The *Arabidopsis thaliana* Homeobox Gene **ATHB12** Is Involved in Symptom Development Caused by Geminivirus Infection

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**Abstract**

**Background:** Geminiviruses are single-stranded DNA viruses that infect a number of monocotyledonous and dicotyledonous plants. *Arabidopsis* is susceptible to infection with the Curtovirus, *Beet severe curly top virus* (BSCTV). Infection of *Arabidopsis* with BSCTV causes severe symptoms characterized by stunting, leaf curling, and the development of abnormal inflorescence and root structures. BSCTV-induced symptom development requires the virus-encoded C4 protein which is thought to interact with specific plant-host proteins and disrupt signaling pathways important for controlling cell division and development. Very little is known about the specific plant regulatory factors that participate in BSCTV-induced symptom development. This study was conducted to identify specific transcription factors that are induced by BSCTV infection.

**Methodology/Principal Findings:** *Arabidopsis* plants were inoculated with BSCTV and the induction of specific transcription factors was monitored using quantitative real-time polymerase chain reaction assays. We found that the ATHB12 and ATHB7 genes, members of the homeodomain-leucine zipper family of transcription factors previously shown to be induced by abscisic acid and water stress, are induced in symptomatic tissues of *Arabidopsis* inoculated with BSCTV. ATHB7 expression is correlated with an array of morphological abnormalities including leaf curling, stunting, and callus-like structures in infected *Arabidopsis*. Inoculation of plants with a BSCTV mutant with a defective c4 gene failed to induce ATHB12. Transgenic plants expressing the BSCTV C4 gene exhibited increased ATHB12 expression whereas BSCTV-infected ATHB12 knock-down plants developed milder symptoms and had lower ATHB12 expression compared to the wild-type plants. Reporter gene studies demonstrated that the ATHB12 promoter was responsive to BSCTV infection and the highest expression levels were observed in symptomatic tissues where cell cycle genes also were induced.

**Conclusions/Significance:** These results suggest that ATHB7 and ATHB12 may play an important role in the activation of the abnormal cell division associated with symptom development during geminivirus infection.

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**Introduction**

Viral pathogens can induce dramatic morphological and developmental changes in plants [1,2]. Geminiviruses are small, generally phloem-limited, ssDNA viruses with a genome size of 2.6–5.2 kb that infect a wide range of both monocotyledonous and dicotyledonous plants, including several economically important crop species [3]. *Beet curly top virus* (BCTV) and *Beet severe curly top virus* (BSCTV) are Curtoviruses, which are monopartite, leafhopper-transmitted viruses that infect only dicotyledonous plants. These two viruses are very similar in both genome structure and pathogenesis, but BSCTV causes more severe symptoms than BCTV [4,5,6]. The viral genome of BSCTV encodes seven open reading frames (ORFs). The protein products of these genes are involved in viral structure and insect vector transmission (capsid protein, V1), replication (C1), pathogenicity (C2) replication enhancement (C3), movement (V1, V3), ssDNA accumulation (V2), and symptom development (C4) [7,8,9,10,11].

In *Arabidopsis*, symptoms of BCTV and BSCTV infection can range from mild to severe and are dependent on the interactions between the virus strain and *Arabidopsis* ecotype that is infected [4,5]. Symptoms in *Arabidopsis* include stunting of the plant, leaf curling, malformation of floral tissue, swelling of inflorescence bolts, anthocyanin production, and in some cases death of the
plant [4,5]. Because of its limited coding capacity, BSCTV must depend on the host cell to supply most of the proteins that are necessary for it to complete its life cycle, especially those proteins involved in the replication of viral DNA. BSCTV must therefore either infect cells already in S-phase or alter the state of the cells so that they are competent to synthesize DNA, a strategy employed by vertebrate RNA and DNA transforming viruses [12,13,14]. Several studies of the role of BCTV ORF C4 in symptom development and its potential interaction with host cell proteins have been completed [11,15,16,17]. Mutations in BCTV ORF C4 have a pronounced effect on symptom development in several hosts, including Nicotiana benthamiana and Beta vulgaris. N. benthamiana plants infected with ORF C4 mutant viruses showed greatly reduced or mild symptoms [17] whereas infected B. vulgaris plants were asymptomatic [11].

