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Plasmodiophora brassicae Infection Modulates Expansin Genes of *Brassica rapa* ssp. *pekinensis*

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Abstract: Clubroot is a soil-borne disease of cruciferous crops, including *Brassica rapa* ssp. *pekinensis*, and causes substantial yield losses. In an attempt to develop clubroot-resistant *B. rapa* cultivars, we investigated the role of a root-abundant expansin-like B1 *(EXLB1)* during *Plasmodiophora brassicae* inoculation. The histochemical analyses of infected transgenic reporter lines showed a role for *BrEXLB1* in disease response as early as 3 dpi. The transgenic overexpression of *EXLB1* in *B. rapa* conferred disease-sensitive phenotypes and was comparable to non-transgenic controls at 30 dpi. In contrast, the heterogeneous population of antisense *BrEXLB1*-overexpressing lines conferred disease resistance against highly pathogenic *P. brassicae* race 2 inoculations under greenhouse conditions. Additionally, we profiled the relative expression of 32 other *BrEXPs* in wild-type seedlings, sampled on different days (1–10) after inoculation using qRT-PCR. The results indicate that the expression pattern of most *BrEXP* genes was significantly altered during different infection times, suggesting their participation in clubroot responses. In particular, the expressions of *EXPA20*, *EXPA21*, and *EXPA34* were consistently downregulated, while the expression of *EXPA5* was upregulated ($\log_2$FC ≥ 2) compared to controls. Altogether, our study showed that *BrEXPs* participate in clubroot disease response, and their genetic manipulation is likely to provide clubroot disease resistance.

Keywords: Chinese cabbage; clubroot; expansin; expansin-like B1 (EXLB1); cell wall

Citation: Muthusamy, M.; Park, S.R.; Park, J.-I.; Lee, S.I. *Plasmodiophora brassicae* Infection Modulates Expansin Genes of *Brassica rapa* ssp. *pekinensis*. *Agriculture* **2022**, *12*, 1416. https://doi.org/10.3390/agriculture12091416

Academic Editors: Renata Bažok and Maja Čačija

Received: 16 August 2022
Accepted: 6 September 2022
Published: 8 September 2022

1. Introduction

Clubroot is a soil-borne disease of cruciferous crops, including *Brassica rapa* ssp. *pekinensis* (popularly known as Chinese cabbage), and one of the characteristic symptoms is the formation of large galls on the host plant's roots, which leads to significant yield (20–100%) losses by impeding root functions [1]. The clubroot pathogen *Plasmodiophora brassicae* is an obligate biotrophic protist that can survive as resting spores in the infested soil for long periods. Therefore, the identification and development of clubroot resistance is an ideal choice and is unavoidable, given the global agro-economic losses in Brassica crops. However, decades-long research in clubroot disease resistance has identified several useful major loci, quantitative trait loci (QTL), and genes for developing disease-resistant plants [2–6]. The emergence of new pathotypes in *P. brassicae* and the report of resistance breakdowns mandates additional sources of resistance [7]. Additionally, different *P. brassicae* pathotypes exist in different hosts [8].

On the other hand, plants have evolved a plethora of constitutive and pathogen-induced resistance mechanisms to cope with continuous pathogen threats, depending on their genotypes [9]. Plant cell walls are complex, and the dynamic structures act as a physical barrier to pathogen invasion, while the cell's bioactive constituents play a crucial role in defense responses [10]. Wall structural modifications can influence cell wall integrity and disease-resistance phenotypes [11,12]. The infectivity of the pathogens is partly attributable to their ability to degrade the cell wall of the host plants. In some cases, the pathogen can

hijack endogenous host mechanisms in cell wall loosening to create an optimal cellular environment for its intracellular proliferation [12]. In fact, clubroot pathogen infection in Arabidopsis triggered changes in the expression of cell wall metabolic genes [13]. Therefore, engineering plants with altered cell wall structural characteristics that prevent pathogen entry is an exciting strategy for developing clubroot resistance. Approximately 10% of plant genes participate in the biosynthesis, transport, deposition, remodeling, and turnover of cell walls during plant development and biotic/abiotic threats [14]. Additionally, the cell wall's constant assembly, remodeling, and disassembly are essential for cell growth and stress adaptation [15]. Several structural and functional components, including expansins, are secreted into the cell wall space to regulate cell wall metabolism. Expansins are pH-dependent cell wall loosening proteins that play a crucial role in cell growth and division. Cell wall-localized expansins can disrupt the extracellular matrix for cell wall relaxation and expansion, thereby contributing to plant growth and development. Cell wall relaxation was shown to be useful in conferring tolerance to abiotic stress conditions, and it can pave easy access to plant pathogens [16]. Plant pathogens were sometimes shown to hijack host regulatory pathways associated with cell growth [17] and cell wall loosening proteins such as expansins. The *EXPA4* overexpression in tobacco increased its susceptibility against *Tobacco mosaic virus* and *Pseudomonas syringae* by reducing the transcript expression levels of defense genes [18], suggesting expansin would also regulate plant defense mechanisms. In another study, suppressing expansin genes (*EXPA1*, *EXPA5*, *EXPA10*, *EXPB3*, *EXPB4*, and *EXPB7*) in rice was attributable to bacterial blight resistance in rice lines [19]. The results indicate that enhanced cell wall loosening/cell wall relaxation activities by expansin genes in hosts might favor pathogen entry/attacks. In addition, it is worth noting that expansin-mediated disease response is specific to phytopathogens [20]. So far, sixteen races of *P. brassicae* have been known/identified; of these, almost all of the races, except races 10 and 12, are known to be present in Korea, and race 4 was found to have the high pathogenicity that is widely distributed among all races [21].

