

**Supporting information**

S1 Fig. *H₂O₂* accumulation in *Arabidopsis* leaves of *Hrip1*-overexpression transgenic plants and WT. Accumulation of *H₂O₂* in leaves of transgenic plants of *Hrip1*-overexpression and WT which grew on MS medium for 7 days and 14 days. Compared with WT, *Hrip1*-overexpression plants are shown to accumulate same levels of *H₂O₂*. (DOCX)

S2 Fig. Rosette leaves number of wild type and transgenic lines of *Hrip1*-overexpression. Timing of floral initiation in transgenic *Arabidopsis* plants was determined by counting the number of rosette leaves formed at the time of bolting (mean ± SE, n = 30 plants per treatment), each experiment was repeated more than thrice. Asterisks indicate significant differences between transgenic and WT *Arabidopsis* (Student’s *t*-test: **P < 0.01). (DOCX)

S3 Fig. Gene Ontology (GO) classification of the DEGs. The unigenes were classified in three main categories: biological process, cellular location, and molecular function. (DOCX)

S4 Fig. Scattered Plot of KEGG pathway terms of differentially expressed genes. Dot size represents the number of different genes and rich factor indicates the value of *p*corrected. (DOCX)

S1 Table. The quality of sequencing data. 1) Sample name: the names of samples. 2) Raw reads: the original sequencing reads counts. 3) Clean reads: number of reads after filtering. 4) Clean base: clean reads number multiply read length, saved in G unit. 5) Error rate: average sequencing error rate, which is calculated by Qphred = -10log10(e). 6) Q20: percentages of bases whose correct base recognition rates are greater than 99% in total bases. 7) Q30: percentages of bases whose correct base recognition rates are greater than 99.9% in total bases. 8) GC content: percentage of G and C in total bases. (DOCX)
S2 Table. Overview of mapping status. 1) Total number of filtered reads (Clean data). 2) Total number of reads that can be mapped to the reference genome. In general, this number should be larger than 70% when there is no contamination and the correct reference genome is chosen. 3) Number of reads that can be mapped to multiple sites in the reference genome. This number is usually less than 10% of the total. 4) Number of reads that can be uniquely mapped to the reference genome. 5) Number of reads that map to the positive strand (+) or the minus strand (-). 6) Splice reads can be segmented and mapped to two exons (also named junction reads), whereas non-splice reads can be mapped entirely to a single exon. The ratio of splice reads depends on the insert size used in the RNA-seq experiments.

S3 Table. The number of genes with different expression levels.

S4 Table. List of differential genes between AtHrip1 and AtWT.

S5 Table. Primer sequence designed for this study.

Author Contributions

Conceptualization: Xin-yue Miao.
Data curation: Xin-yue Miao, Cong-fen He, Zhi-wei Cheng.
Funding acquisition: Zhi-wei Cheng.
Investigation: Xin-yue Miao, Hong-pan Qu.
Methodology: Xin-yue Miao, Cong-fen He.
Supervision: Zhi-wei Cheng.
Writing – original draft: Ya-lei Han, Zhi-wei Cheng.
Writing – review & editing: De-wen Qiu, Zhi-wei Cheng.

References
1. Kissoudis C, van de Wiel C, Visser R, van der Linden G. Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. Front Plant Sci. 2014; 5:207. https://doi.org/10.3389/fpls.2014.00207 PMID: 24904607.
2. Hirayama T, Shinozaki K. Research on plant abiotic stress responses in the post-genome era: past, present and future. Plant J. 2010; 61(6):1041–52. https://doi.org/10.1111/j.1365-313X.2010.04124.x PMID: 20409277.
3. He T Ba SY. Innate immunity in plants: an arms race between pattern recognition receptors in plants and effectors in microbial pathogens. Science. 2009; 324(5928):742–4. https://doi.org/10.1126/science.1171647 PMID: 19423812.
4. Cramer GR, Urano K, Delrot S, Pezzotti M, Shinozaki K. Effects of abiotic stress on plants: a systems biology perspective. BMC Plant Biology. 2011; 11(1):163. https://doi.org/10.1186/1471-2229-11-163 PMID: 22094046.
5. Cheng Z, Sun L, Qi T, Zhang B, Peng W, Liu Y, et al. The bHLH transcription factor MYC3 interacts with the Jasmonate ZIM-domain proteins to mediate jasmonate response in Arabidopsis. Mol Plant. 2011; 4(2):279–88. https://doi.org/10.1093/mp/ssq073 PMID: 21242320.
6. Penninckx IA, Eggemont K, Terras FR, Thomma BP, De Samblanx GW, Buchala A, et al. Pathogen-induced systemic activation of a plant defensin gene in Arabidopsis follows a salicylic acid-independent pathway. The Plant Cell. 1996; 8(12):2309–23. https://doi.org/10.1105/tpc.8.12.2309. PMID: 8989885.