Expression of the BCTV and BSCTV C4 proteins in transgenic Arabidopsis results in phenotypes that mimic symptoms seen during viral infection [18,19]. Taken together, these results demonstrate that C4 plays an important role in symptom development in plants and that the induction of aberrant cell division is a key component of BCTV- and BSCTV-induced developmental abnormalities.

It is currently not clear how geminivirus-encoded proteins interact with host components to induce the pathomorphogenic phenotypes associated with virus infection. Homeobox genes that are key regulators of plant development have been implicated to be involved in the process of pathomorphogenesis [20]. However, a direct correlation between pathomorphogenesis and homeobox genes has not yet been established. The Arabidopsis genome encodes a large number of homeobox genes that can be categorized into six major families [21]. A family unique to plants is the homeodomain-leucine zipper (HD-Zip) transcription factors that include both a homeodomain and a leucine zipper motif [22,23,24,25,26,27,28]. HD-Zip proteins are important regulators of plant development, including the integration of environmental stimuli with developmental pathways [21,29].

Arabidopsis contains 47 HD-Zip genes that are grouped into four distinct subfamilies based upon gene structure and function. The expression of the Arabidopsis Class II ATHB2 and ATHB4 genes increases considerably after treatment with far-red-rich light [30]. The ATHB7 (Class I) and ATHB8 (Class III) genes are induced by exogenous plant hormones including abscisic acid (ABA) and auxin [31,32], and ATHB7 has been shown to be induced by drought [33,34]. ATHB12, isolated from A. thaliana in 1998 [34] and characterized as a member of the Class I HD-Zip family [21,33], is induced by ABA and water stress [33,34,35,36,37]. The ATHB12 gene also is expressed in all organs of the plant and is similar to the paralogous gene, ATHB7. ATHB12 and ATHB7 share similarities in sequence, a common intron-exon organization, and similar specificities in DNA binding that distinguish the two paralogs from other Class I HD-Zip genes in Arabidopsis [34,37] and appear to be key factors in the integration of development with abiotic stress.

In this study, we found that both ATHB12 and ATHB7 expression were induced by BSCTV infection of Arabidopsis and that BSCTV-induced symptom development was tightly correlated with the transcriptional activation of ATHB12. Infection of an ATHB12 T-DNA insertion mutant demonstrated that ATHB12 is necessary for complete symptom development. These results demonstrate that unique HD-Zip proteins are involved in the pathomorphogenesis caused by the biotic stress imposed on Arabidopsis during BSCTV infection and identifies a new class of host proteins that regulate host responses to virus infection.

Results

Expression of ATHB12 in BCTV-infected Arabidopsis

Since BCTV- and BSCTV-induced symptom development likely depends on changes in gene expression, we conducted a preliminary screen using DNA microarrays to compare the gene expression patterns in inflorescence tissues from mock-inoculated and BSCTV-infected plants. This preliminary screen suggested that ATHB12 was strongly induced in symptomatic tissues of BSCTV-infected Arabidopsis plants and further studies focused on confirming this result.

BSCTV-infected Arabidopsis showed the typical phenotypes of stunted inflorescence stems, leaf and stem curling, and malformation of inflorescence shoot tip and auxiliary bud structures caused by abnormal cell division (Figure 1). Quantitative real-time polymerase chain reaction (RT-PCR) assays revealed that ATHB12 transcript levels were lowest in the inflorescence shoot tip and highest in the root in uninfected plants (Figure 2A). BSCTV infection significantly modulated the transcript levels of ATHB12 in different organs within three weeks post-infection (Figure 2B). ATHB12 transcript levels in the inflorescence shoot tip were approximately 2-fold higher in BSCTV-infected plants compared to mock-inoculated plants. At the same time, ATHB12 expression in the inflorescence stems or roots of BSCTV-inoculated plants was reduced 2-4 fold. Analysis of ATHB12 transcript levels using semi-quantitative PCR detected gene expression 7 days after BSCTV infection in the shoot tips of Arabidopsis, and expression continued to rise for up to 21 days in parallel with increasing symptom development (Figure 2C).