B. rapa is an economically important vegetable and, more importantly, has expanded the expansin superfamily with 53 members, including several segmental and eight tandem duplicates [22,23]. In some cases, the retention of duplicate gene pairs was known to offer neofunctionalization under certain physiological conditions in plants. In the *B. rapa* expansin superfamily, only five genes, including *BrEXLB1*, retained all three duplicates, which draws attention to functional characterization. Additionally, in a previous study dealing with the root-abundant, expansin-like B1 (*EXLB1*) gene of *Brassica rapa* ssp. *Pekinensis*, it was shown to influence primary root growth by altering the root elongation size in Arabidopsis [24,25]. Herein, we investigated the role of *BrEXLB1* in clubroot disease response through transgenic approaches. For this purpose, we used BrELXB1promoter::*GUS*, *BrEXLB1* sense (BrEXLB1-S), as well as antisense (BrEXLB1-AS), overexpressing *B. rapa* transgenic lines developed previously [25,26]. Additionally, we profiled the expression changes of 32 *B. rapa* expansin genes under *P. brassicae* race 2 inoculations by qRT-PCR assays. The present study showed that *BrEXP* genes play a role in clubroot disease responses, and manipulating root-abundant *BrEXPs* would alter the degree of resistance to *P. brassicae* race 2 in Chinese cabbage.

2. Materials and Methods

2.1. Plant Material, Treatments, Early Detection, and GUS Assay

Seeds of *B. rapa* ssp. *pekinensis* ('DH03') and transgenic seedlings overexpressing BrEXLB1promoter::*GUS*, developed previously [26], were used. The seeds were stratified at 4 °C for a day, and then sown on compost soil (Baroker, Seoulbio, Seoul, Korea) composed of cocopeat (65–70%), peat moss (8–12%), vermiculite (10–14%), zeolite (3–5%), and perlite (5–8%), containing a multi-well plastic tray in greenhouse conditions (23°/20 °C and 14-h photoperiod). One set of control and transgenic seedlings (5 d-old) were inoculated with 4.5 mL of *P. brassicae*, race 4 (1×10^6 resting spores/mL of H_2O) suspension (supplied by the National Institute of Horticultural and Herbal Science, Wanju, Korea), sampled at 1,

3, 5, 7, and 10 days post-inoculation (dpi), and designated as inoculated, while the other set of plants, mock-inoculated with 4.5 mL of sterile H_2O, were designated as controls. The inoculated and control seedlings were used for GUS assay, PCR-based early detection, and qRT-PCR-based *BrEXP* expression profiling. The whole seedlings of treated and controls collected at 1, 3, 5, 7, and 10 dpi were thoroughly rinsed with distilled water and subjected to a GUS staining assay, which was performed using a β-Glucuronidase Reporter Gene Staining Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions. For phenotyping, the leaf growth parameters of the fully expanded top leaf (one per plant) were considered, and the mean values from five or more plants were plotted in graphs, while the root growth parameters, such as maximum width and depth, were calculated at 28 dpi for each line by image analysis. The roots of three independent plants were photographed with a scale, and the parameters such as root depth (length/height) and root width (at the widest portion) were calculated using ImageJ software.

To confirm the infection at early time points where no characteristic symptoms are visible and to assess the uniform infection in all the plants, a pair of PCR primers specific to *P. brassicae* [27] was used. The primers targeting either the 18S ribosomal RNA (*18S rRNA*) gene (TC1 primer pair), or a fragment of the *18S rRNA* and internal transcribed spacer 1 (ITS1) region of the rDNA repeat (TC2 primer pair) of the clubroot pathogen, were used with genomic DNA (10 ng) derived from each treatment and control mentioned above. A positive control was included by performing PCR on the genomic DNA of the pathogen and DNA derived from infected tissues with typical symptoms. The genomic DNA was extracted from frozen gall tissues and young seedlings, using the DNeasy Plant Pro Kit (Qiagen, Hilden, Germany), and the amplification cycle conditions were followed, as reported by Cao et al. [27]. Furthermore, the PCR amplicons at the expected molecular size were gel-purified and subcloned into a pGEM-T-easy vector to facilitate Sanger sequencing with universal *T7* and *SP6* primers. The homology of the resultant sequences with existing clubroot sequences at NCBI was analyzed using the BLAST tool.

2.2. Screening of BrEXLB1 Transgenic Seedlings for Clubroot Resistance against P. brassicae Race 2

The seeds of BrEXLB1-S- and BrEXLB1-AS-overexpressing lines, non-transgenic controls, and *P. brassicae* resistant *B. rapa* cultivars, were sown on compost soil (Baroker, Seoulbio, Seoul, Korea) containing a multi-well plastic tray in greenhouse conditions (23°/20 °C and 14-h photoperiod). At 8 days post-sowing, uniform-sized, visually healthy seedlings were treated with 5 mL of spore (10^6 spores mL^{-1} H_2O) suspension (Asia Seed company, Seoul, Korea) of clubroot pathogen *P. brassicae* (race 2, Yeoncheon isolate, Gyeonggi) in the soil at the base of each plant and designated as treated plants. The other plants were mock-inoculated with 5 mL of H_2O and were as designated controls (*B. rapa* ('DH03')) for phenotyping. Inoculated seedlings were maintained at a high soil moisture level for one week to facilitate pathogen proliferation. At 30 days post-inoculation, the plants were removed, and the roots were washed thoroughly before assessing the clubroot symptoms. A score was assigned based on the presence or absence of galls on the roots. Plants with clubs on primary or lateral roots were considered susceptible, while plants with no galls were designated as resistant to *P. brassicae* race 2. Disease incidence (DIC) was recorded as the percentage of diseased plants (with club roots) in the total number of inoculated plants.

2.3. Relative Quantification of BrEXP Genes in P. brassicae Treated Young B. rapa Seedlings at Different Developmental Stages

Total RNA (5 μg) was extracted from control (mock) seedlings and seedlings (three independent biological replicates) inoculated with *P. brassicae* race 4, collected at 1, 3, 5, 7, and 10 dpi using RNeasy Plant Mini Kits (Qiagen, Germany), and cDNA was prepared in 20 μL reactions with amfiRivert cDNA Synthesis Platinum Master Mix according to the manufacturer's protocol (GenDEPOT, Baker, TX, USA). For *BrEXP* gene expression profiling, qRT-PCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with gene-specific primers (Table S1), AccuPower®2X Green-

Star Master Mix (Bioneer, Daejeon, Korea), and 1:20 diluted cDNA of each treatment as a template. The PCR conditions were as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 30 s, followed by a melting curve step to confirm the specificity of the amplified products. *BrActin2* was used as an internal control for expression normalization. The relative quantity of target gene transcripts was determined by applying the $2^{-\Delta\Delta CT}$ method [28]. The expression changes of *EXP* genes ($\log_2$ FC) at different sampling intervals post-inoculation were calculated in relation to their expression values observed in equivalent mock samples/controls (1, 3, 5, 7, and 10 dpi) and directly presented as a heatmap. To understand the trend in expression changes among *BrEXPs* or sampling times, principal component analysis (PCA) was performed using Tbtools [29].

