



Article

Expression Profiling of Autophagy Genes *BxATG1* and *BxATG8* under Biotic and Abiotic Stresses in Pine Wood Nematode *Bursaphelenchus xylophilus*

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Received: 26 September 2017; Accepted: 27 November 2017; Published: 6 December 2017

Abstract: The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is the pathogen of pine wilt disease (PWD) and causes huge economic losses in pine forests and shows a remarkable ability to survive under unfavorable and changing environmental conditions. This ability may be related to autophagy, which is still poorly understood in *B. xylophilus*. Our previous studies showed that autophagy exists in PWN. Therefore, we tested the effects of autophagy inducer rapamycin on PWN and the results revealed that the feeding rate and reproduction were significantly promoted on fungal mats. The gene expression patterns of *BxATG1* and *BxATG8* under the different stress were determined by quantitative reverse transcription PCR (qRT-PCR). We tested the effects of RNA interference on *BxATG1* and *BxATG8* in PWN during different periods of infection in *Pinus thunbergii*. The results revealed that *BxATG1* and *BxATG8* may play roles in allowing PWN to adapt to changing environmental conditions and the virulence of PWN was influenced by the silence of autophagy-related genes *BxATG1* and *BxATG8*. These results provided fundamental information on the relationship between autophagy and PWN, and on better understanding of gene function of *BxATG1* and *BxATG8* in PWN.

Keywords: *Bursaphelenchus xylophilus*; autophagy; autophagy-related genes; quantitative reverse transcription PCR; RNA interference

1. Introduction

Pine wilt disease (PWD) was considered as “pine cancer”, which is caused by pine wood nematode (PWN), *Bursaphelenchus xylophilus*. The first case of PWD caused by *B. xylophilus* in Europe was reported in 1999 in Portugal [1], and was soon after found in Spain [2]. The PWD was introduced to Japan a century ago and spread to China and Korea in the 1980s [3]. *B. xylophilus* usually damages exotic pine trees and causes serious damage and great economic loss to the resources of pine trees and natural landscapes in most areas of China [4]. At present, research on PWN mainly focuses on the pathogenic mechanism, inspection, quarantine and how to identify PWN and other species of *Bursaphelenchus* [5]. A number of studies have focused on the pathogenic and growth related genes in PWN [6]. However, the pathogenic mechanism of PWN is not yet clear. As an invasive species, how PWN can fit some environmental conditions, such as high and low temperatures, and response to the resistance reaction of pine tree becomes a key reason for the death of pine trees. These are still open questions in PWD research.

Autophagy is a self-protection for an eukaryotic cell to adapt poor conditions. Several studies have shown that autophagy plays significant roles in physiological and pathophysiological processes in eukaryotic cells [7,8]. Autophagy related genes are indispensable for the ability to drop in each growing phase of *Pyricularia grisea*, such as production of spores, turgor of appressorium, pathogenicity and sexual reproduction [9–12]. Interference of some autophagy related genes will produce some growth defects. For example, the shape of dauer larvae will be affected; the life-span will be shortened; survival rate at starvation will be reduced; and the cell will be boosted to die [13]. In recent years, some functional genes similar to autophagy genes have been identified in *Caenorhabditis elegans* [14–16]. Atg1 is a protein kinase, which can activate the key inducing factor for autophagy [17]. However, the autophagy gene *Atg8* participates in two ubiquitin similar integration systems and it is the most direct proof to be always used to test the autophagic activity [18]. Target of rapamycin (TOR) is an evolutionarily conserved serine/threonine protein kinase that was first found in yeast and subsequently its homologs were found in mammals. They were collectively referred to as mammalian target of rapamycin (mTOR). The mTOR signaling pathway plays an important role in the regulation of cell growth, proliferation, apoptosis and autophagy. Under normal conditions, the mTOR pathway is activated and autophagy is inhibited. When this pathway is repressed, autophagy is activated [19]. Rapamycin (RAPA) is the first known mTOR pathway inhibitor. The study found that rapamycin (RAPA) was a potential autophagy inducer that could induce and promote autophagy by inhibiting the mTOR pathway [20]. However, there are no previous reports on mTOR in PWN. Induced autophagy and impact on the PWN remain poorly understood. In natural conditions, autophagy occurs at a low level. When the external environment changes, the level of autophagy will change to regulate the homeostasis of the organism [8]. This process is likely to have a certain impact on the environmental adaptability and pathogenicity of PWN. In recent research in our laboratory, the phenomenon of autophagy was found to exist in PWN and two autophagy genes *BxATG1* and *BxATG8* of PWN were cloned and analyzed biologically [21]. However, reports on autophagy of PWN are scarce and the relationship between autophagy genes and the pathogenicity of PWN is still unclear. Based on previous research, an exogenous autophagy inducer rapamycin was introduced in this study to explore the relationship between feeding, reproduction and autophagy in PWN. Quantitative reverse transcription PCR (qRT-PCR) was used to quantify the expression of autophagy genes *BxATG1* and *BxATG8* of PWN when temperature changes and oxidation occurs. The aim of the study was to reveal the relationship between autophagy and anti-reversion force of PWN, and to provide a reference to elucidate the pathogenesis of PWN.