7. Creelman RA, Mullet JE. Oligosaccharins, brassinolides, and jasmonates: nontraditional regulators of plant growth, development, and gene expression. The Plant Cell. 1997; 9(7):1211–23. https://doi.org/10.1105/tpc.9.7.1211 PMID: 9254935.

8. McConn M, Browse J. The Critical Requirement for Linolenic Acid Is Pollen Development, Not Photosynthesis, in an Arabidopsis Mutant. The Plant Cell. 1996; 8(3):403–16. https://doi.org/10.1105/tpc.8.3.403 PMID: 12239389.

9. Pieterse CMJ, van Wees SCM, van Pel J, Knolle M, Laan R, Gerrits H, et al. A Novel Signaling Pathway Controlling Induced Systemic Resistance in Arabidopsis. The Plant Cell. 1998; 10(9):1571–80. https://doi.org/10.1105/tpc.10.9.1571. PMID: 9724702.

10. Overmyer K, Tuominen H, Kettunen R, Betz C, Langebartels C, Sandermann H, et al. Ozone-Sensitive Arabidopsis rcd1 Mutant Reveals Opposite Roles for Ethylene and Jasmonate Signaling Pathways in Regulating Superoxide-Dependent Cell Death. The Plant Cell. 2000; 12(10):1849–62. https://doi.org/10.1105/tpc.12.10.1849. PMID: 11041881.

11. Farmer EE. Surface-to-air signals. Nature. 2001; 411(6839):854–6. https://doi.org/10.1038/35081189 PMID: 11459069.

12. Rao MV, Lee H-I, Davis KR. Ozone-induced ethylene production is dependent on salicylic acid, and both salicylic acid and ethylene act in concert to regulate ozone-induced cell death. The Plant Journal. 2002; 32(4):447–56. https://doi.org/10.1046/j.1365-313x.2002.01434.x. PMID: 12445117.

13. Turner JG, Ellis C, Devoto A. The Jasmonate Signal Pathway. The Plant Cell. 2002; 14:S153–S64.

14. Bu Q, Jiang H, Li C-B, Zhai Q, Zhang J, Wu X, et al. Role of the (+)-7-iso-Jasmonoyl-L-isoleucine amidohydrolase 1 in Arabidopsis. J Biol Chem. 2000; 275(25):19132–8. https://doi.org/10.1074/jbc.M002133200 PMID: 11459069.

15. Scott TF, Staswick PE. Allene oxide synthase: unstable intermediates in the metabolism of lipid hydroperoxides. Proceedings of the National Academy of Sciences of the United States of America. 1988; 85(10):3382–6. https://doi.org/10.1073/pnas.85.10.3382. PMID: 2835769.

16. Sun J, Xu Y, Ye S, Jiang H, Chen Q, Liu F, et al. Arabidopsis ASA1 is important for jasmonate-mediated regulation of auxin biosynthesis and transport during lateral root formation. The Plant Cell. 2009; 21(5):1495–511. https://doi.org/10.1105/tpc.10.8.064303 PMID: 19435934.

17. Wasternack C, Song S. Jasmonates: biosynthesis, metabolism, and signaling by proteins activating and repressing transcription. J Exp Bot. 2017; 68(6):1303–21. https://doi.org/10.1093/jxb/erw443 PMID: 27940470.