2.4. Promoter Motif Analysis

To identify putative cis-acting elements of downregulated genes (*EXPA20/21/34*), the promoter/upstream region (−1500 bp to +1 bp) of each gene was manually retrieved from the NCBI gene database (Table S1) and searched against known motifs of the plantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) database (accessed on 29 March 2022) [30].

3. Results

3.1. Experimental Design and Molecular Detection of Clubroot Pathogens

In our preliminary studies to decide on the inoculum dosage and pathogen proliferation and to confirm the uniform infection in experimental seedlings, a PCR with primers, which specifically amplifies the 18S rRNA or a fragment of the 18S rRNA and ITS region rDNA repeat of *P. brassicae* from plant-derived genomic DNA, was performed. As mentioned in the methods section, mock-inoculated and clubroot pathogen-inoculated wild-type seedlings sampled at 1, 3, 5, 7, and 10 dpi were analyzed. As shown in Figure 1, PCR amplicons were present in all treated plants, starting as early as 1 dpi. The amplicon intensity from the positive controls was relatively higher than that of the test samples. In comparison, TC2 performed better in detecting Korean *P. brassicae* isolates than TC1, as it showed better amplification efficiency on targets. Contrastingly, non-infected plants did not contain any PCR amplicons, thus validating the uniform infection in treated plants. The resolved PCR amplicons were subcloned and sequenced to verify and know their relatedness to other pathotypes based on 18S rRNA sequence similarities. The resultant sequences had 100% sequence similarity (as queried on 21.1.2022, 4.48 pm) with the 18S rRNA and ITS region of *P. brassicae* isolate Yeoncheon 2. Interestingly, they also matched other Korean isolates, such as Hoengseong 1, Haenam 1, Pyeongchang 2, Seosan 1, and Gangneung 1.

3.2. BrEXLB1 Promoter Is Activated during P. brassicae (Race 4) Inoculations in Young B. rapa Seedlings

A GUS reporter-aided approach was adopted to evaluate the role of BrEXLB1 promoter activity during clubroot infection. The histochemical localization of BrEXLB1 promoter-driven GUS activity was observed prominently in the roots and leaves of *P. brassicae*-infected plants (Figure 2). The expression was especially strong at 3 and 5 dpi. Interestingly, at 1 dpi, GUS expression was found in root tissues. In contrast, control seedlings showed no notable GUS activity in any part of the seedlings, indicating that the early response is exclusive to the clubroot pathogen. At 3 to 5 dpi, GUS activity was extended to hypocotyls and primary leaves and maintained until 10 dpi. No GUS activity was observed in untreated plants, except for some root-specific activities (at the 3rd and 5th days). The in silico analysis of *cis*-regulatory elements in BrEXLB1 putative promoter motifs revealed that BrEXLB1*p* has wounding and pathogen responses, i.e., W box (TTGACC), defense and stress responsiveness motifs (TC-rich repeats; ATTTTCTTCA/GTTTTCTTAC), ABRE4 (CACGTA), and unnamed_4 motifs (Table S2). The potential roles of those cis-elements in clubroot disease responses are not known.

3.3. Enhanced Growth in P. brassicae-Inoculated BrEXLB1-AS Lines

P. brassicae race 4 inoculations in BrEXLB1-S, BrEXLB1-AS, and non-transgenic control plants did not develop typical clubroot symptoms for 1–28 days (Figure 3). The relative expression of *BrEXLB1* in test lines prior to infections was measured to confirm the transgenics (Figure 3A). To confirm the biomasses of *P. brassicae*, a conventional PCR with primers (TC2) specific to *P. brassicae* was carried out, and the PCR amplicons were seen in most of the inoculated seedlings (Figure 3E), although the band intensity between sense and antisense lines appeared to be different. Interestingly, BrEXLB1-AS lines had notably more enhanced leaf and root growth than wild types and sense lines under similar conditions (Figure 3B–D). While the leaf growth of BrEXLB1-S lines seemed to be comparable with wild lines (Figure 3C), it was significantly different from BrEXLB1-AS lines. In most cases, the enhanced leaf growth of AS lines was restricted to 19 dpi. At 28 dpi, the leaf growth of AS was not significantly different from wild lines (except AS-9), possibly indicating that the leaf growth in AS lines is transient. However, as shown in Figure 3D, the root growth/root biomass in AS lines was visibly more enhanced at 28 dpi than that of wild and S-lines. The root growth of AS was comparable to S-lines under normal/control conditions (data not shown), suggesting that the enhanced root growth of EXLB1-AS lines is attributable to clubroot infection.

