AaERF1 Positively Regulates the Resistance to Botrytis cinerea in Artemisia annua

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Abstract

Plants are sessile organisms, and they can not move away under abiotic or biotic stresses. Thus plants have evolved a set of genes that respond to adverse environment to modulate gene expression. In this study, we characterized and functionally studied an ERF transcription factor from Artemisia annua, AaERF1, which plays an important role in biotic stress responses. The AaERF1 promoter had been cloned and GUS staining results of AaERF1 promoter-GUS transgenic A. annua showed that AaERF1 was expressed ubiquitously in all organs. Several putative cis-acting elements such as W-box, TGA-box and Py-rich element, which are involved in defense responsiveness, are present in the promoter. The expression of AaERF1 can be induced vigorously by methyl jasmonate as well as by ethephon and wounding, implying that AaERF1 may activate some of the defense genes via the jasmonic acid and ethylene signaling pathways of A. annua. The results of electrophoretic mobility shift assay (EMSA) and yeast one-hybrid experiments showed that AaERF1 was able to bind to the GCC box cis-acting element in vitro and in yeast. Ectopic expression of AaERF1 could enhance the expression levels of the defense marker genes PLANT DEFENSIN1.2 (PDF1.2) and BASIC CHITINASE (ChiB), and increase the resistance to Botrytis cinerea in the 35S::AaERF1 transgenic Arabidopsis. The down-regulated expression level of AaERF1 evidently reduced the resistance to B. cinerea in A. annua. The overall results showed that AaERF1 positively regulated the resistance to B. cinerea in A. annua.

Jasmonic acid (JA) and ethylene (ET) are two important hormones that act synergistically during plant resistance to necrotrophic pathogens such as B. cinerea by activating some ERF genes, which are responsive to both JA and ET treatment. For example, in Arabidopsis, two ERF genes, ERF1 and ORA19, are induced by JA and ET [7,8]. Overexpression of either ERF1 or ORA19 resulted in constitutive expression of the defense marker genes, PLANT DEFENSIN1.2 (PDF1.2) and BASIC CHITINASE (ChiB), thus enhancing the resistance to B. cinerea [7,8]. A recent study also showed that another two ERF genes, ERF5 and ERF6, play redundant roles as positive regulators of JA/ET-mediated defense against B. cinerea in Arabidopsis [9]. Thus, at least four ERFs play key role in integrating the JA and ET signal in disease resistance. In previous studies, our group also showed that two ERFs from Gossypium barbadense, showed positive effects on disease resistance [10,11]. ERF genes from other species, such as tomato (TARF1 and PiRF), soybean (GmERF5) and Bupleurum kasi (BkERF1, BkERF2.1 and BkERF2.2) also showed positive effects on disease resistance, suggesting the ERF genes have a conserved role in diverse species to counteract with plant pathogens [5,12–16].

Artemisia annua L. is an important medicinal plant that produces artemisinin, which is widely used in malaria treatment. A recent study has shown that JA can increase artemisinin production by inducing two ERF genes in A. annua. AsAERF1 and AsAERF2, both of which directly bind to the CTRDREHYCBF2 (CBF2) and RAV1AAAT (RAA) motifs present in both ADS and CYP71AV1
promoters to activate those key enzymes in artemisinin biosynthesis pathway [17]. Our previous study showed that wounding stress also significantly elevated the artemisinin content by increasing ADS and CYP71AV1 expression levels [18]. Compared to the great effort in artemisinin biosynthesis pathway, little is known about the disease resistance in *A. annua*. Since ERFs are key regulators that integrate JA and ET signals in disease resistance, it is attempted to establish whether *AaERF1* has a role in disease resistance. Thus, our research focused on the function of *AaERF1* in plant antifungal field and illustrated that *AaERF1*, which could bind to GCC box in *in vitro* and in yeast, positively regulated the resistance to *B. cinerea* in *A. annua*.