18. Vick BA, Zimmerman DC. The biosynthesis of jasmonic acid: a physiological role for plant lipoxygenase. Biochemical and biophysical research communications. 1983; 111(2):470–7. https://doi.org/10.1016/0006-291x(83)90330-3. PMID: 6404266.

19. Brash AR, Baertschi SW, Ingram CD, Harris TM. Isolation and characterization of natural allene oxides: unstable intermediates in the metabolism of lipid hydroperoxides. Proceedings of the National Academy of Sciences of the United States of America. 1988; 85(10):3382–6. https://doi.org/10.1073/pnas.85.10.3382. PMID: 2835769.

20. Ziegler J, Stenzel I, Hause B, Maucher H, Hamberg M, Grimm R, et al. Molecular cloning of allene oxide cyclase. The enzyme establishing the stereochemistry of octadecanoids and jasmonates. J Biol Chem. 2000; 275(25):19132–8. https://doi.org/10.1074/jbc.M002133200 PMID: 10764787.

21. Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science. 2001; 294(5548):1871–5. https://doi.org/10.1126/science.294.5548.1871 PMID: 11729303.

22. Koo AJK, Chung HS, Kobayashi Y, Howe GA. Identification of a peroxisomal acyl-activating enzyme involved in the biosynthesis of jasmonic acid in Arabidopsis. J Biol Chem. 2006; 281(44):33511–20. https://doi.org/10.1074/jbc.M607854200 PMID: 16963437.

23. Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, et al. (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol. 2009; 5(5):344–50. https://doi.org/10.1038/nchembio.161 PMID: 19349968.

24. Yan J, Zhang C, Gu M, Bai Z, Zhang W, Qi T, et al. The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. The Plant cell. 2009; 21(8):2220–36. https://doi.org/10.1105/tpc.109.065730 PMID: 19717617.

25. Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA. COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. Proceedings of the National Academy of Sciences of the United States of America. 1999; 96(12):6573–6. https://doi.org/10.1073/pnas.85.10.3382. PMID: 19717617.
26. Sheard LB, Tan X, Mao H, Ben-Nissan G, Hinds TR, et al. Jasmonate perception by inositol-phosphate-potentiated COI1-JAZ co-receptor. Nature. 2010; 468(7322):400–5. https://doi.org/10.1038/nature09430 PMID: 20927106.

27. Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, et al. JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature. 2007; 448(7154):661–5. https://doi.org/10.1038/nature05960 PMID: 17637677.

28. Chini A, Fonseca S, Fernandez G, Adie B, Chico JM, Lorenzo O, et al. The JAZ family of repressors is the missing link in jasmonate signalling. Nature. 2007; 448(7154):666–71. https://doi.org/10.1038/nature06006 PMID: 17637675.

29. Yan J, Yao R, Chen L, Li S, Gu M, Nan F, et al. Dynamic perception of jasmonates by the F-box protein COI1. Molecular Plant. 2018; 11(10):1237–47. https://doi.org/10.1016/j.molp.2018.07.007 PMID: 30092285.

30. Xie DX, Feys BF, James S, Nieto-Rostro M, Turner JG. COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. Science. 1998; 280(5366):1091–4. PMID: 9582125.

31. Huang H, Gao H, Liu B, Qi T, Tong J, Xiao L, et al. Arabidopsis MYB24 Regulates Jasmonate-Mediated Stamen Development. Front Plant Sci. 2017; 8:1525. https://doi.org/10.3389/fpls.2017.01525 PMID: 28929760.

32. Zhang L, Zhang F, Melotto M, Yao J, He SY. Jasmonate signaling and manipulation by pathogens and insects. J Exp Bot. 2017; 68(1):1371–85. https://doi.org/10.1093/jxb/erw478 PMID: 28069779.

33. Zipfel C, Felix G. Plants and animals: a different taste for microbes? Curr Opin Plant Biol. 2005; 8(4):353–60. https://doi.org/10.1016/j.pbi.2005.04.004 PMID: 15922649.

34. Dangl JL, Jones JD. Plant pathogens and integrated defence responses to infection. Nature. 2001; 411(6839):826–33. https://doi.org/10.1038/35081161 PMID: 11459065.