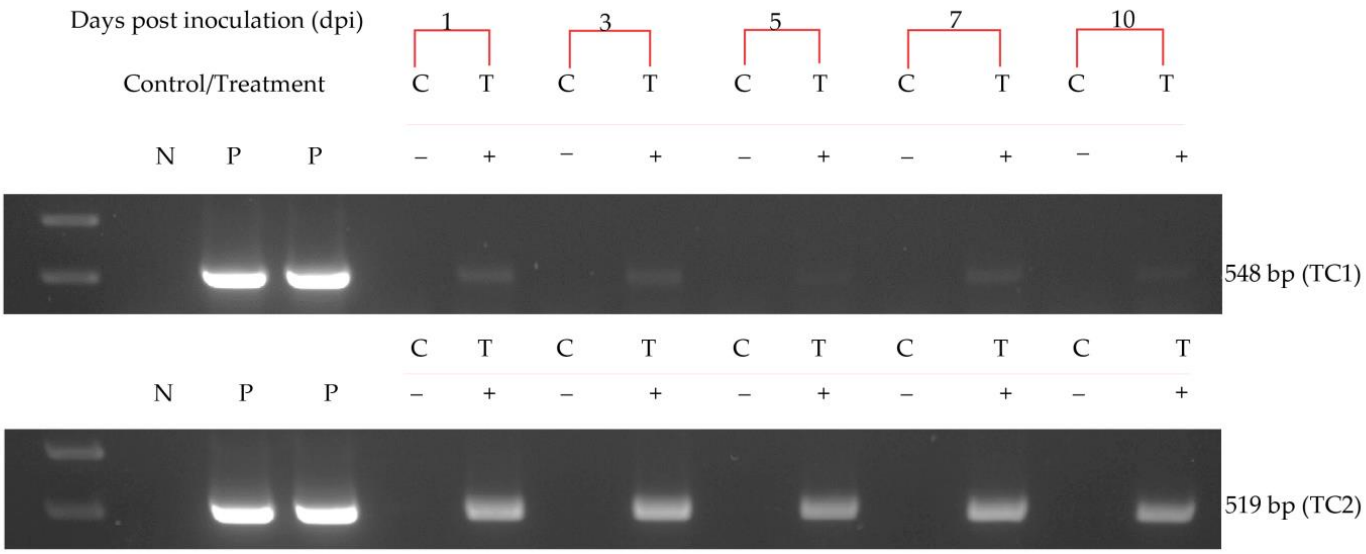

Figure 1. PCR-based early detection of *P. brassicae* in young *B. rapa* seedlings. N, negative control; P, genomic DNA derived either from the pathogen or infected plants with typical clubroot symptoms as positive control; C, DNA template from control seedlings; T, seedlings treated with *P. brassicae* race 4; 1, 3, 5, 7, and 10 represent the sampling time (in days) after inoculation; "+" or "−" indicates the presence or absence, respectively, of PCR amplicons in 1.5% agarose gel, representing the 18S ribosomal RNA (TC1) and a fragment of 18S rRNA and internal transcribed spacer 1 (ITS1) region of the rDNA repeat (TC2) of *P. brassicae*, as mentioned in the methods section.

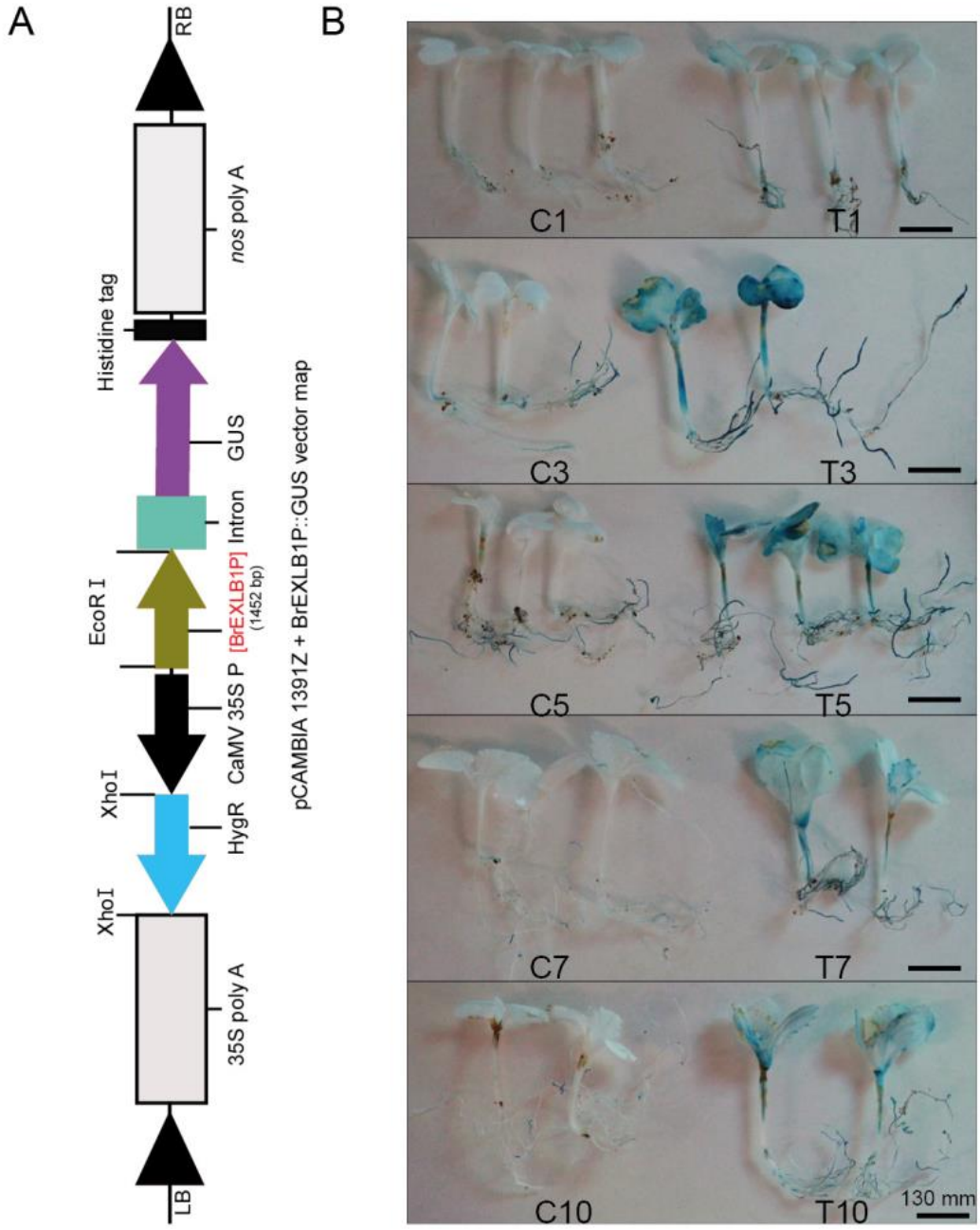

Figure 2. Histochemical localization of GUS activity in BrEXLB1promoter::*GUS*-overexpressing transgenic *B. rapa* lines. (**A**) represents the simplified vector map comprised BrEXLB1promoter::*GUS* expression cassette. (**B**) shows the GUS activity after clubroot pathogen inoculation in young *Brassica rapa* seedlings. In (**B**), C denotes controls/mock-inoculated transgenic lines; T stands for treated and denotes *P. brassicae* race 4-inoculated lines; 1, 3, 5, 7, and 10 represent sampling time points (in days) post-inoculation. Scale bar = 130 mm.