**Results**

*AaERF1* is Ubiquitously Expressed in *A. annua*

The promoter sequence of *AaERF1* (JQ513909) was cloned by genomic walking (Figure 1A). To observe the expression pattern of *AaERF1* in details, the *AaERF1* promoter was subcloned to the pCAMBIA1391Z vector (Figure 1B) and then *AaERF1* promoter-GUS transgenic *A. annua* plants were generated. Six lines of the transgenic *A. annua* plants expressing the GUS and three lines for wild-type background were prepared. All the lines showed similar fusion protein expression. GUS activity was detected in all tissues examined, including roots, stems, leaves and flowers (Figure 2A, 2B, 2C and 2D). In 1-month-old plants, GUS activity was high in root tips, stems and leaves (Figure 2A, 2B and 2C). During the flowering period, GUS activity was also detected in flower buds. So, *AaERF1* is ubiquitously expressed in *A. annua*. From Figure 2B and 2C, GUS expression was also detected in the glandular trichomes and T-shaped trichomes. No signals were observed in the negative control plants transformed with pCAMBIA1391 empty vector (Figure S1).

Prediction of cis-acting Elements of Promoter Region of *AaERF1*

Putative cis-acting elements of the promoter were predicted using the PLANTCARE software [http://bioinformatics.psb.ugent.be/webtools/plantcare/html/](http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Figure 1A; Table 1). A putative TATA box sequence was found at -27 bp, and the putative CAAT box sequence was located at -38 bp. The 5'-UTR pyrimidine-rich stretch site is a cis-acting element conferring high transcription levels. Such an element was found at position -1345 to -1336 as shown in Figure 1A. A TC-rich repeat, which is involved in defense and stress response, was localized to position -590 to -581. A TGA-box element (TGACGTCA), which is involved in plant defense responsiveness, was found at position -209 to -201. A G/C-box element (CAGCTG), which is involved in light-induction or hormone control, was found at position -1458 to -1453. The W box is a fungal elicitor responsive element, which was present at positions -547 to -542 bp and -336 to -332 bp in *AaERF1* promoter. A search for the regulatory elements in *AaERF1* promoter also carried EIRE box. The above cis-acting elements are summarized in Table 1. Nearly all these cis-acting elements are related to defense responsiveness. Consequently, *AaERF1* may be a defense responsiveness transcription factor in *A. annua*.

Expression Profiling Analysis of *AaERF1* after Hormone and Stress Treatments

In this study, RT-Q-PCR analysis was used to obtain the expression pattern of *AaERF1* after hormone and stress treatments including MeJA (100 μM), ethephon (500 μM) and wound treatments. The transcript level of *AaERF1* was increased rapidly and peaked within 1 h after MeJA treatment, followed by a gradually decline (Figure 3A). The treatment with ethephon shows a similar expression pattern with the treatment of MeJA (Figure 3B). The transcript level of *AaERF1* was also sensitive to stress treatments. Wounding could induce a significant accumulation of *AaERF1* transcript in a short time period (0.5 h). Then the transcript level was quickly decreased (Figure 3C). The statistics analysis showed that the observed differences were statistically significant.

Comparative and Bioinformatic Analyses of *AaERF1*

The results of the BLAST-Protein (BLASTP) online [http://www.ncbi.nlm.gov/blast] showed that the *AaERF1* protein had a highly conserved AP2 domain with other ERF proteins, including Arabidopsis AtERF1, AtERF2, ORCA3, LeERF1, NtERF1, TaERF3 and ORA59 (Figure S2A). This domain is divided into two conserved segments of YRG and RAYG, in which a β-sheet and α-helix are predicted (β-α motif; see Figure S2A). A phylogenetic tree of ERF proteins was drawn using the CLUSTAL X program. The phylogenetic tree demonstrated that ERF proteins originated from a common ancestor and diverged into several groups (Figure S2B). According to the phylogenetic tree, the protein of *AaERF1* had close evolutionary relationships to *AaERF2*, *LeERF1*, *NtERF1* and *TaERF3* which showed that they might share similar functions in disease resistance (Figure S2B).