35. Breen S, Williams SJ, Outram M, Kobe B, Solomon PS. Emerging Insights into the Functions of Pathogenesis-Related Protein 1. Trends Plant Sci. 2017; 22(10):871–9. https://doi.org/10.1016/j.tplants.2017.06.013 PMID: 28743380.

36. van Loon LC, Rep M, Pieterse CM. Significance of inducible defense-related proteins in infected plants. Annu Rev Phytopathol. 2006; 44:135–62. https://doi.org/10.1146/annurev.phyto.44.070505.143425 PMID: 16602946.

37. Glazebrook J. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual review of phytopathology. 2005; 43:205–27. https://doi.org/10.1146/annurev.phyto.44.040204.135923 PMID: 16078883.

38. Dunaevskii I, Tsybina TA, Beliakova GA, Domash VI, Shapno TP, Zabreiko SA, et al. Proteinase inhibitors as antistress proteins in higher plants. Prikl Biokhim Mikrobiol. 2005; 41(4):392–6. PMID: 16212034.

39. Gutierrez-Campos R, Torres-Acosta JA, Saucedo-Arias LJ, Gomez-Lim MA. The use of cysteine proteinase inhibitors to engineer resistance against potyviruses in transgenic tobacco plants. Nat Biotechnol. 1999; 17(12):1223–6. https://doi.org/10.1038/70781 PMID: 10585723.

40. Urwin PE, Lilley CJ, McPherson MJ, Atkinson HJ. Resistance to both cyst and root-knot nematodes conferred by transgenic Arabidopsis expressing a modified plant cystatin. The Plant Journal: for cell and molecular biology. 1997; 12(2):455–61. https://doi.org/10.1046/j.1365-313x.1997.12020455.x PMID: 9301094.

41. Vila I, Quilis J, Meynard D, Breitler JC, Marfà V, Murillo I, et al. Expression of the maize proteinase inhibitor (mpi) gene in rice plants enhances resistance against the striped stem borer (Chilo suppressalis): effects on larval growth and insect gut proteinases. Plant Biotechnology Journal. 2005; 3(2):187–202. https://doi.org/10.1111/j.1467-7652.2004.00117.x PMID: 17173619.
46. Zarate SI, Kempema LA, Walling LL. Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant physiology. 2007; 143(2):866–75. https://doi.org/10.1104/pp.106.09035 PMID: 17189328.

47. Haq SK, Atif SM, Khan RH. Proteinase inhibitor genes in combat against insects, pests, and pathogens: natural and engineered phytoprotection. Arch Biochem Biophys. 2004; 431(1):145–59. https://doi.org/10.1016/j.abb.2004.07.022 PMID: 15464737.

48. Chassot C, Nawrath C, Metraux JP. Cuticular defects lead to full immunity to a major plant pathogen. Plant J. 2007; 49(6):972–80. https://doi.org/10.1111/j.1365-313X.2006.03017.x PMID: 17257167.

49. Lai CP, Huang LM, Chen LO, Chan MT, Chan JF. Genome-wide analysis of GDSL-type esterases/lipases in Arabidopsis. Plant Mol Biol. 2017; 95(1–2):181–97. https://doi.org/10.1007/s11103-017-0648-y PMID: 28840447.

50. Oh IS, Park AR, Bae MS, Kwon SJ, Kim YS, Lee JE, et al. Secretome analysis reveals an Arabidopsis lipase involved in defense against Alternaria brassicicola. Plant Cell. 2005; 17(10):2832–47. https://doi.org/10.1105/tpc.105.034819 PMID: 16126835.

51. Kim HG, Kwon SJ, Jang YJ, Nam MH, Chung JH, Na YC, et al. GDSL LIPASE1 modulates plant immunity through feedback regulation of ethylene signaling. Plant Physiol. 2013; 163(4):1776–91. Epub 2013/10/31. https://doi.org/10.1104/pp.113.225649 PMID: 24170202.