2. Results

2.1. Effect of Rapamycin on *B. xylophilus* Feeding Rate and Reproduction on Fungal Mats

The effect of rapamycin on PWN reproduction was tested on Potato Dextrose Agar (PDA) plates inoculated with *Botrytis cinerea* at 25 °C. After the nematode was treated by rapamycin, the feeding rate of nematode was faster than control. The feeding rate increased with an increase of rapamycin concentration and the treatment of 50 mM was the fastest. These results showed that the feeding rate of PWN was significantly influenced by rapamycin treatment, and PWN increases its feeding rate at the autophagy induction at 50 mM concentration of rapamycin solution (Figure 1).

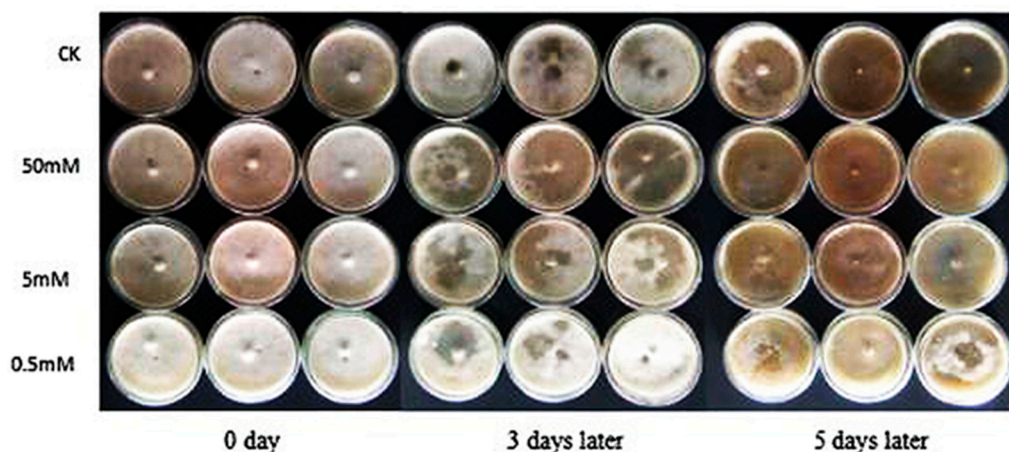


Figure 1. Effect of rapamycin on the pine wood nematode (PWN) inoculated onto *B. cinerea*. The nematodes treated by ddH₂O control solution (CK) and 50, 5, and 0.5 mM rapamycin were grown in *B. cinerea*.

After treated with rapamycin solution, the PWN grew more rapidly than that of the control, and the one, which was treated with 50 mM rapamycin solution was the highest, and other treatments were slightly higher, and the amplitude of fluctuation was about 5000 pieces of nematodes (Figure 2). The result manifested autophagy plays a boost role in the reproduction of PWN. This was the same trend of feeding rate.

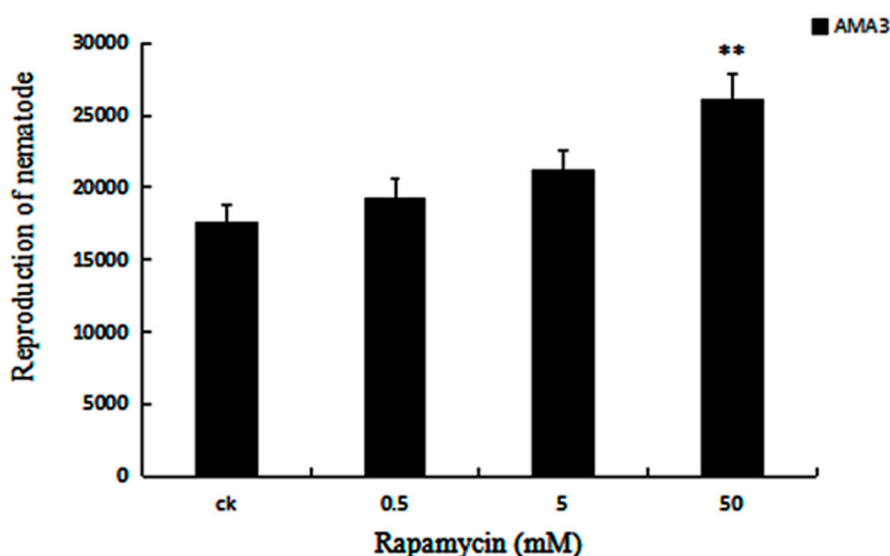


Figure 2. Effect of Rapamycin on reproduction of PWN. Reproduction rate of PWN washed from PDA plate of *B. cinerea* (ddH₂O and 0.5, 5, and 50 mM Rapamycin). Data represent mean \pm SD from three independent experiments. Bars show standard deviations of the mean. Asterisks on top of the bars indicating statistically significant differences between the Rapamycin-treated (0.5, 5 and 50 mM Rapamycin) nematodes and controls (** $p < 0.01$, Student's *t*-test).

2.2. Expression Level of Autophagy Genes *BxATG1* and *BxATG8* in *B. xylophilus* after Treated with Rapamycin

Rapamycin was used to induce Autophagy. Quantitative reverse transcription PCR (qRT-PCR) was performed to determine the effect of rapamycin on the expression of *BxATG1* and *BxATG8* expression levels. The gene expression of *BxATG1* and *BxATG8* significantly increased after PWN was soaking in a 50 mM concentration of rapamycin solution compared with those after PWN was soaking in ddH₂O control solution. When the expression level of the control was considered as 100%,

the mean expression level of rapamycin-treated samples rose by 1.45 times and 4.60 times (Figure 3). The autophagy-related gene *Atg8* was the marker of autophagy. Thus, these results showed that rapamycin can induce autophagy of PWN effectively.