*AaERF1* Protein Interacts with the GCC Box *in vitro*

Since the AP2 domain of *AaERF1* contained the key amino acids to bind the GCC box, the recombinant MBP-*AaERF1* protein was constructed and overexpressed in *E. coli* BL21, purified, and used to examine the DNA binding ability *in vitro*. The purified MBP-*AaERF1* protein was mixed, respectively, with the labeled wild-type GCC probe or a mutated GCC probe in the binding reaction. The results of EMSA showed that the gel mobility shift was specific to the MBP-*AaERF1* protein with the labeled GCC probe (lane 2 in Figure 4A). As expected, there were no shifted bands in the combination of MBP-*AaERF1* plus the mutated GCC (mGCC) probe (lane 5 in Figure 4A) and in the negative controls, including MBP with the labeled GCC probe (lane 1) or mGCC probe (lane 4), and only the labeled GCC probe (lane 3) or mGCC probe (lane 6) (Figure 4A). The results demonstrated that *AaERF1* was able to bind to the GCC box cis-acting element, but not to the mutated GCC box *in vivo*.

*AaERF1* can Bind to the GCC Box in Yeast

The yeast one-hybrid system is a stable system to study the DNA binding ability of transcription factors [19]. The results of yeast one-hybrid and β-galactosidase activity assays indicated that only the hybrid cells containing the combination of pB42AD-*AaERF1* and p176-4×GCC-LacZ showed β-galactosidase activity compared with other combinations, including pB42AD with p176-LacZ, pB42AD-*AaERF1* with p176-4×GCC-LacZ, pB42AD-*AaERF1* with p178-LacZ, and pB42AD with p176-4×GCC-LacZ. The results demonstrated that *AaERF1* could bind to the GCC box cis-acting element in yeast cells (Figure 4B).

*AaERF1*-overexpression in Arabidopsis Causes the Increase of Disease Resistance to *B. cinerea*

The transgenic Arabidopsis plants were first confirmed by kanamycin-resistant screening and genomic DNA-based PCR, and then three transgenic lines were chosen for further analysis. The control experiment involving the transfer of empty plasmid
The transcript levels of AaERF1 had a significant increase in AaERF1-overexpression lines (Figure 5A). Correspondingly, Chi-B was shown to be elevated between 2.3- and 7.7-fold in AaERF1-overexpression lines (Figure 5B). The transcript levels of PDF1.2 were elevated between 15- and 1269-fold than that of the control (Figure 5C). The statistics analysis showed that the observed differences were statistically significant.

The AaERF1-overexpression lines were observed following inoculation with B. cinerea. For each of the AaERF1-overexpression lines, we observed a significant reduction in the development of disease symptoms in independent inoculation experiments. Four days following inoculation with B. cinerea, 79% of the control plants showed symptoms of infection, whereas only between 32% and 42% of the leaves from AaERF1-overexpression lines were symptomatic (Figure 6A, 6C). The statistics analysis showed that the observed differences were statistically significant. The three independent AaERF1i lines were inoculated with B. cinerea. The results showed that each of the AaERF1i lines had a significant reduction in the disease symptoms in three independent inoculations. Six days following inoculation with B. cinerea, most of the leaves in AaERF1i lines were dry and dead, while most of the control plants were growing well (Figure 7B). The results showed that AaERF1 was a positive regulator to the disease resistance to B. cinerea in Arabidopsis.
Discussion

The putative cis-acting elements of *AaERF1* promoter were predicted as shown in Figure 1A and summarized in Table 1. The W box (TTGAC) is the binding site for members of the WRKY family of transcription factors [20]. The importance of W boxes was illustrated by studies on Arabidopsis transcription during systemic-acquired resistance [21]. Previous reports indicated that the G-box-related hexamers CACNGTG, CACATG and (T/C)ACGTG are the binding sites of MYC2 [22–24]. MYC2 is a negative regulator of the JA-responsive pathogen defense genes PDF1.2 and B-CHI [25]. At -209bp of *AaERF1* promoter, there is