52. Kim HG, Kwon SJ, Jang YJ, Chung JH, Nam MH, Park OK. GDSL lipase 1 regulates ethylene signaling and ethylene-associated systemic immunity in Arabidopsis. FEBS Lett. 2014; 588(9):1652–8. https://doi.org/10.1016/j.febslet.2014.02.062 PMID: 24631536.

53. Lee DS, Kim BK, Kwon SJ, Jin HC, Park OK. Arabidopsis GDSL lipase 2 plays a role in pathogen defense via negative regulation of auxin signaling. Biochem Biophys Res Commun. 2009; 379(4):1038–42. https://doi.org/10.1016/j.bbrc.2009.01.006 PMID: 19146828.

54. Ebel J. Oligoglucoside elicitor-mediated activation of plant defense. Bioessays. 1998; 20(7):569–76. https://doi.org/10.1002/(SICI)1521-1878(199807)20:7<569::AID-BIEE8>3.0.CO;2-F PMID: 9723006.

55. B Beissmann HR. Isolation and purity determination of a glycoprotein elicitor from wheat stem rust by medium-pressure liquid chromatography. Journal of Chromatography A. 1990; 521(2):187–97. https://doi.org/10.1016/0021-9673(90)85043-U.

56. Peng XC, Qiu DW, Zeng HM, Guo LH, Yang XF, Liu Z. Inducible and constitutive expression of an elicitor gene Hrip1 from Alternaria tenuissima enhances stress tolerance in Arabidopsis. Transgenic Res. 2015; 24(1):135–45. https://doi.org/10.1007/s11248-014-9824-x PMID: 25120219.

57. Nurnberger T. Signal perception in plant pathogen defense. Cell Mol Life Sci. 1999; 55(2):167–82. https://doi.org/10.1007/s000180050283 PMID: 24481912.

58. Nurnberger T, Scheel D. Signal transmission in the plant immune response. Trends Plant Sci. 2001; 6(8):372–9. https://doi.org/10.1016/S1360-1385(01)02019-2. PMID: 11495791.

59. Novacky JSJWA. Rapid and Transient Activation of a Myelin Basic Protein Kinase in Tobacco Leaves Treated with Harpin from Erwinia amylovora. Plant Physiology. 1997; 115(2):853–61. https://doi.org/10.1101/tpc.11248-014-9824-x PMID: 25120219.

60. Peng JL, Bao ZL, Ren HY, Wang JS, Dong HS. Expression of harpin(xoo) in transgenic tobacco induces pathogen defense in the absence of hypersensitive cell death. Phytopathology. 2004; 94(10):1048–55. https://doi.org/10.1094/PHYTO.2004.94.10.1048 PMID: 18943792.

61. Shao M, Wang J, Dean RA, Lin Y, Gao X, Hu S. Expression of a harpin-encoding gene in rice confers durable nonspecific resistance to Magnaporthe grisea. Plant Biotechnol J. 2008; 6(1):75–81. https://doi.org/10.1111/j.1467-7652.2007.00304.x PMID: 18005094.

62. Chuang H-w CP-Y, Syu Y-y. Harpin Protein, an Elicitor of Disease Resistance, Acts as a Growth Promoter in Phalaenopsis Orchids. J Plant Growth Regul. 2014; 133(4):788–97. https://doi.org/10.1007/s00344-014-9425-1.

63. Chen M, Zeng H, Qiu D, Guo L, Yang X, Shi H, et al. Purification and characterization of a novel hypersensitive response-inducing elicitor from Magnaporthe oryzae that triggers defense response in rice. PLoS One. 2012; 7(5):e37654. https://doi.org/10.1371/journal.pone.0037654 PMID: 22624059.

64. Wang B, Yang X, Zeng H, Liu H, Zhou T, Tan B, et al. The purification and characterization of a novel hypersensitive-like response-inducing elicitor from Verticillium dahliae that induces resistance responses in tobacco. Appl Microbiol Biotechnol. 2012; 93(1):191–201. https://doi.org/10.1007/s00253-011-3405-1 PMID: 21691787.