Activation of ATHB12 promoter activity by BSCTV infection

To determine if the increased ATHB12 transcript levels observed after BSCTV infection were due to transcriptional activation, we utilized a reporter gene approach based on histochemical staining of transgenic plants expressing an ATHB12 promoter-gusA fusion. Figure 3A depicts the constructs used in these experiments. Three different putative promoter regions comprised of 236, 1,472, and 2,124 bp of the 5’ upstream region of the ATHB12 gene transcriptionally fused to the E. coli gusA gene encoding β-glucuronidase (GUS) [38] were introduced into Arabidopsis. Evaluation of GUS activity in uninoculated seedlings demonstrated that GUS activity increased depending on the size of the ATHB12 promoter (Figure 3B). The shortest promoter (p0.3) exhibited very little expression which was localized to disrect regions at the tips of the cotyledons. The 1,472 bp (p1.6) promoter conferred significant expression in the roots, but not in the shoot. The 2,124 bp promoter (p2.2) conferred the highest level of GUS activity in seedlings, with significant expression in both the root and the shoot. To test the effects of BSCTV infection on ATHB12 promoter activity, transgenic plants containing the p2.2 promoter were inoculated with BSCTV. Histochemical localization of GUS activity was examined in inflorescence stems from plants showing varying levels of symptom development ranging from moderate to severe. The intensity of GUS activity correlated with the severity of symptoms in the shoot tips (Figure 3C). To determine if ATHB12 promoter activity was associated with induction of cell cycle genes, similar studies were conducted with transgenic Arabidopsis plants that expressed a reporter gene composed of the CDC2a promoter fused to the gusA gene [39]. Histochemical staining for GUS activity showed that cells in the symptomatic tissues at the shoot tip of the transgenic plants expressed high GUS activity and the overall pattern of expression was similar to that observed in the ATHB12-gusA
plants (Figure 3C). In mock-inoculated control plants, CDC2a and ATHB12 promoter activity was limited to the elongation region of the inflorescence stem and in developing flower buds at three weeks after inoculation (Figure 3C and 3D).

BSCTV-induced expression of ATHB12 requires C4

Previous studies in N. benthamiana and B. vulgaris suggest that the C4 gene is required for symptom development in BCTV-infected plants. To determine if the BCTV C4 gene was required for the induction of ATHB12, ATHB12 accumulation was compared in BSCTV-infected plants, BSCTV c4 mutant-infected plants, and transgenic plants expressing the BSCTV C4 gene under the control of the Cauliflower mosaic virus (CaMV) 35S promoter (Figure 4A). The expression of ATHB12 increased significantly in BSCTV-infected plants and transgenic Arabidopsis constitutively expressing the BSCTV C4 gene. In contrast, the level of ATHB12 increased only slightly in BSCTV c4-mutant infected Arabidopsis. Since previous studies have shown that ATHB7 is co-regulated with ATHB12 in response to ABA and water stress, we also examined the expression of ATHB7 in this experiment (Figure 4B). The expression pattern of ATHB7 was very similar to that observed for ATHB12; ATHB7 transcripts were higher in BSCTV infected plants and transgenic lines expressing C4 whereas plants inoculated with the BSCTV c4 mutant showed little induction.

To determine if ATHB12 expression is required for symptom development in BSCTV-infected plants, an ATHB12 knock-down (KD) mutant [40] and the wild-type background ecotype Ws-O were inoculated with BSCTV. Inoculated plants were then assessed for symptom severity. ATHB12 KD plants developed fewer symptoms in response to BSCTV infection (Table 1). Of the inoculated ATHB12 KD plants, 14% were asymptomatic, 44% had mild symptoms and 42% developed severe symptoms. In contrast, a larger number of inoculated wild-type ecotype Ws-O plants displayed severe (68%) and mild symptoms (31%) and only 1% of Ws-O plants were asymptomatic after BSCTV infection.