![Image A](image_url_a)

![Image B](image_url_b)

![Image C](image_url_c)

![Image D](image_url_d)

**Table 1.** Putative cis-acting regulatory elements involved in defense responsiveness in *AaERF1* promoter.

| Cis-elements | Motif and position | Putative function |
|--------------|--------------------|------------------|
| 5-UTR pyrimidine-rich stretch consensus: TTTCTTCTCCT | -1345 AGAGAAGAA -1336 | cis-acting element conferring high transcription |
| EIRE-box: TTGACC | -336 TTGACC -331 | elicitor responsive element |
| W-box consensus: TTGAC | -547 TTGAC -542; -336 TTGAC -332 | fungal elicitor responsive element |
| TGA-box: TGACGTCA | -209 TGACGTCA -201 | cis-acting element conferring plant defense responsiveness |
| G/C-box consensus: CACGTC | -1458 CACGTC -1453 | cis-acting element involved in light-induction or hormone control |
| TC-rich repeats: ATTTCTTCA | -590 ATTAGAGAAT -581 | cis-acting element involved in defense and stress responsiveness |

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a TGA motif, which is a perfect binding site for TGA transcription factors. TGA transcription factors are essential for the activation of JA and ET dependent defense mechanisms in Arabidopsis [26]. In addition, we identified the TC-rich repeats (ATTTTCTCCA) in the promoter of \textit{AaERF1}, which was previously described in tobacco (\textit{Nicotiana tabacum}) as cis-acting elements involved in defence and stress responsiveness [27]. All above elements are related to the disease resistance, implying that \textit{AaERF1} may have a similar function.

The results of RT-Q-PCR showed that the transcript level of \textit{AaERF1} was increased rapidly and peaked within 1 h after MeJA, ethylene or wound treatments, followed by a gradual decline. Jasmonates and ethylene are considered the major signal compounds for wound-induced gene expression in plants [28]. Methyl jasmonate was reported as a volatile compound emitted from the leaves of \textit{A. tridentata} subspecies \textit{tridentata} resulting in the induction of defense-related genes in nearby tomato plants [29]. So, the transcript of \textit{AaERF1} can be induced vigorously by MeJA, ethylene or wound treatments, implying that \textit{AaERF1} may play an important role in the JA and ET signaling pathways and have some function in disease resistance of \textit{A. annua}.

The bioinformatic analysis showed that the AP2/ERF domain of \textit{AaERF1} contained a $\beta$-sheet and $\alpha$-helix ($\beta$-$\alpha$ motif; see Figure S2A), all of which are important for DNA binding with the GCC Box [30,31]. The phylogenetic tree analysis of \textit{AaERF1} showed that \textit{AaERF1} had close relationship with \textit{AtERF2} and \textit{TaERF3}.
AtERF2 and TaERF3 have been well characterized and their functions were mainly related to disease resistance, at least in part, via binding to the GCC box in the promoter region of downstream genes [19,32–34]. So, all above analysis implied that the protein of AaERF1 has a function in disease resistance and may have the GCC Box binding ability.

From the results of EMSA and yeast one-hybrid experiment, we know that AaERF1 was able to bind to the GCC box cis-acting element in vitro and in yeast cells. The ERF subfamily of proteins recognizes the cis-acting element GCC box, which is mainly involved in the response to biotic stresses like pathogenesis [5]. Enhancement of disease resistance in plants has been achieved by overexpressing ERF proteins, such as Arabidopsis AtERF1 [8,35], AtERF2 [31] and rice OsBIERF3 [36]. So, we infer that the overexpression of AaERF1 could enhance the disease resistance in plants.