65. Bu B, Qiu D, Zeng H, Guo L, Yuan J, Yang X. A fungal protein elicitor PevD1 induces Verticillium wilt resistance in cotton. Plant Cell Rep. 2014; 33(3):461–70. https://doi.org/10.1007/s00299-013-1546-7 PMID: 24337817.
66. Liu M, Khan N, Wang N, Yang X, Qiu D. The Protein Elicitor PevD1 Enhances Resistance to Pathogens and Promotes Growth in *Arabidopsis*. Int J Biol Sci. 2016; 12(8):931–43. https://doi.org/10.7150/ijbs.15447 PMID: 27489497.

67. Kulye M, Liu H, Zhang Y, Zeng H, Yang X, Qiu D. Hrip1, a novel protein elicitor from necrotrophic fungus, *Alternaria tenuissima*, elicits cell death, expression of defence-related genes and systemic acquired resistance in tobacco. Plant, Cell & Environment. 2012; 35(12):2104–20. https://doi.org/10.1111/j.1365-3040.2012.02539.x PMID: 22591019.

68. Peng X, Qiu D, Zeng H, Guo L, Yang X, Liu Z. Inducible and constitutive expression of an elicitor gene Hrip1 from *Alternaria tenuissima* enhances stress tolerance in *Arabidopsis*. Transgenic Res. 2015; 24(1):135–45. https://doi.org/10.1007/s11248-014-9824-x PMID: 25120219.

69. Clough SJ, Bent AF. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. 1998; 16(6):735–43. https://doi.org/10.1046/j.1365-313x.1998.00343.x PMID: 10069079.

70. Davletova S, Rizhsky L, Liang H, Shengqiang Z, Oliver DJ, Coutu J, Shulaev V, Schlauch K, Mittler R. Cytosolic ascorbate peroxidase 1 is a central component of the reactive oxygen gene network of *Arabidopsis*. The Plant Cell. 2005; 17(1):268–81. https://doi.org/10.1105/tpc.104.026971 PMID: 15608336.

71. Hu P, Zhou W, Cheng Z, Fan M, Wang L, Xie D. JAV1 controls jasmonate-regulated plant defense. Mol Cell. 2013; 50(4):504–15. https://doi.org/10.1016/j.molcel.2013.04.027 PMID: 23706819.

72. Mortazavi A, Williams BA, McCue K, Schaef er L, Wold B. Mapping and quantifying mammalian transcripts by RNA-Seq. Nat Methods. 2008; 5(7):621–8. https://doi.org/10.1038/nmeth.1226 PMID: 18516045.

73. Peng X, Qiu D, Zeng H, Guo L, Yang X, Liu Z. Inducible and constitutive expression of an elicitor gene Hrip1 from *Alternaria tenuissima* enhances stress tolerance in *Arabidopsis*. Transgenic Res. 2015; 24(1):135–45. https://doi.org/10.1007/s11248-014-9824-x PMID: 25120219.
86. Dong HP, Peng J, Bao Z, Meng X, Bonasera JM, Chen G, et al. Downstream divergence of the ethylene signaling pathway for harpin-stimulated Arabidopsis growth and insect defense. Plant Physiol. 2004; 136(3):3628–38. https://doi.org/10.1104/pp.104.048900 PMID: 15516507.

87. Liu R, Lü B, Wang X, Zhang C, Zhang S, Qian J, et al. Thirty-seven transcription factor genes differentially respond to a harpin protein and affect resistance to the green peach aphid in Arabidopsis. Journal of Biosciences. 2010; 35(3):435–50. https://doi.org/https://doi.org/10.1007/s12038-010-0049-8 PMID: 20826953.

88. Chunling Zhang HS, Leı Chen1, Xiaomeng Wang, Beibei Lü, Shuping Zhang, Yuan Liang, Ruoxue Liu, Jun Qian, Weiwei Sun, Zhenzhen You, Hansong Dong. Harpin-induced expression and transgenic overexpression of the phloem protein gene AtPP2-A1 in Arabidopsis repress phloem feeding of the green peach aphid Myzus persicae. BMC Plant Biology. 2011; 11(11):1471–2229. https://doi.org/10.1186/1471-2229-11-11 PMID: 21226963.