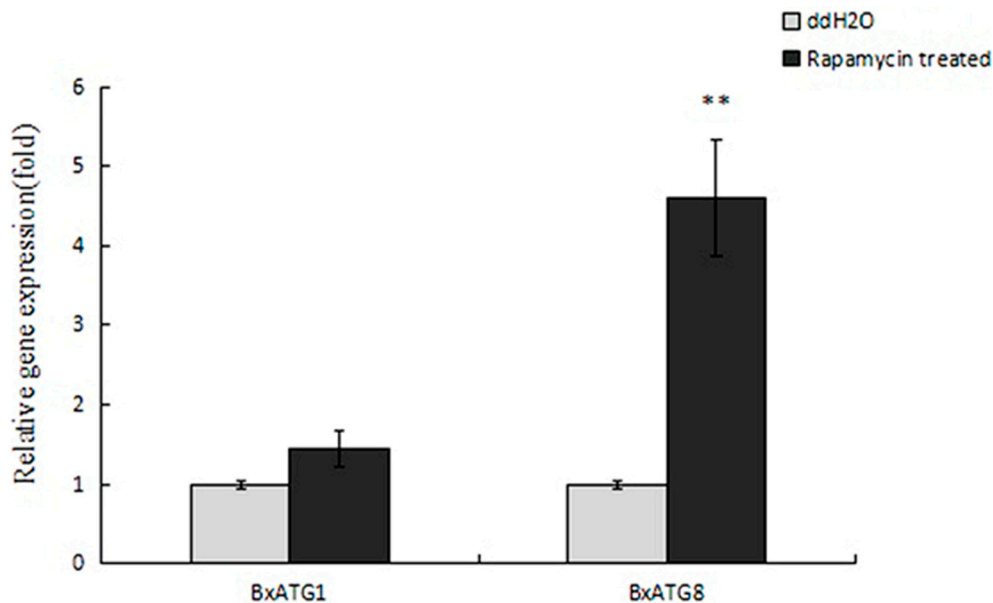


Figure 3. The expression level of *BxATG1* and *BxATG8* after treatment with a 50 mM concentration of rapamycin. Data represent mean \pm SD from three independent experiments. Bars show standard deviations of the mean. Asterisks on top of the bars indicate statistically significant differences (** $p < 0.01$, Student's *t*-test).

2.3. Response and Expression of *B. xylophilus* Autophagy Genes *BxATG1* and *BxATG8* at Temperature Changes

The expression level of gene *BxATG1* of PWN AMA3 increased with increasing temperature and reached the peak when the temperature was at 35 °C. It showed that the autophagy reaction was most fiercely enabled when the nematode was at 35 °C. It increased by 4.2 times of the expression level when at 25 °C. When the temperature was 40 °C, the expression level of gene *BxATG1* declined suddenly (Figure 4A).

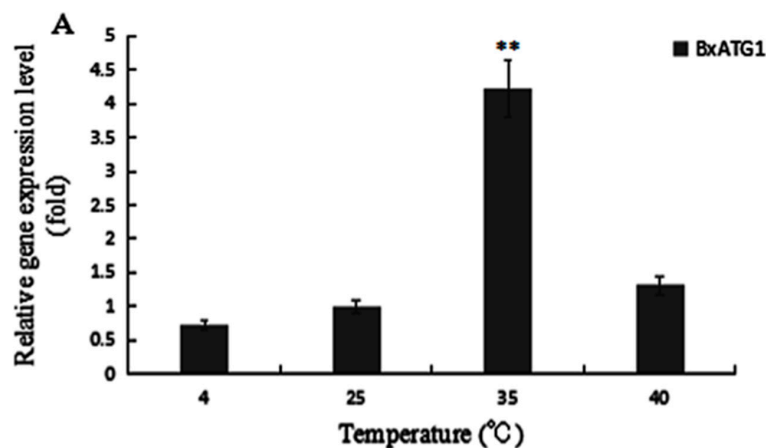


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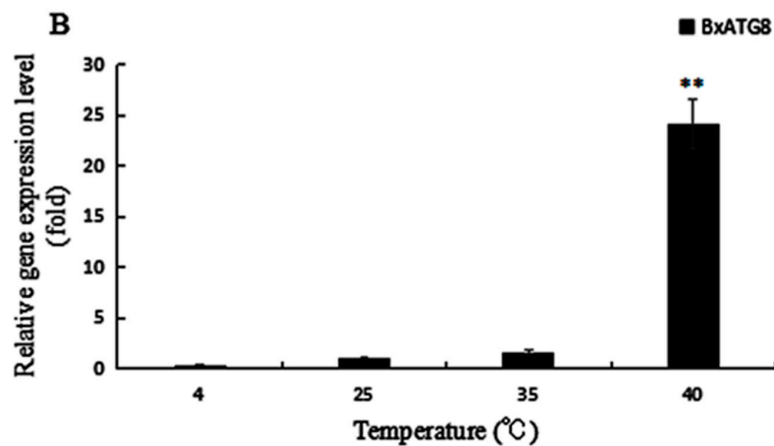


Figure 4. Quantitative reverse transcription PCR (qRT-PCR) analysis of: *BxATG1* (A); and *BxATG8* (B), in PWN after treated at different temperatures. The expression level of 25 °C was set as 100%. Data represent mean \pm SD from three independent experiments. Bars show standard deviations of the mean. Asterisks on top of the bars indicate statistically significant differences (** $p < 0.01$, Student's *t*-test).