**Figure 4. The DNA binding ability of AaERF1 via GCC box.** A. Electrophoretic mobility shift assays on DNA binding of AaERF1. Lane 1: negative controls contain MBP plus labelled GCC probe; lane 2: the MBP–AaERF1 protein plus labelled GCC probe; lane 3: only labelled GCC probe; lane 4: negative controls contain MBP plus labelled mutated GCC probe; lane 5: MBP–AaERF1 plus labelled mutated GCC probe; lane 6: only labelled mutated GCC probe. The protein–GCC probe complex and free probes are indicated respectively by two arrows. B. GCC box binding analysis of AaERF1 using the yeast one-hybrid system. Sketch maps show the construction of vectors used in this experiment. Photographs show the growth behavior of transformants on SD/Trp–Ura–/X-gal medium. Sector 1: p178-4×GCC-LacZ+pB42AD-AaERF1; sector 2: p178+pB42AD-AaERF1; sector 3: p178-4×GCC-LacZ+pB42AD; sector 4: p178+pB42AD. doi:10.1371/journal.pone.0057657.g004
of RT-Q-PCR showed that the transcripts of *AaERF1*, *Chi-B* and *PDF1.2* showed an obvious correlated increase in *AaERF1*-overexpression lines, which were similar with the overexpression of *ORA59* in Arabidopsis [8] (Figure 5A, 5B and 5C). After the inoculation with *B. cinerea*, the control lines dried and died, while most of the *AaERF1*-overexpression lines were growing well (Figure 6). The results showed that overexpression of *AaERF1* could increase the resistance to *B. cinerea* in Arabidopsis.

Six days after inoculated with *B. cinerea*, nearly all the *AaERF1* transgenic *A. annua* showed symptoms of infection, while the control plant were growing well (Figure 7B). Yu *et al.* showed that *AaERF1* could directly bind to the CBF2 and RAA motifs present in both *ADS* and *CYP71AV1* promoters [17]. In the *AaERF1*-transgenic lines, as a result of reduced *ADS* and *CYP71AV1* gene expression, the contents of artemisinin and artemisinic acid were decreased to 76-58% and 55-30% of the wild-type level, respectively [17]. For large amounts of specialized metabolites are considered briefly and related to demonstrated or presumed roles in plant defense [38,39], the reduction of artemisinin and artemisinic acid may result in reduction of the resistance to *B. cinerea* in *A. annua*. From the above results, we conclude that *AaERF1* is a positive regulator of the resistance to *B. cinerea* in *A. annua*. 

Figure 5. The expression levels of *AaERF1*, *Chi-B* and *PDF1.2* in 35S::*AaERF1* transgenic Arabidopsis analyzed by RT-Q-PCR. Vertical bars represent standard deviation. A. The expression of *AaERF1* in the control and transgenic Arabidopsis plants. Values indicate the mean fold relative to sample the *AaERF1*-5 transgenic plants. B. The expression of *Chi-B* in the control and transgenic Arabidopsis plants. Values indicate the mean fold relative to sample the pCAMBIA2300+ empty vector transgenic plants. C. The expression of *PDF1.2* in the control and transgenic Arabidopsis plants. Values indicate the mean fold relative to sample the pCAMBIA2300+ empty vector transgenic plants. *Actin* is used as a control for normalization. Data are averages ± SE from three independent experiments. doi:10.1371/journal.pone.0057657.g005
In conclusion, the promoter of AaERF1 was cloned by genomic walking and the GUS staining results of AaERF1 promoter-GUS transgenic A. annua showed that AaERF1 is ubiquitously expressed in A. annua. The expression of AaERF1 can be induced vigorously by MeJA, ethephon and wound treatments, implying that AaERF1 may activate some of the defense genes via the JA and ET signaling pathways of A. annua. Electrophoretic mobility shift assay (EMSA) and yeast one-hybrid results showed that AaERF1 was able to bind to the GCC box cis-acting element in vitro and in yeast. The overexpression of AaERF1 could enhance the expression levels of Chi-B and PDF1.2 and increase the resistance to B. cinerea in the 35S::AaERF1 transgenic Arabidopsis. The downregulated expression level of AaERF1 evidently reduced the resistance to B. cinerea in A. annua. These data suggested that AaERF1 could not only regulate the artemisinin biosynthetic pathway, but also play important roles as a positive regulator of the resistance to B. cinerea in A. annua.