89. Choi MS, Kim W, Lee C, Oh CS. Harpins, multifunctional proteins secreted by gram-negative plant-pathogenic bacteria. Mol Plant Microbe Interact. 2013; 26(10):1115–22. https://doi.org/10.1094/MPMI-02-13-0050-CR PMID: 23745678.

90. Dong Y, Li P, Zhang C. Harpin Hpa1 promotes flower development in Impatiens and Parochetus plants. Bot Stud. 2016; 57(1):22. https://doi.org/10.1186/s40529-016-0132-z PMID: 28597432.

91. Kobayashi Y, Kaya H, Goto K, Iwabuchi M, Araki T. A pair of related genes with antagonistic roles in mediating flowering signals. Science. 1999; 286(5446):1960–2. E PMID: 10583960.

92. Kardailsky I, Shukla VK, Ahn JH, Dagenais N, Christensen SK, Nguyen JT, et al. Activation tagging of the floral inducer FT. Science. 1999; 286(5446):1962–5. PMID: 10583961.

93. Giakountis A, Coupland G. Phloem transport of flowering signals. Curr Opin Plant Biol. 2008; 11(6):687–94. https://doi.org/10.1016/j.pbi.2008.10.003 PMID: 18977685.

94. An H, Roussos C, Suarez-Lopez P, Corbesier L, Vincent C, Pineiro M, et al. CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. Development. 2004; 131(15):3615–26. https://doi.org/10.1242/dev.01231 PMID: 15229176.

95. Samach A, Onouchi H, Gold SE, Ditta GS, Schwarz-Sommer Z, Yanofsky MF, et al. Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science. 2000; 288(5471):1613–6. https://10.1126/science.288.5471.1613 PMID: 10834834.

96. Takada S, Goto K. Terminal flower2, an Arabidopsis homolog of heterochromatin protein1, counteracts the activation of flowering locus T by constans in the vascular tissues of leaves to regulate flowering time. Plant Cell. 2003; 15(12):2856–65. https://doi.org/10.1105/tpc.016345 PMID: 14630968.

97. Jaeger KE, Wigge PA. FT protein acts as a long-range signal in Arabidopsis. Current Biology. 2007; 17(12):1050–4. https://doi.org/10.1016/j.cub.2007.05.008 PMID: 17540569.

98. Mathieu J, Warthmann N, Kuttner F, Schmid M. Export of FT protein from phloem companion cells is sufficient for floral induction in Arabidopsis. Current Biology. 2007; 17(12):1055–60. WOS:000247409000028. https://doi.org/10.1016/j.cub.2007.05.009 PMID: 17540570.

99. Notaguchi M, Abe M, Kimura T, Daimon Y, Kobayashi T, Yamaguchi A, et al. Long-Distance, Graft-Transmissible Action of Arabidopsis FLOWERING LOCUS T Protein to Promote Flowering. Plant and Cell Physiology. 2008; 49(12):1922–30. https://doi.org/10.1093/pcp/pcn176 PMID: 19649187.

100. Liu M, Khan NU, Wang N, Yang X, Qiu D. The Protein Elicitor PevD1 Enhances Resistance to Pathogens and Promotes Growth in Arabidopsis. Int J Biol Sci. 2016; 12(8):931–43. https://doi.org/10.7150/ijbs.15447 PMID: 27489497.

101. Chini A, Gimenez-Ibanez S, Goossens A, Solano R. Redundancy and specificity in jasmonate signaling.Curr Opin Plant Biol. 2016; 33:147–56. https://doi.org/10.1016/j.pbi.2016.07.005 PMID: 27490895.

102. Fernandez-Calvo P, Chini A, Fernandez-Barbero G, Chico JM, Gimenez-Ibanez S, Geerink J, et al. The Arabidopsis bHLH transcription factors MYC3 and MYC4 are targets of JAZ repressors and act additively with MYC2 in the activation of jasmonate responses. Plant Cell. 2011; 23(2):701–15. https://doi.org/10.1105/tpc.110.080788 PMID: 21335373.

103. Pauwels L, Goossens A. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. Plant Cell. 2011; 23(9):3089–100. https://doi.org/10.1105/tpc.111.089300 PMID: 21963667.