Discussion

Since geminiviruses have a very compact genome that does not encode essential replication proteins, they are highly dependent on the recruitment of plant-host factors to replicate and complete their life cycle [41]. Earlier studies have shown that Tomato golden mosaic virus (TGMV) induces the transcription and accumulation of proliferating cell nuclear antigen (PCNA) in cells that normally do not express PCNA [42,43]. These studies provided one of the first indications that geminiviruses have the ability to reprogram plant cells to support viral replication. A more recent study demonstrates that geminivirus expression can affect the expression of over 3,000 genes [44]. The BCTV/BSCTV interaction with Arabidopsis provides an excellent system to study this reprogramming since in this case, viral infection leads to the induction of the cell cycle and aberrant cell divisions [4,5,18,19,45]. How BCTV/BSCTV infection results in aberrant cell division is not clear, however it is likely that it requires the activation of specific genes in infected cells. RKP, a putative RING finger E3 ligase protein with homology to the human cell cycle regulator KPC1 has been shown to be induced by the BSCTV symptom determinant C4 [15]. The BCTV and BSCTV C4 proteins have been shown to interact with Arabidopsis shaggy-related protein kinases [16,46] and the AL1 protein interacts with the kinases GRIK1 and GRK2 [47], suggesting that effects on key signal transduction pathways may be important in symptom development. Similarly, the BCTV and Spinach curly top virus (SCTV) C2 proteins and the TGMV AL2 protein interact with Arabidopsis AKIN11, a SNF1-related kinase, and adenosine kinase 2 (ADK2) [48,49,50]. Interactions have also been identified that appear to affect hormonal regulation of plant cell division and development. The Tomato yellow leaf curl China virus (TYLCCNV) BC1 protein has been shown to interact with ASYMMETRIC LEAVES 1 (AS1) and to partially suppress JA-controlled responses [51]. The TGMV AL2 and SCTV C2 proteins have recently been shown to induce cytokinin-responsive
genes [52]; this may be a result of the previously described interaction with ADK2 [48]. This is consistent with other studies showing that geminivirus-induced symptoms may involve changes in hormone signaling [1,5,45].

Our current study demonstrates that the ATHB7 and ATHB12 homeobox genes that are known to be regulated by ABA and water stress [33,34,35] are induced in Arabidopsis by BSCTV infection. The induction of these homeobox genes was correlated with the induction of aberrant cell division and the development of severe symptoms such as leaf curling, stunting, and formation of callus-like structures. Many plant homeobox genes are related to hormonal signaling [31,33,34,53,54] and alteration of hormonal balance is a well-known feature of a number of plant-pathogen interactions [35]. BCTV and BSCTV induces typical symptoms similar to those caused by an imbalance of auxin concentration in symptomatic tomato and Arabidopsis tissues and is correlated with increased promoter activity of auxin-induced genes [1,5,45]. BSCTV induction of ATHB7 and ATHB12 is not likely due to effects on auxin. Previous studies have shown that hormonal control of these genes is regulated primarily by ABA in Arabidopsis [33,34,35], which is consistent with our studies of p2.2-gusA transgenic plants treated with auxin that showed auxin does not induce ATHB12 promoter activity (data not shown). The induction of ATHB12 and ATHB7 suggests that BSCTV infection also may alter ABA levels or that there is an ABA-independent mechanism for activating ATHB7 and ATHB12 expression. Other studies [16] have shown that the BCTV C4 protein binds to Arabidopsis shaggy-related kinase AtSK1, a negative regulator of the brassinosteroid signaling pathway, although there is no evidence that brassinosteroid levels are affected by BSCTV infection. We found that brassinosteroid treatment of wild-type seedlings or ATHB12-promoter-gusA transgenic plants did not induce ATHB12 transcripts or GUS expression, respectively (data not shown). Thus, the induction of ATHB12 expression probably does not directly involve brassinosteroids.