Materials and Methods

Plant Materials

The seeds of A. annua were obtained from the School of Life Sciences, Southwest University in Chongqing, P.R. China. The plants of A. annua were grown in a greenhouse. Arabidopsis thaliana

Figure 6. The 35S: AaERF1 lines show increased disease resistance. A. The numbers of control and the three independent 35S: AaERF1 transgenic Arabidopsis lines showing disease symptoms 4 d after inoculation with Botrytis cinerea. Average data with standard errors from three biological replicates are shown. B. The control and 35S: AaERF1 lines, without inoculation with Botrytis cinerea. C. The control and 35S: AaERF1, 4 d after inoculation with Botrytis cinerea, with 35S: AaERF1 plants showing reduced disease symptoms (see “Materials and Methods” for description). doi:10.1371/journal.pone.0057657.g006
ecotype Columbia-0 was used in this study and grown under 16 h light \((70 \text{ mmol m}^{-2}\text{s}^{-1})\) and 8 h dark cycle at 22 \(\text{C}\). Different tissues of \textit{A. annua} and Arabidopsis plants were collected for RNA extraction using plant RNA isolation reagent (Tiangen Biotech, Beijing) following the manufacturer’s instructions. The concentration of the purified RNA was quantified with a nucleic acid analyser (Nanodrop-1000, Nano).

**Isolation and Analysis the \textit{AaERF1} Promoter**

The upstream region of \textit{AaERF1} was amplified from the genomic DNA using the Genome Walker Kit (Clontech, Canada). The \textit{AaERF1}-specific primers (\textit{AaERF1-sp1, AaERF1-sp2, Adaptor Prime1} and Adaptor Prime2) were used following the manufacturer’s recommended procedures. The products amplified from the final reaction products were electrophoresed in 1% agarose gel, and a 1543 bp fragment was eluted from the gel and cloned into the pMD18-T-simple vector. The insert DNA was sequenced by Shenzhen Genomics Institute. The sequence obtained was searched for putative cis-acting elements previously characterized using the PlantCare software (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

**β-galactosidase (GUS) Expression in Transgenic \textit{A. annua}**

To generate the \textit{AaERF1} promoter-GUS construct, the 5’-flanking DNA of the \textit{AaERF1} coding region was amplified with \textit{AaERF1-PF} and \textit{AaERF1-PR}. The 1.5 kb PCR fragment was cloned into the pCAMBIA1391Z vector for sequence confirmation. The construct was transformed into \textit{A. annua} plants as described previously [40]. Histochemical staining for GUS activity in transgenic plants was performed as the protocol described.
previously [41]. Plants transformed with pCAMBIA1391Z were used as a parallel negative control.

**Hormonal and Stress Treatments**

*Arabidopsis annua* plants grown in MS medium for 2 weeks were treated with solutions of 100 μM MeJA (Sigma Aldrich, USA) and 500 μM Ethenoph. Ethenoph, an ethylene releaser, was used as ethylene replacement [42]. Since ethephon on hydrolysis releases ethylene and phosphorus, therefore the disodium hydrogen phosphate buffer (Na2HPO4, 5 mM) was prepared for the decomposition of ethephon. 100 μl ethephon solution was mixed with 100 μl disodium hydrogen phosphate (3 mM). Ten plants were transferred to new petri dishes and pooled for each treatment. For wound induction, the same mixed plants of *A. annua* were cut some 2–3 mm nicks and kept at 22°C under humidified conditions. For the hormonal treatments, all the plants were collected before treatment (0 h) and at different time point after treatment (1 h, 3 h, 6 h, 12 h, 24 h) for the gene expression analysis. For the wound treatments, a time point of 0.5 h was added.