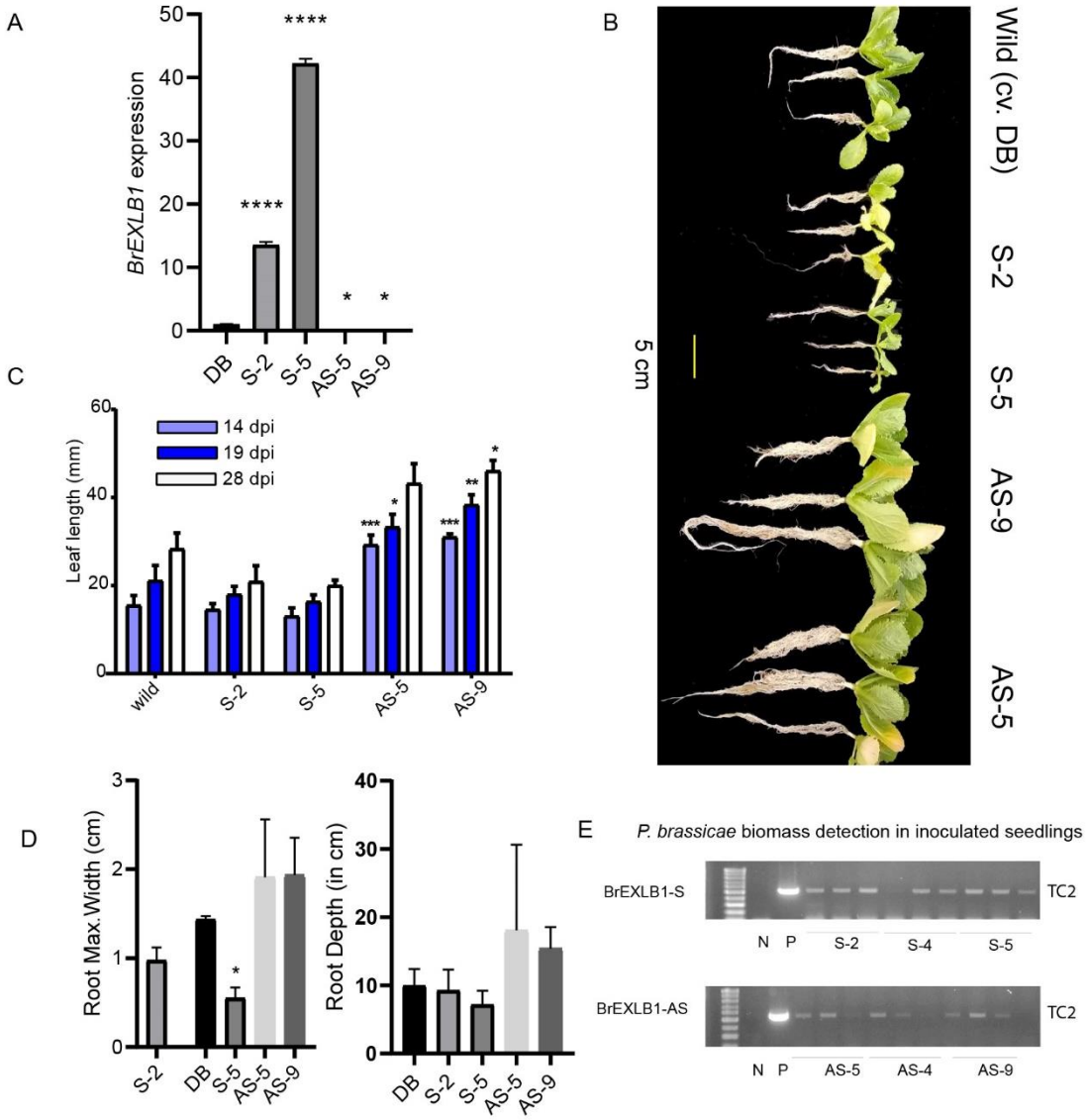

Figure 3. The root and leaf traits in *P. brassicae*-inoculated BrEXLB1 transgenic and wild-type *B. rapa* spp. *pekinensis* lines at greenhouse conditions. The 5-d-old *B. rapa transgenic* seedlings overexpressing *BrEXLB1* sense (S-2, 4, 5, and 6), antisense (AS-2, 5, 9, and 10), and wild type (DB) were inoculated with *P. brassicae* race 4. The leaf growth was measured randomly at 14, 19, and 28 dpi, while the root growth was photographed at 28 dpi; 3A denotes the three representative lines from treatments. In 3B, the relative quantification (in folds) of *BrEXLB1* in transgenic lines is mentioned in 3A over the controls before infection, while 3C indicates the mean leaf growth (mm) parameters measured from *P. brassicae* race 4-infected plants (n = 5) at different times; 3D shows the mean root growth parameters (maximum root width and depth) measured at 28 dpi; 5E shows the *P. brassicae* presence in inoculated seedlings (BrEXLB1 sense and antisense OX lines) through PCR amplicons of TC2. * indicates that the mean differences between treatment and controls are statistically significant (statistics by ANOVA test are shown; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$).

3.4. Antisense-Mediated Suppression of BrEXLB1 Likely to Improve P. brassicae Resistance in Transgenic B. rapa Lines

To investigate the *BrEXLB1* effect on disease incidence upon *P. brassicae* race 2 infections, 8-d-old BrEXLB1-S and -AS transgenic seedlings were inoculated, and the experimental plants were evaluated and compared with controls, resistant *B. rapa* cultivars for clubroot symptoms at 30 dpi (Figure 4). Disease incidence (DIC) was recorded as the percentage of diseased plants (with club roots) in the total number of inoculated plants (10–14 seedlings

per treatment). Unlike race 4 infections, race 2 strains developed typical disease symptoms in BrEXLB1-S lines (100 DIC) and non-transgenic control (DB) lines (100 DIC), indicating that the control and BrEXLB1-overexpressing lines were highly susceptible to the clubroot pathogen, and all lines had characteristic gall formation on their roots. However, the antisense-mediated suppression of *BrEXLB1* at transcript levels showed no gall formation/disease symptoms in some seedlings derived from the heterogeneous populations (50 DIC), while few others had symptomatic roots, indicating moderate resistance to the pathogen. The resistant cultivar had the lowest disease incidence of 7.69, which indicates their high resistance to race 2 *P. brassicae* infection.