A role for ATHB12 induction in symptom development is supported by our studies of transgenic Arabidopsis expressing the BSCTV C4 gene and the ATHB12 KD line. The BSCTV ORF C4 gene is an important factor in symptom development [11,17] and C4-expressing transgenic plants show systemically abnormal development that is similar to BSCTV-induced symptoms in inflorescence shoot tips. We found that the expression level of ATHB12 was correlated with the severity of abnormal development in C4 transgenic plants and that infection of Arabidopsis with a BCTV c4 mutant did not induce significant symptom development or ATHB12 gene expression, implying a relationship between the functions of the BCTV C4 protein and the effects of ATHB12 induction on symptom development. This is consistent with the observation that BSCTV-infected ATHB12 KD plants with reduced ATHB12 transcript levels exhibited significantly milder symptoms than wild-type plants. The fact that the ATHB12 KD plants exhibited some symptom development is likely due to the fact the KD plants express approximately 20–30% of normal levels of ATHB12 transcripts and that ATHB7 expression is not affected in the KD plants. Taken together, our data suggest that the ATHB7 and ATHB12 genes may be important factors in BSCTV-induced symptom development. This is particularly interesting since these two homeobox genes have previously been found to be important in coordinating abiotic stress signals with growth and development [21,29]. The fact that ATHB7 and ATHB12 are induced by BSCTV infection suggest that alterations in ABA biosynthesis or localization may be involved in symptom development. Further genetic and biochemical studies using mutant Arabidopsis ATHB7 and ATHB12 plants, hormone metabolism mutants, and BSCTV mutants are needed to determine the precise connection between BSCTV C4 and ATHB7/ATHB12 induction with the activation of abnormal cell division that is associated with BSCTV-induced symptom development.

Materials and Methods

Plant growth and virus inoculation

Arabidopsis ecotype Col-0 was obtained from the Arabidopsis Biological Resource Center at the Ohio State University. The pCDC2 transgenic line, which was made by fusion of the CDC2 promoter with the gusA gene, was provided by Dr. Von Montague [39] and the transgenic Col-0 constitutively expressing BSCTV ORF C4 was previously described [19]. The ATHB12 KD mutant [40] was kindly provided by Dr. Cheon, Sookmyung Women’s University, Korea. Seeds were planted in flats containing artificial soil, covered with plastic domes, and then placed and maintained in growth chambers at 18–22°C and 50–80% relative humidity, with a day length of 10 or 12 hrs from fluorescent bulbs at an
The intensity of 100–200 uE m$^{-2}$ s$^{-1}$ [4]. Plastic domes were removed 10–14 days after sowing when seedlings were well established. Four- to five-week-old plants were inoculated with infectious viral DNA by agroinoculation of wounds produced in the crown of the rosette by needle puncture, as previously described [4,56]. Control mock-inoculated plants were agroinoculated with *Agrobacterium tumefaciens* GV3101 harboring an empty pMon521 vector.

**BSCTV strain**

The isolation and characterization of full-length infectious clones of BSCTV has been previously described [6]. The BSCTV clone was provided by Dr. Drake Stenger (Agricultural Research Service, United States Department of Agriculture, Parlier, California, USA) and the BSCTV leftward ORF C4 mutant clone was provided by Dr. Kenneth Buckley (Ohio State University, Columbus, Ohio, USA).

**Construction of ATHB12 promoter-gusA transgenic Arabidopsis lines**

A putative full-length promoter that is responsive to ABA [34,35] was generated by constructing a transcriptional fusion of the 2.1 kb-5' upstream of *ATHB12* to the β-glucuronidase reporter gene (*gusA*) in vector pBI121 [57]. To define the possible location of the cis-acting region responsible for BSCTV infection, smaller segments of this 2.1 kb region were generated by PCR and fused with GUS. All vectors were confirmed by DNA sequencing and transferred to *A. tumefaciens* strain GV3101. The resulting *A. tumefaciens* strains were used to transform Arabidopsis plants as described [19]. The following primer sequences were used: p2.2, forward: 5'-GAA TCT CTG AGT TGC TGA TAT TGG C-3' and reverse: 5'-GGA TCT CTG AGT TGC TGA TAT TGG C-3'; p1.6, forward: 5'-GAA TCT CTG AGT TGC TGA TAT TGG C-3' and reverse: 5'-TTG ACT AAT AAA AGT ATC GCA AGG C-3'; p0.3, forward: 5'-GAA TCT CTG AGT TGC TGA TAT TGG C-3' and reverse: 5'-ATG GCT TAA GGA TAA AGT GAC GTT G-3'; and p0.2, forward: 5'-GAA TCT CTG AGT TGC TGA TAT TGG C-3' and reverse: 5'-CTA CTT GTC TGA AAT GTC TAC AGA T-3'.