**Expression Pattern Analysis of AaERF1 by RT-Q-PCR**

Expression patterns of AaERF1 in response to hormonal and stress treatments of *A. annua* were analyzed using the RT-Q-PCR method. The expression levels of AaERF1, Chi-B and PDF1.2 in *AaERF1*-overexpressing Arabidopsis were also analysed by this method. All RNA samples were digested with DNase I (RNase-free) prior to use. Aliquots of 0.4 μg total RNA were employed in the reverse transcription reaction using random hexamer primers for the synthesis of first strand cDNA. The amplification reactions of qRT-PCR were performed on an iCycler iQTM Real-Time PCR Machines (Bio-Rad, Watford, UK) with gene-specific primers (Table S1), and the SYBR ExScript RT-PCR kit (Takara, Shiga, Japan) protocol to confirm changes of gene expression. The DDCT method. All experiments were repeated three times.

**Comparative and Bioinformatic Analysis**

Based on the sequence of AaERF1 [JN162091], one pair of primers (AaERF1-F and AaERF1-R) were designed, synthesized and used to amplify the full-length sequence of AaERF1 from *A. annua*. Comparative and bioinformatic analysis of AaERF1 protein were carried out online at the websites, http://www.ncbi.nlm.nih.gov and http://cm.expasy.org. Sequence analysis was performed using DNAMAN software (Lynnon Biosoft, USA) and Vector NTI software (Invitrogen). The phylogenetic analysis of AaERF1 protein and ERF proteins from other species was carried out by Clustal X (1.81) using default parameters. A phylogenetic tree was constructed by neighbor-joining method using software MEGA version 3.1 [43,44].

**Electrophoretic Mobility Shift Assay**

The AaERF1 cDNA sequence was cloned into the EcoRI and PstI sites of the pMAL-C2 vector to produce a MBP-AaERF1 fusion construct (New England BioLabs). The sequenced pMAL-C2-AaERF1 construct was introduced into *E. coli* BL21 for expression. Fusion proteins were expressed in BL21 cells by adding 0.5 mM IPTG to culture medium for 7 h at 28°C and purified using amylose resin (New England BioLabs). The purified recombinant protein was quantified using the Bradford assay (2-D Quant Kit, Amersham Biosciences Corp., San Francisco, CA, USA). The 3′ end biotin-labelled oligonucleotides for the GCC box and the mutated mGCC box were synthesized (Sangon) and equimolar pairs were annealed using the protocol provided by Sigma (Table 1). The binding reaction, gel preparation and electrophoretic mobility-shift assay (EMSA) were performed following the protocol of Zhang et al. [45].

**Yeast one-hybrid**

To analyze the GCC-binding activity of AaERF1 protein, the entire encoding regions of *AaERF1* was fused into the *BamHI*-Xhol sites of the activation domain of the pB42AD vector (pAD). Reporter vectors containing the 4 × GCC lacZ was prepared and integrated into the yeast strain EGY48, inserted upstream of the minimal promoter element (MP) and the reporter gene lacZ existed in the vector p178. Vectors were introduced into yeast strain through LiAc mediated transformation method (Clontech, Shanghai, China). The cells were grown on tryptophan- and uracil-deficient SD medium for 2–3 days at 30°C, and then transferred to 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) containing plates for color change observation [46].

**Overexpression in *A. thaliana***

The full-length AaERF1 coding sequence was amplified with primers AaERF1-F and AaERF1-R by Platinum PrimeSTAR HS DNA polymerase (Takara, Shiga, Japan) and subcloned into pMD18-T simple vector. The pMD18-T-AaERF1 vector was digested by *SacI* and *BamHI*. The full-length ORF of AaERF1 was cloned into the *BamHI* and *SacI* sites of the pCAMBIA2300+ vector under the 35S promoter to generate pCAMBIA2300-35S::AaERF1::NOS. The construct was transferred into *Agrobacterium tumefaciens* GV3101, and then introduced into *A. thaliana* (ecotype Columbia) plants using the floral dip method [47]. Transgenic plants were selected on MS plates containing 50 μg/mL kanamycin. PCR was performed to verify the transgenic status of the screened plants.