Figure 4. Clubroot pathogen *P. brassicae* race 2-infected BrEXLB1 transgenic, wild-type, and resistant *B. rapa* spp. *pekinensis* cultivars. (**A**) BrEXLB1-S plants; (**B**) BrEXLB1-AS; (**C**) wild type; (**D**) clubroot-resistant *B. rapa*. The plus (+) and minus (−) symbols indicate the presence and absence, respectively, of the clubs in roots.

3.5. Expression Changes of BrEXP Genes in P. brassicae-Inoculated Seedlings

The expression pattern of 32 *BrEXPs* was investigated at transcript levels in 5-d-old-*B. rapa* seedlings inoculated with *P. brassicae* and sampled at different time intervals (1, 3, 5, 7, and 10 dpi). The overall results showed that most *Br*EXPs responded significantly to the clubroot pathogen by expression changes as early as 1 dpi, suggesting its potential as early response genes for clubroot disease (Figure 5).

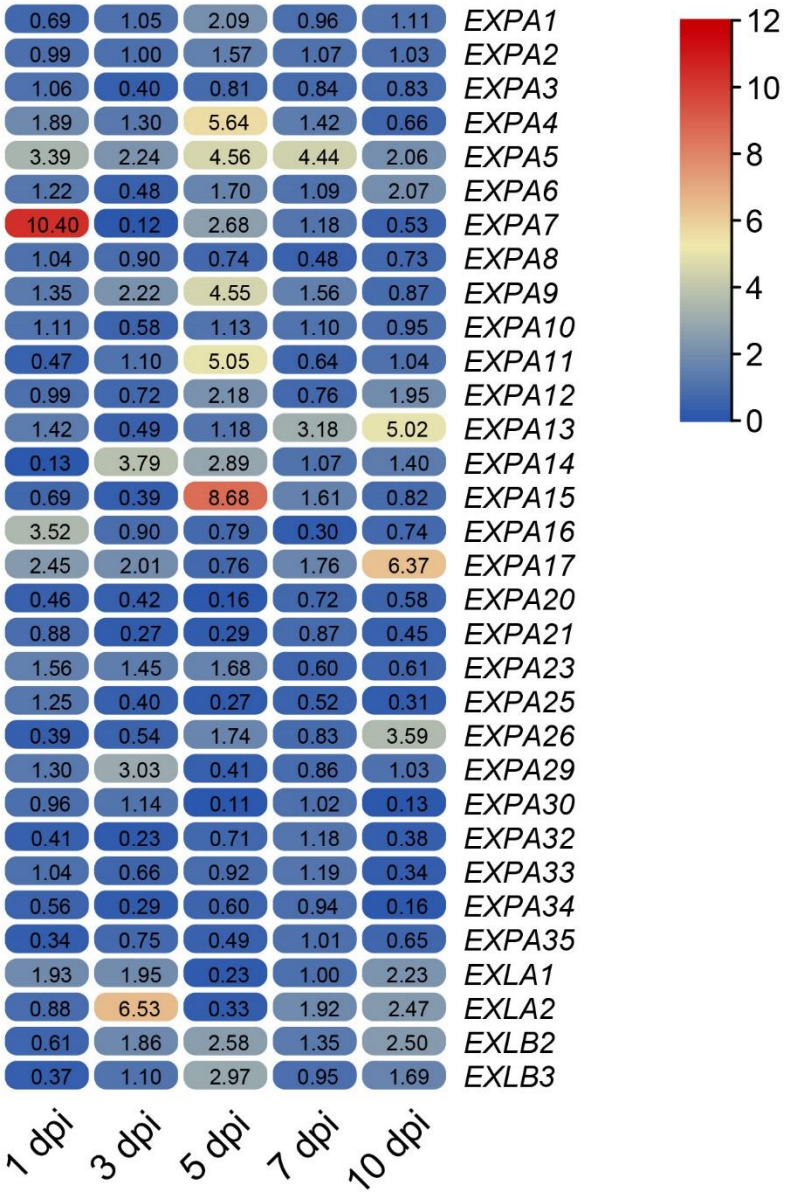

Figure 5. The expression pattern of *B. rapa* expansin (*BrEXP*) genes during *P. brassicae* race 4 infections. The expression changes in folds (Log_2FC) (depicted by color keys) were derived from qRT-PCR-based relative quantification of biological replicates; 1, 3, 5, 7, and 10 dpi represent the sampling times (in days) post-inoculation. The changes in expression (folds) of *EXP* genes during inoculation (displayed in heatmap blocks) were calculated in relation to their expression values in equivalent mocked samples/controls. BrActin2 was used for expression normalization.

Altogether, 28 α-expansin (*EXPA*), 2 β-expansin (*EXPB*), 1 expansin-like A (*EXLA*), and expansion-like B (*EXLB*) genes showed differential expression in any one of five samples collected during *P. brassicae* inoculations. Among these, *EXPA20/21/34* was consistently downregulated compared to their respective equivalent controls at all sampling intervals, while the *EXPA5* expression showed constant upregulation ($log_2FC \geq 2$) compared to equivalent controls. Additionally, the expression pattern of most genes was relatable to sampling/infection times. The highest number of genes (12) showed the peak of their induced expression at 5 dpi, followed by 1 and 10 dpi. Similarly, the maximum number of genes (nine) showed their peak of reduced expression was also 5 dpi, suggesting that major transcriptional reprogramming of *BrEXPs* occurs at 5 dpi. *EXLB1* and *EXPA16* showed some unique trends in expression changes. Their expression was slightly upregulated

at 1 dpi; however, a gradual reduction in expression was observed during the disease progression (3, 5, 7, and 10 dpi). Most other genes showed no clear trend in expression changes, and it seems the plant *EXP* response to pathogens is also governed by the growth stages of host plants or infection times, as indicated by the qRT-PCR results.