**Histochemical staining for GUS activity**

Tissues were stained for GUS activity in solutions containing 100 mM sodium phosphate, pH 7.0, 10 mM EDTA, 0.1% Triton X-100, 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-GlucU, cyclohexylammonium salt (X-Gluc), 100 μg/mL chloramphenicol, 2 mM potassium ferricyanide, and 2 mM potassium ferrocyanide, as previously described [58]. In brief, tissues were harvested directly into a volume of staining solution sufficient to cover the tissue and placed under a house vacuum for 10 min. Staining was carried out at 37°C in the dark for 48 hr. After staining, the tissue was incubated in 70% ethanol to remove the chlorophyll. The ethanol was changed several times until the tissue was clear.

**Total RNA isolation and quantitative RT-PCR**

RNA isolation and analysis was performed as described by [59]. To analyze the quantitative expression pattern of each gene by RT-PCR, the cDNA strand was synthesized from 2 μg of total RNA using oligo (dT) primers and Moloney murine leukemia virus (MMLV) reverse transcriptase (SuperBio Co., Korea). Primers...
ATHB12 and Geminivirus Symptoms

Figure 4. Expression of ATHB12 and ATHB7 in plants infected with wild-type BSCTV or a BSCTV c4 mutant. Quantitative RT-PCR was used to determine the relative expression levels of ATHB12 in the inflorescence shoot tips of infected plants and mock-inoculated Col-0 compared to the transcript levels observed in a transgenic line expressing BSCTV C4. Note that both ATHB12 and ATHB7 transcript levels are significantly induced by wild-type BSCTV whereas no significant induction was seen in plants inoculated with the c4 mutant virus. Transgenic Arabidopsis that constitutively expressed BSCTV C4 exhibited significantly higher expression of ATHB12 and ATHB7 than wild-type mock inoculated plants. The data shown represent the mean ± S.D. of three or four experiments; different letters above the bars refer to significant differences between mean values as determined using a Tukey’s Multiple Comparison Test (P<0.05).

Author Contributions
Conceived and designed the experiments: KRD SL CIC. Performed the experiments: JP HJL YSH. Analyzed the data: KRD SL CIC. Wrote the paper: KRD SL.

References
1. Smith S, McCall S, Harris J (1968) Auxin transport in curly top virus-infected tomato. Phytopathology 58: 1669–1670.
2. Lucas W, Balachandran S, Park J, Wolf S (1996) Plasmodesmal companion cell-mesophyll communication in the control over carbon metabolism and phloem transport: insights gained from viral movement proteins. J Exp Bot 47: 1119–1128.
3. Matthews R (2001) Plant virology, Fourth Edition. San Diego: Academic Press 1056 p.
4. Lee S, Stenger DG, Bisaro DM, Davis KR (1994) Identification of loci in Arabidopsis that confer resistance to geminivirus infection. Plant J 6: 525–535.
5. Park J, Hwang H, Shin H, Im K, Auh CK, et al. (2004) Altered cell shapes, hyperplasia, and secondary growth in Arabidopsis caused by beet curly top geminivirus infection. Mol and Cells 17: 117–124.
6. Stenger DC, Carbonaro D, Duffus JE (1990) Genomic characterization of phenotypic variants of beet curly top virus. J of Gen Virol 71: 2211–2215.
7. Briddow RW, Watts J, Markham PG, Stanley J (1988) The coat protein of beet curly top virus is essential for infectivity. Virology 172: 620–633.
8. Frischmuth S, Frischmuth T, Latham JR, Stanley J (1993) Transcriptional analysis of the virion-sense genes of the geminivirus beet curly top virus. Virology 197: 312–319.
9. Hormuzdi SG, Bisaro DM (1995) Genetic analysis of beet curly top virus: evidence for three virion sense genes involved in movement and regulation of single- and double-stranded DNA levels. Virology 193: 900–909.
10. Hormuzdi SG, Bisaro DM (1995) Genetic analysis of beet curly top virus: examination of the roles of L2 and L3 genes in viral pathogenesis. Virology 206: 1044–1054.
11. Stanley J, Latham JR, Pinter MS, Bedford I, Markham PG (1992) Mutational analysis of the monopartite geminivirus beet curly top virus. Virology 191: 396–405.
12. Hunter T (1991) Cooperation between oncogenes. Cell 64: 249–270.