**RNAi in *A. annua***

The 201 bp fragment of AaERF1, corresponding to AaERF1 cDNA from nucleotides 179–378, was cloned from A. annua by RT–PCR. In RT–PCR, AaERF1i-F (with *Xhol* and *XhoI* sites) and AaERF1i-R (with *BamHI* and *HindIII* sites) were used as the forward and reverse primers, respectively. The amplified fragment was cloned into pMD18-T simple vector and sequenced. After confirmation by sequencing, the fragments were forwardly and reversely placed on the two end sides of the GUS intron in pBlueScript SK+ to construct the hp structure. Then the expression cassette was excised with *SacI* and *KpnI* from pBlueScript SK+ containing the AaERF1 hp structure and ligated into the expression vector pCAMBIA2300+ to get the final hp AaERF1-containing vector pCAMBIA2300:: p35S::hairpin AaERF1-nos and pCAMBIA2300:: vector containing only nptII (neomycin phosphotransferase gene conferring resistance to kanamycin) was used as the control vector in transformation. The pCAMBIA2300:: p35S::hairpin AaERF1-nos vector was then transformed into A. tumefaciens strain EHA105 by a conventional freezing and thawing method, and the resulting strains were used in the transformation of A. annua. The transformation of A. annua was performed following the protocol of Zhang et al. [40]. All the primers used in this study are in the Table S1.
Pathogen Infections

*Botrytis cinerea* was grown on potato dextrose agar plates for about 2 weeks at 26°C. Spores were collected from the cultures and washed twice with sterile water. Washed spores were suspended by 10 mL of sterile water, and the suspension was filtered through miracloth to remove mycelia. Four-week-old plants were spray with spore suspensions (2 × 10^5 spores mL^-1) and maintained under high humidity. Disease development was observed over the following 6 d. Inoculated plants were scored based on the presence under high humidity. Disease development was observed over the following 6 d. Inoculated plants were scored based on the presence of any disease symptoms after 4 d inoculation with *B. cinerea*, including chlorosis of the leaves, curling and necrosis of the leaves. For each of the control and transgenic plant lines, three biological replications were performed in parallel.

Supporting Information

Figure S1 Gus-staining of transgenic *A. annua* using the pCAMBIA1391Z empty vector plasmid. (TIF)

Figure S2 Comparison of AP2/ERF domain sequences and dendrogram of ERF proteins. A. Amino acid alignment of the AP2/ERF domains between *AeERF1* and ERF proteins. Highly conserved residues in all the sequences are indicated in white with black background and only partially conserved residues in ERF proteins are showed in black with grey background. One α-helix and three β-sheets are marked above the corresponding sequences. The YRG and RAYD elements are indicated with solid lines below the consensus sequence. B. A phylogenetic tree of the ERF proteins was constructed. Alignments were made in Clustal X using the default parameters. Accession numbers for the AP2/ERF proteins are showed in black with grey background. One white with black background and only partially conserved residues Highly conserved residues in all the sequences are indicated in black with white background. (DOC)

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**AeERF1 Regulates the Resistance to *B. cinerea***

**Figure S3 Analysis of transgenic *A. annua* plants by PCR.** A. PCR analysis of 35S forward primer and *AeERF1* reverse primer in *AeERF1*-RNAi transgenic plants. M: DNA size marker DL2000, V: empty-vector transgenic *A. annua*, C: water control, P: positive control. B. PCR analysis of 35S forward primer and the reverse primer of kanamycin-resistant gene in transgenic *AeERF1*-RNAi transgenic plants. (TIF)

**Table S1** Primers used in this study. (DOC)

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Author Contributions

Conceived and designed the experiments: XL KXT. Performed the experiments: XL WMJ GFW. Analyzed the data: XL WMJ LZ. Contributed reagents/materials/analysis tools: FZ FYZ QS. Wrote the paper: XL.
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