To investigate the possible molecular basis of the clubroot pathogen regulating *BrEXP* expressions (*EXPA20/21/34*), we analyzed the promoter motifs of those genes. There were nine putative *cis*-regulatory elements (CREs), such as TCT-motif, ethylene response element (ERE), MyB binding site response element (MRE), unnamed__4, MYC, MYB-like sequence, and core promoter motifs (TATA-box and CAAT-box), that were commonly found in *EXPA20/21/34* genes (Table S2).

The gene expression data collected at different intervals were also analyzed by principal component analysis (PCA) to understand the expression/variation trends among *BrEXP* genes (Figure 6A,B). The PCA score plot shows the overlapping and clustering of 15 genes (*EXPA2/6/8/10/12/20/21/23/25/30/32/33/34/35* and *EXLB3*), mostly belonging to the α-EXPs in the negative axis of PC1, with the total variance accounting 34.1% as the first principal component (PC1) and 23.8% as the second principal component (PC2). The clubroot response expression patterns of *EXLA2* and *EXPA5/7/13/15/17* are unique and distinct from others. Among sampling times, 5 dpi stand out different from the others, while the expression pattern of most *EXP* genes at 3 dpi and 10 dpi looks similar. These results also indicate the critical influence of infection times. The correlation analysis between *EXLB1* and other expansin genes across different sampling times (1, 3, 5, 7, and 10 dpi) showed that their expression pattern positively correlated with *EXLA1* and *EXPA7/8/20/25/16*, and it negatively correlated with *EXLA2*, *EXLB2/3*, and *EXPA1/2/4/9/11/12/14/15* genes (Table S3).

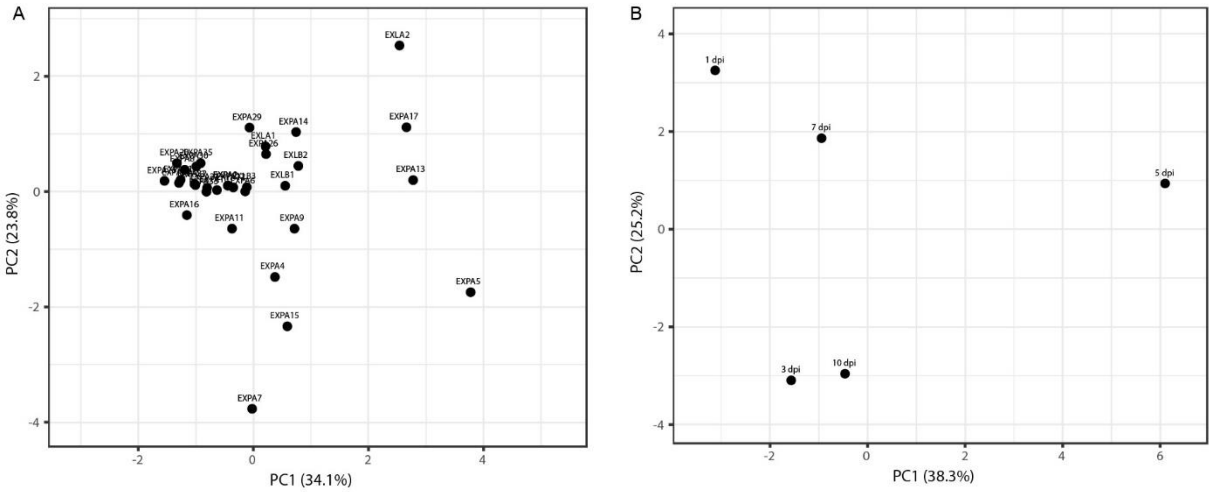

Figure 6. Principal component analysis performed among differentially expressed *BrEXP* genes (**A**) with their expression changes in folds and sampling times (dpi) after inoculation of *P. brassicae* (**B**).

4. Discussion

Modifications of cell wall composition and structure occur throughout the plant lifecycle. It directly affects cell wall integrity (CWI), which determines the plant defense responses against pathogen attacks by multiple modes of action [9,11]. The impairment or enhancement of the expression of wall-related genes can alter the CWI. Although the emphasis was given to plant cell walls, cell wall-associated genes, and wall structural modification in pathogen invasion and disease resistance responses [10–12], the resources demonstrate that the role of expansin/cell wall loosening proteins in plant disease responses is relatively limited. The current climatic changes are favoring the widespread incidence of clubroot disease in the Brassica species [31]; hence, it is important to develop new resistant resources. Until recently, the role of expansin in clubroot disease responses in *B. rapa* was not available, although the central mechanism by which EXPs disrupt the extracellular matrix for cell wall relaxation and expansion, and thereby contribute to plant growth and

development, was clearly known for a long time in terrestrial plants [15,16,32]. Herein, the histochemical analyses of BrEXLB1 promoter-driven GUS activity during clubroot infection confirm *BrEXPs*' participation in clubroot disease. Prominent GUS activity in transgenic reporter lines and qRT-PCR-based expression patterns revealed that *BrEXPs* respond to the clubroot pathogen as early as 1–3 dpi, suggesting that *BrEXPs* are crucial in early responses, while the minimal GUS activity found in non-infected control roots at 3, 5 dpi can be attributed to *BrEXLB1* in root development, as reported previously [25]. The GUS assay results with BrEXLB1 promoter indirectly imply that *BrEXLB1* might play a role, either positively or negatively, in clubroot disease responses. The expression pattern of *BrEXLB1* during early clubroot infection (1, 3, 7, and 10 dpi) showed induced expression, which was different from later infections, where a drastic reduction was observed as the disease progressed [25]. Additionally, *BrEXLB1* is one of five EXP genes that retained all three gene copies in the Brassica genome after the triploidization event [23]. The retention of gene duplicates could lead to neofunctionalization; hence, it draws attention to crop improvement programs. Some of the EXLB class genes were shown to enhance the abiotic stress tolerance, in particular, drought [25,33] and heat stress conditions [34]. Therefore, the identification of EXLB roles in disease resistance would strengthen both biotic and abiotic stress protection in crop plants. Additionally, the instant availability of BrEXLB1 transgenic lines and the possibility of them being a potential candidate gene for clubroot disease experiments made us investigate the impact of transgenic overexpression and the antisense-mediated suppression of *BrEXLB1* on clubroot disease resistance.