Table 1. Symptom severity and infectivity of BSCTV in the ATHB12 KD and Ws-O.

| Plant Line | Symptom Severity | *Asymptomatic | *Mild Symptoms | *Severe Symptoms |
|------------|-----------------|---------------|----------------|-----------------|
| Ws-O       | n/N (%)         | 23/74 (31)    | 50/74 (68)     |                 |
| ATHB12 KD  | n/N (%)         | 31/71 (44)    | 30/71 (42)     |                 |

*Asymptomatic: inoculated plants were indistinguishable from mock-inoculated plants and inflorescence bolts did not exhibit any abnormal structures.
*Mild symptoms: Mild curling of inflorescence shoot tips and inflorescence bolt height was comparable to control plants.
*Severe symptoms: Severe curling of bolts, malformed inflorescence structures, and severe stunting of plants.

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Australia) included 30 cycles of 10 sec denaturation at 94°C, 15 sec annealing at 60°C, and 20 sec polymerization at 72°C. The products of quantitative RT-PCR assays were analyzed by 1% agarose gel electrophoresis and showed a single band with the size predicted in the template sequence. Two negative controls, one without the total RNA template or reverse transcriptase, were included in each experiment. The housekeeping Arabidopsis actin2 gene (U41990) was used as an internal normalization standard to quantify the target mRNA (forward: 5’GAAAGATATGCATCGACTTTCCTA’3’ and reverse: 5’ACATACATAGGG-GAGCGTTAAAGG3’). Each data point represents the average of three or four experiments and the error bars represent the standard deviation. Statistical analyses were done using GraphPad Prism (GraphPad Software, USA) and included a one-way ANOVA with a Tukey’s Multiple Comparison Test for significant differences.

For semi-quantitative PCR, cDNA synthesis was done as described for RT-PCR and the subsequent PCR reactions with a gene-specific primer set were carried out with the following cycling conditions: 94°C for 5 min, 25 cycles of 94°C for 1 min, annealing temperature as specified for 1 min, and 72°C for 1–2 min, with a single final extension at 72°C for 5 min. The actin2 gene was used as an internal control. Gene expression patterns between mock and infected groups were compared following electrophoresis on 1.5% agarose gels.

used for the quantitative RT-PCR were designed by Primer3 (http://www-genome.wi.mit.edu/cgi bin/primer/ primer3.cgi) and synthesized by Bioneer (Bioneer Co., Korea). The primers used were ATHB12, forward: 5’-GGTTAGACCAAGGGAGGTTCTTATGTGTA-3’ and reverse: 5’-CAATTCCTCAGAAAGATGCTAAAGCAACT-3’; ATHB7, forward: AAGATGAATGGGGTCACTCA, and reverse: ACACTTTAGCAGCATGGTTCAACATG. Quantitative RT-PCR amplification was done using the modification of Schefc et al. [60]. PCR conditions using a Rotor-Gene 3000 (Corbett Research Co., Australia) included 30 cycles of 10 sec denaturation at 94°C, 15 sec annealing at 60°C, and 20 sec polymerization at 72°C. The products of quantitative RT-PCR assays were analyzed by 1% agarose gel electrophoresis and showed a single band with the size predicted in the template sequence. Two negative controls, one without the total RNA template or reverse transcriptase, were included in each experiment. The housekeeping Arabidopsis actin2 gene (U41990) was used as an internal normalization standard to quantify the target mRNA (forward: 5’GAAAGATATGCATCGACTTTCCTA’3’ and reverse: 5’ACATACATAGGG-GAGCGTTAAAGG3’). Each data point represents the average of three or four experiments and the error bars represent the standard deviation. Statistical analyses were done using GraphPad Prism (GraphPad Software, USA) and included a one-way ANOVA with a Tukey’s Multiple Comparison Test for significant differences.

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Author Contributions
Conceived and designed the experiments: KRD SL CIC. Performed the experiments: JP HJL YSH. Analyzed the data: KRD SL CIC SHK. Contributed reagents/materials/analysis tools: GKA KHI DJV. Wrote the paper: KRD SL.