The expression level of gene *BxATG8* of AMA3 increased in a trapezoid with increasing temperature. The expression level of gene *BxATG8* reached the highest at 40 °C and rose by 24.1 times of the one at 25 °C. *ATG8* is the main gene to be measured for autophagy activity (Figure 4B). When the two genes were compared with each other, the expression level of gene *BxATG8* was much higher than that of gene *BxATG1*. When both were at peaks, the peak expression level of gene *BxATG8* of nematode AMA3 was 24 times of the one at the most suitable growing temperature, but the gene *BxATG1* was only four times. The results showed that PWN goes through the high temperature stress by regulating autophagy activity and *ATG8* plays an important role in the process of regulating autophagy.

2.4. Expression Level of *B. xylophilus* Autophagy Genes *BxATG1* and *BxATG8* under Oxidative Stress

Compared to CK (0 mM H₂O₂), with an increase of H₂O₂ concentration, the expression level of autophagy related gene *BxATG1* of PWN increased first and then declined. When the concentration reached 15 mM, the starting reaction of autophagy was the strongest. The expression level of *BxATG1* increased by 2.87 times, compared with the counterpart, then it descended (Figure 5A).

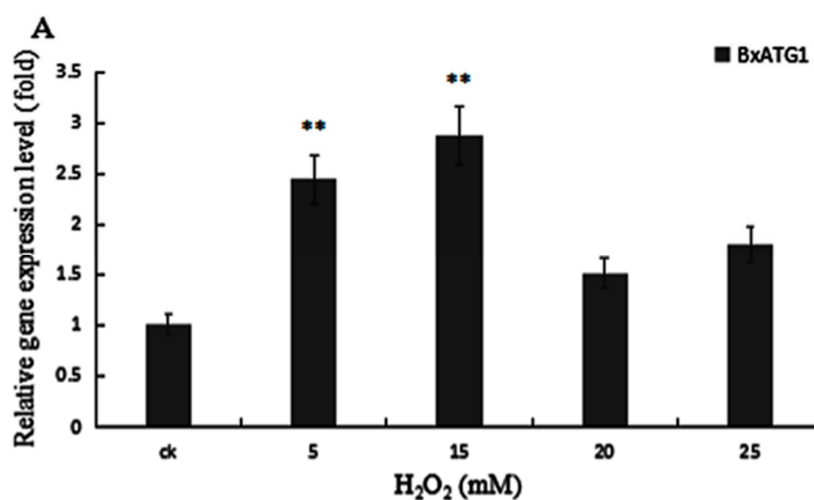


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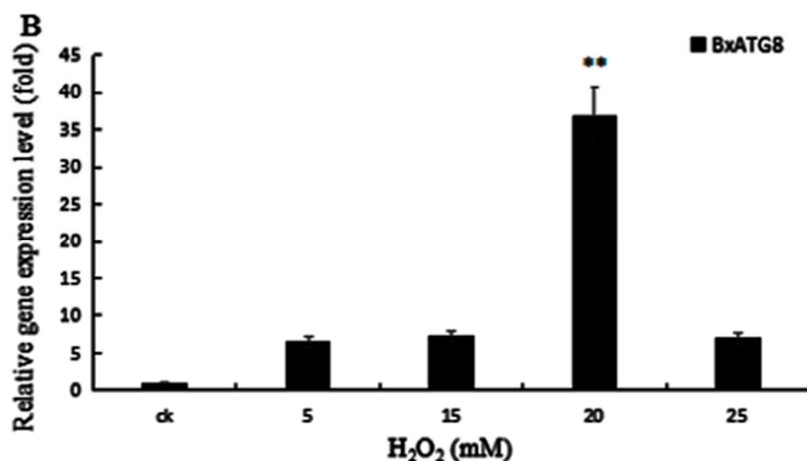


Figure 5. Quantitative reverse transcription PCR (qRT-PCR) analysis of: *BxATG1* (A); and *BxATG8* (B), in PWN after treated with oxidative stress. The expression level of the control was set as 100%. Data represent mean \pm SD from three independent experiments. Bars show standard deviations of the mean. Asterisks on top of the bars indicate statistically significant differences (** $p < 0.01$, Student's *t*-test).

The expression level of autophagy related gene *BxATG8* of PWN ascended with the increase of H₂O₂ concentration and then decreased. When the concentration reached 20 mM, the autophagy response was strongest. The expression level of *BxATG8* increased 36.9 times compared to the check (Figure 5B). The results showed that PWN helped themselves through the oxidative stress by regulating autophagy activity.

2.5. Expression Level of *B. xylophilus* Autophagy Genes *BxATG1* and *BxATG8* after Pine Trees Inoculated with *B. xylophilus*

Two-year-old *Pinus thunbergii* seedlings were inoculated with PWN. Expression of autophagy genes *BxATG1* and *BxATG8* in PWN reached the highest level after the nematode invading pine trees for seven days. Gene *BxATG1* increased by 19.3 times and gene *BxATG8* increased by 36.9 times. After 14 days, the expression of genes *BxATG1* and *BxATG8* were only 2.4 times higher for gene *BxATG1* and 0.89 times lower for gene *BxATG8*. After pine trees were inoculated for 30 days, expression of *BxATG1* and *BxATG8* presented second peaks, which were 13.7 and 17.0 times, respectively. Meanwhile, the expression of *BxATG8* at peak was higher than that of gene *BxATG1* (Figure 6). The result showed autophagy was strong in the beginning and the last period of invading pine tree.

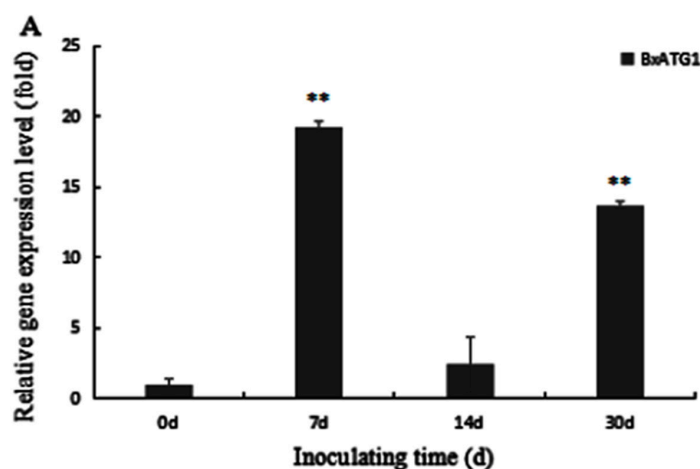


Figure 6. Cont.

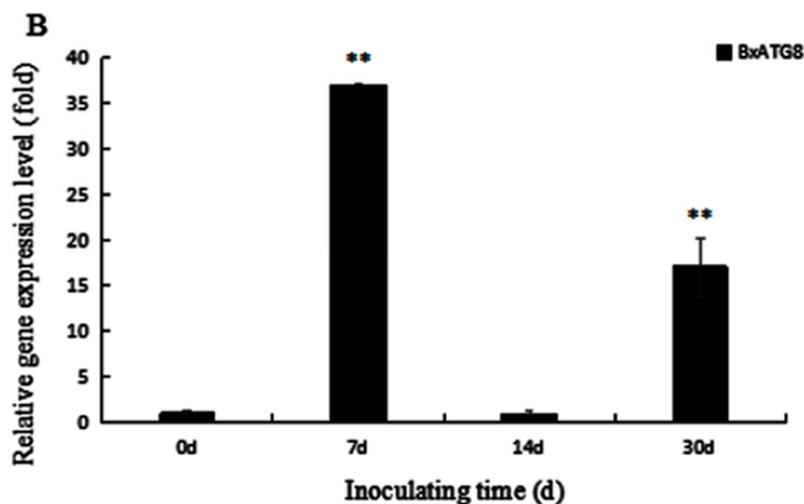


Figure 6. Quantitative reverse transcription PCR (qRT-PCR) analysis of: *BxATG1* (A); and *BxATG8* (B), in PWN after inoculating trees. The expression level of the control was set as 100%. Data represent mean \pm SD from three independent experiments. Bars show standard deviations of the mean. Asterisks on top of the bars indicate statistically significant differences (** $p < 0.01$, Student's *t*-test).

2.6. Virulence of Pine Wood Nematode (PWN) after RNAi

Two-year-old *Pinus thunbergii* seedlings were inoculated with dsRNA- and ddH₂O-treated nematodes (Figure 7A). After 14 days, wilting appeared in the seedlings inoculated with nematodes soaked in the negative controls (dsGFP-treated and ddH₂O-treated solution) (Figure 7B), and the infection rates were both 50%. The disease severity indices (DSI) were both 12.5. After 20 days, the seedlings inoculated with nematodes soaked in ds*BxATG1* and ds*BxATG8* solutions began to wilt (Figure 7C); the infection rates of *P. thunbergii* inoculated with nematodes soaked in ds*BxATG1*, ds*BxATG8* and dsGFP and ddH₂O were 50%, 75%, 100% and 100%, respectively, and the DSI were 12.5, 18.8, 50 and 50, respectively. After 30 days, all of the seedlings inoculated with dsGFP-treated and ddH₂O-treated nematodes turned red-brown (Figure 7D), and DSI were both 100, and the disease severity indices of *P. thunbergii* seedlings inoculated with nematodes soaked in ds*BxATG1* and ds*BxATG8* were 56.3 and 87.5, respectively (Table 1). The wilting processes of *P. thunbergii* seedlings were different among the treatments. *P. thunbergii* seedlings inoculation with dsGFP-treated and ddH₂O-treated nematodes started to wilt in 12 days, and were dead in 29 and 28 days, respectively. Delayed symptom development was observed by silencing *BxATG1* and *BxATG8*, respectively (Table 1). The results indicated that the virulence of PWN was influenced by the RNAi of autophagy genes *BxATG1* and *BxATG8*.

Table 1. The symptoms of *P. thunbergii* inoculated with PWN.

Nematodes	Infection Rates			Disease Severity Indices			Days of Symptoms Appeared (d)	Days of <i>P. Thunbergii</i> Wilted (d)
	14th day	20th day	30th day	14th day	20th day	30th day		
nematodes soaked in ddH ₂ O	50	100	100	12.5	50	100	12	28
nematodes soaked in dsGFP	50	100	100	12.5	50	100	12	29
nematodes soaked in ds <i>BxATG1</i>	0	50	100	0	12.5	56.3	17	45
nematodes soaked in ds <i>BxATG8</i>	0	75	100	0	18.8	87.5	16	38

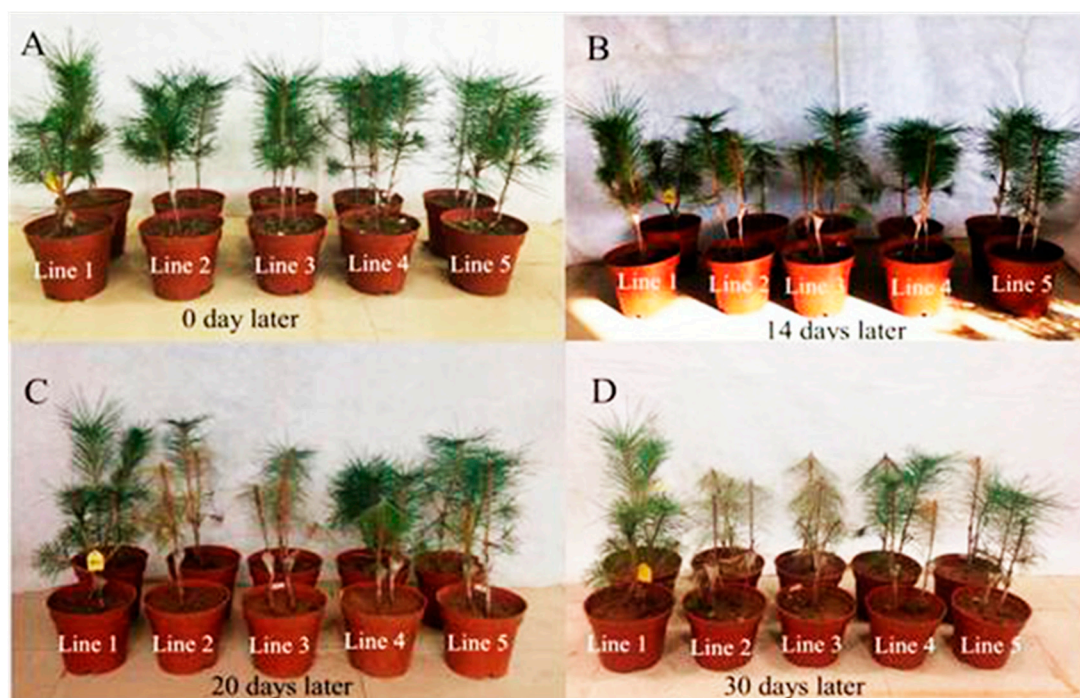


Figure 7. Symptoms in *P. thunbergii* seedlings: 0 day (A); 14 days (B); 20 days (C); and 30 days (D) after inoculation with nematodes soaked in ddH₂O (line 2), dsGFP (line 3), ds*BxATG1* (line 4), and ds*BxATG8* (line 5) solutions. *P. thunbergii* seedlings treated with ddH₂O alone were considered as controls (line 1). Each pot contains two *P. thunbergii* seedlings.

3. Discussion

Recently, the function of autophagy in eukaryote is explored and indicated that it played an important biological role. Reports showed that autophagy can boost reproduction of *Aedes aegypti*, *Pyricularia grisea* and *Colletotrichum orbiculare*, and play a role in promoting adaptive ability in stress environment [22,23]. In this study, the feeding rate of PWN increased in trapezoid with the ascending of induction rapamycin concentration and the trend of reproduction is very similar with the feeding rate. The result confirmed that autophagy plays a role in promoting the feeding and reproductive abilities of PWN.

Autophagy is helpful for organism to live in an adverse environment and improve its adaptability [24]. As a defensive reaction, the process of autophagy will degrade the damaged protein, organelle and cytoplasmic components under environmental stress, provide nutrition, keep living for cells, and provide raw materials for self-repair and existing cells [25]. Autophagy occurs due to the stimulation of ambient environment such as temperature changes [13]. Active oxygen is also one of the induction factors of autophagy [26,27]. At the beginning of PWN invading pine tree, active oxygen increased sharply in the pine trees [28–30]. *Atg* genes can perform different functions [31,32]. Quantitative reverse transcription PCR (qRT-PCR) was performed to analyze the effect of temperature change and oxidative stress on the *BxATG1* and *BxATG8* expression levels. Based on our results, with the increase of temperature, the expression level of autophagy genes *BxATG1* and *BxATG8* of PWN ascended and reached peak, and high temperature stimulated autophagy of PWN. The results also suggested PWN adapted to the environment of oxidative stress through autophagy and the expression level of genes *BxATG1* and *BxATG8* reached the peak with the ascending of H₂O₂ concentration. PWN could die later for the possibilities of too much autophagy and high concentration of active oxygen. It is not difficult to find that, whether the nematode was under stress of high temperature or oxidation, the peak of expression level of gene *BxATG8* always occurred after gene *BxATG1*. This conforms to the sequence found in eukaryotes (e.g., yeast) and *C. elegans* that gene *Atg1*

participated in the induction of autophagy and gene *Atg8* participated in ubiquitin similar integrative system [33]. Of course, this is only speculated from the sequence of gene expressions. Whether upstream and downstream signal channels, similar to that of *C. elegans*, exist in PWN, and the complex signal transmission and molecular regulation mechanism in autophagy of PWN [34] should be further studied.

To resist the invasion by pine wood nematodes, the pine induces defense mechanisms against the pathogen and numerous defense molecules are generated, such as cyclic aromatics, terpenoids and reactive oxygen species [35–37]. At the same time, PWN must also mobilize reciprocal defensive reactions to avoid damage from the complex compounds [38]. Autophagy is part of the cellular response, which can change rapidly in a cell due to changing environmental and physiological conditions. Therefore, it plays an important role in the cellular response to stress [39]. Autophagy is a means of clearance that reduces damage caused by the plant's molecular responses [24,40]. In the present study, we found that autophagy in PWN reacted strongly at the beginning and at the end of infecting *P. thunbergii*. We presumed that pine wood nematode adapted a variety of resistance of pine by strong autophagy in the early stages of infection. The autophagy reaction reached a second peak because of lack of food and high population density in the late stage of infection. RNAi was developed as an effective tool in plants and animals to study gene functions and for genetic manipulation [41,42]. Moreover, RNAi has also been used to assess the pathogenic and molecular effects of silenced *B. xylophilus* genes [43–46]. In the present study, *BxATG1* and *BxATG8* were shown to likely be associated with virulence in *B. xylophilus* by eliminating damage caused by *P. thunbergii* defense mechanisms and promoting the synthesis of toxic metabolites to help *B. xylophilus* colonize the pine trees. However, the potential role of autophagy in PWN needs to be further investigated.

In summary, autophagy can improve the feeding and breeding abilities of PWN. We found from the molecular level that anti-adverse force and pathogenicity of PWN are related with the regulation mechanism of autophagy genes. It is speculated that, by autophagy, PWN can regulate its internal environment to survive adverse conditions such as high temperatures or oxidative stress, minimize the damage resulted from defensive mechanism of pine and promote synthesis of its toxin metabolite, to help successfully invade and establish colonies that damage pine trees. The discovery of these relationships between autophagy and PWN will assist us to understand the biological suitability mechanism of PWN under adverse conditions and the function of autophagy genes in pine wilt disease.

4. Materials and Methods

4.1. PWN Growth Conditions and Experimental Organisms

The highly virulent AMA3 strain of PWN was isolated from wood chips of infested *P. thunbergii* Parl from Anhui, China. The nematodes were grown in colonies of *B. cinerea* Pers [47] on PDA plates for 7 days at 25 °C. Then, they were extracted overnight from PDA plates using the Baermann funnel method [48]. Two-year-old *P. thunbergii* seedlings were obtained from the greenhouse at Nanjing Forestry University (Nanjing, China).

4.2. Preparation of Autophagy Inducer

Fifty milligrams of rapamycin were poured into 1 mL DMSO to be 50 mg/mL (about 50 mM) solution, which was then diluted with ddH₂O (Double-distilled H₂O) into 5 mM and 0.5 mM for use [49,50].

4.3. Analysis of Feeding and Reproduction of PWN after Autophagy Induction

One thousand nematodes from different treatments (ddH₂O and 50, 5, 0.5 mM rapamycin) were picked and transferred onto a PDA plate with *B. cinerea* and cultured at 25 °C for 5 days. Three biological replicates were used in each treatment. The growth condition of nematodes was

photographed daily. Subsequently, the nematodes were extracted from PDA plates using the Baermann funnel method and the nematodes were counted.

4.4. RNA Extraction and cDNA Synthesis of PWN

The total RNA of collected nematodes (a mixture of adults and juveniles) was extracted using TRIzol reagent (Invitrogen, Waltham, MA, USA). The RNA was quantified using a spectrophotometer and examined by electrophoresis on a 1% agarose gel. cDNA was synthesized from 2 µg of total RNA using the TransScript II One-Step gDNA Removal and cDNA Synthesis SuperMix according to the manufacturer's instructions (Trans Gen Biotech, Beijing, China).

4.5. Quantitative Reverse Transcription PCR (qRT-PCR)

Quantitative reverse transcription PCR (qRT-PCR) was performed to determine the expression levels of *BxATG1* and *BxATG8*. qRT-PCR was then carried out using SYBR Green Master Mix (Vazyme, Nanjing, China). The Actin gene of PWN was used as an internal control, with the primers listed in Table 2. Relative expression levels were determined using the ABI Prism 7500 software (Applied Biosystems, Foster City, CA, USA) and the $2^{-\Delta\Delta Ct}$ method. qRT-PCR was conducted with three biological and technical replicates [51].

Table 2. PCR primers used in the study.

Name of Primers	Sequence (5'-3')
<i>BxATG1-T7I-F</i>	TAATACGACTCACTATAGGGAAGGCAGAAATCGGACA
<i>BxATG1-I-R</i>	AATCGGCTCATGGAAAA
<i>BxATG1-I-F</i>	AAGGCAGAAATCGGACA
<i>BxATG1-T7I-R</i>	TAATACGACTCACTATAGGGAATCGGCTCATGGAAAA
<i>BxATG8-T7I-F</i>	TAATACGACTCACTATAGGGAACCCAAGTTTGAGACCT
<i>BxATG8-I-R</i>	TAATACGACTCACTATAGGGAAGGAGAAGAAGAACTTTTCAC
<i>BxATG8-I-F</i>	CTGTTACAAACTCAAGAAGG
<i>BxATG8-T7I-R</i>	AAAGGAGAAGAAGAACTTTTCAC
<i>GFP-T7I-F</i>	TAATACGACTCACTATAGGGCTGTTACAAACTCAAGAAGG
<i>GFP-I-R</i>	CGAAAACACTACAATAAGA
<i>GFP-I-F</i>	AACCCAAGTTTGAGACCT
<i>GFP-T7I-R</i>	TAATACGACTCACTATAGGG CGAAAACACTACAATAAGA
<i>Actin F</i>	GCAACACGGAGTTCGTTGTAGA
<i>Actin R</i>	GTATCGTCACCAACTGGGATGA
<i>qBxATG1-F</i>	AGAGTGTGGGTGAGGGA
<i>qBxATG1-R</i>	CTCGGCATTGGTACATTATA
<i>qBxATG8-F</i>	GTCAACGATGTCATTCCCCA
<i>qBxATG8-R</i>	AACTGATCACTCTTCGGCGG

4.6. Analysis of Expression Level of *BxATG1* and *BxATG8* When Temperature Changes

Collected nematodes with different treatment (4, 25, 35 and 40 °C water bath for 15 min) were transferred into Eppendorf (EP) pipes. Then RNA of PWN was extracted and cDNA was synthesized (see Section 4.4). qRT-PCR was performed to determine the expression levels of *BxATG1* and *BxATG8*.

4.7. Analysis of Expression Level of *BxATG1* and *BxATG8* in Oxidative Stress

Nematodes were soaked in different H₂O₂ solutions (1 mL ddH₂O with 10 µL volumes of 5, 15, 20 and 25 mM H₂O₂) for 30 min. RNA was extracted and synthesize cDNA of PWN was synthesized (see Section 4.4). qRT-PCR was performed to determine the expression levels of *BxATG1* and *BxATG8*.

4.8. Analysis of Expression Level of *BxATG1* and *BxATG8* after Pine Was Inoculated with *B. xylophilus*

The nematodes were inoculated to *P. thunbergii* seedlings. One thousand nematodes were injected into each seedling [52]. Nematodes were extracted overnight from *P. thunbergii* seedlings

(after inoculating 7, 14 and 30 days) using the Baermann funnel method. Then, RNA was extracted and cDNA of *PWN* was synthesized (see Section 4.4). Quantitative reverse transcription PCR (qRT-PCR) was performed to determine the expression level of *BxATG1* and *BxATG8*.

4.9. *BxATG1* and *BxATG8* Interference Using Double-Stranded RNA

Double-stranded RNA (dsRNA) was synthesized using the MEG script RNAi Kit (Ambion Inc., Austin, TX, USA) with the primers *BxATG1-T7I-F*, *BxATG1-I-R*, *BxATG1-I-F*, *BxATG1-T7I-R*, *BxATG8-T7I-F*, *BxATG8-I-R*, *BxATG8-I-F*, *BxATG8-T7I-R*, *GFP-T7I-F*, *GFP-I-R*, *GFP-I-F*, and *GFP-T7I-R* (Table 2). The RNAi soaking method was performed according to Urwin et al. [53]. Freshly cultured nematodes were soaked in dsRNA solution (800 ng/ μ L) and incubated at 180 rpm for 48 h at 20 °C. The nematodes soaked in the corresponding non-dsRNA solution were used as controls. Each treatment had three replicates. Samples from each treatment were washed thoroughly with ddH₂O three times after soaking and then used for additional experiments. *BxATG1* and *BxATG8* were silenced by RNAi effectively which was performed according to Deng et al. [21].

4.10. Evaluation of Virulence of *PWN* after RNAi

To determine the virulence of *PWN* after RNAi (*BxATG1* and *BxATG8*), the nematodes were inoculated onto *P. thunbergii* seedlings for virulence determination. One thousand nematodes were injected into each seedling. *PWN* soaked in ds*GFP* and ddH₂O were used as negative controls, ddH₂O alone as a positive control. Eight biological replicates were conducted. Periodically, the seedlings were observed and were photographed to record their state. The disease severity of *P. thunbergii* seedlings was divided into five levels: 0, healthy seedlings with green needles growing well; I, a few needles turning brown; II, half of the needles turning brown and the terminal shoots of seedlings bending; III, most of the needles turning brown, and the terminal shoots of seedlings drooping; and IV, all of the needles turning brown, and the whole seedling wilting. The infection rates and the disease severity index were calculated according to Xiang et al. [47].

$$\text{Infection rates} = \frac{\sum \text{number of infected plants with symptoms}}{\text{Total number of plants}} \times 100\%$$

$$\text{Disease severity index (DSI)} = \frac{\sum \text{number of disease plants} \times \text{symptom stage}}{\text{Total number of plants} \times \text{highest symptom stage}} \times 100$$

4.11. Statistical Analysis

All assays were performed in triplication. The statistical significance was determined using SPSS Statistics 17.0 software (IBM China Company Ltd., Beijing, China) to perform the paired *t*-tests. Asterisks indicate statistically significant differences (** *p* < 0.01, Student's *t*-test).

Acknowledgments: This work was supported by the National Key Research and Development Program of China (2016YFC1202100), Jiangsu Provincial Agricultural Science and Technology Innovation Fund (CX (16) 1005) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank De-Wei Li for critically reviewing the manuscript.

Author Contributions: Fan Wu and Li-Na Deng designed the study, analyzed the data, conducted experiments and prepared manuscript; Xiao-Qin Wu and Jian-Ren Ye designed the study and acquired the data; Hong-Bin Liu and Xiao-Qin Wu analyzed the data; and Xiao-Qin Wu contributed reagents/materials/analysis tools and approved final manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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