Both races (2 and 4) of *P. brassicae* were previously reported in Korea. In this study, the screening of BrEXLB1-S and -AS lines with *P. brassicae* race 4 produced some interesting phenotypes, with none of the plants, including wild-type lines, having typical clubroot symptoms. Although the PCR-based early detection and sequencing confirmed the presence of *P. brassicae* race 4 in all the plants, it is unclear how the plants, including wild-type lines, remained symptomless. The possible reason could be an insufficient proliferation of pathogens under semi-controlled environments. However, we observed differential influences on leaf and root growth parameters of BrEXLB1-AS lines. Considering our previous study, we can rule out the possibility of the transgenic expression of *BrEXLB1* affecting leaf or root growth parameters in AS lines [26]. On the other hand, *P. brassicae* inoculation can transiently enhance plant growth/biomass via enhanced IAA synthesis and XTH action at early infection periods [35]. Nonetheless, in our study, the enhanced growth pattern was observed only in BrEXLB1-AS lines, thus warranting further studies on how BrEXLB1 interaction with pathogens affects growth patterns in *B. rapa*. Phytohormones could also contribute to BrEXLB1-mediated clubroot disease response, as *BrEXLB1* expression was induced by the exogenous application of IAA, ABA, SA, and ethylene in *B. rapa* [25]. In order to know the performances of transgenic lines under another prevalent, *P. brassicae* race 2, we conducted similar screening experiments, and the results revealed that *BrEXLB1* is a negative regulator of clubroot disease resistance in *B. rapa*. One of the possibilities is that enhanced cell wall relaxation by ELXB1 overexpression might favor pathogen entry in host root cells. Nevertheless, a recent study that dealt with one of the *EXLB* class members (*AdEXLB8*) in wild *Arachis* showed that overexpression could offer tolerance to both abiotic and biotic stress conditions by activating hormonal and antioxidant-based defense mechanisms [36]. However, unlike *BrEXLB1* [25], *AdEXLB8* does not significantly contribute to root growth. Although no direct evidence supporting *EXP* in clubroot resistance is available, their expression pattern in infected or resistant cultivars confirms they could participate in plant disease responses through multiple mechanisms [13,18,20,37]. Previous studies by Park et al. [38] and Otulak-Kozieł et al. [39] demonstrated that the overexpression of *NbEXPA1* and potato *EXPA3* leads to disease susceptibility in respective host plants against viral pathogens. Additionally, in another recent study, *at*exp1-1 (loss of function mutant) improved the resistant phenotypes against a necrotrophic fungus, *Plectosphaerella cucumerina*, compared to the wild type [9]. To progress further or to have concrete evidence of clubroot resistance in AS-lines, it is essential to select homozygous/stable transgenic AS popula-

tions. This preliminary screening for resistance showed that the alteration of *BrEXLB1* expression is likely to impart *P. brassicae* disease resistance to *B. rapa*. The symptomatic AS populations can be attributed to possible unstable transgenic phenotypes/overexpression of antisense *BrEXLB1*. Therefore, *BrEXLB1* knockout lines may be developed by applying the CRISPR/Cas system via Agrobacterium-mediated genetic transformation in *B. rapa*. In some cases, the potential suppression of EXP expression can lead to undesirable traits, such as retarded plant growth and development (Ding et al., 2008) in transgenic plants, in addition to disease resistance [40]. However, the suppression of *BrEXLB1* does not display any visible morphological abnormalities in *B. rapa* under normal conditions. Previously, it was shown that the exogenous application of phytohormones, such as indole-3-acetic acid and jasmonic acid, as well as other factors, such as white light and drought stress, induce BrEXLB1 promoter activity [26]. Hence, we presume that the inhibition of *BrEXLB1* might suppress auxin signaling to impart *P. brassicae* resistance [40]. A previous study by Gil et al. [16] identified that beet necrotic yellow vein virus, a causative agent of rhizomania in sugar beets, can hijack auxin-regulated pathways and reduce taproot growth by interacting with host AUX/IAA proteins and thereby inducing the expansin activity. This could be explained as a possible reason why BrEXLB1 OX lines show reduced root growth during clubroot infection. However, further study is required for the clear understanding of clubroot pathogen and expansin interaction in *B. rapa* plants.

In order to understand the role of other *BrEXPs* in clubroot disease response, we quantified the relative expression changes of 32 *BrEXPs* during *P. brassicae* race 4 infections. All the *BrEXPs* were differentially expressed during clubroot pathogen attacks. The early infection led to a consistent reduction in the expression of some α-EXP family members (*EXPA20/21/34)*. Contrastingly, *P. brassicae* infection in Arabidopsis induced the expression of α-expansin (EXP) gene family members, albeit in relatively later stages of infection [13]. Another study dealing with comparative transcriptome analyses of symptomless roots and gall roots of *B. oleracea* revealed that the downregulation of *EXPA20* may help maintain roots free of clubs [37]. A recent tissue-specific transcriptome-wide analysis of *BrEXPs* revealed that *BrEXPA20/21* are abundantly expressed in root tissues [24], while *BrEXPA34* is not abundant, suggesting that *BrEXPs* are also regulated by tissue-specificity. Previously, it was shown that the downregulation of cell wall loosening proteins, such as EXP and XTH, is important for disease resistance or symptomless roots [37]. As shown in PCA analysis, the expression pattern of *BrEXPs* under clubroot infection is likely to be influenced either by infection times or growth stages of plants. Moreover, to understand the clubroot-mediated downregulation of some *BrEXPs*, we scanned the potential promoter motifs that could perceive the pathogen signal and transcribe target genes as a counter-response. The functional characterization of nine putative cis-regulatory elements (CREs) identified in this study might provide clues about clubroot response gene transcription.

5. Conclusions

We found that *BrEXLB1* is response to clubroot disease in *B. rapa* and its effective suppression likely to enhance disease resistance. Most of the *BrEXPs* were differentially expressed during clubroot infection suggesting their participation in the clubroot disease response of *B. rapa*. The future studies dealing genetic manipulation of root-abundant *BrEXPs* could facilitate clubroot disease resistance in Chinese cabbage.

6. Patents

Transgenic reporter *B. rapa* lines overexpressing BrEXLB1*p*:*GUS* constructs for its response to clubroot pathogen inoculation (Application No. 10-2021-0136083) and BrEXLB1 antisense-overexpressing lines for enhanced protection against clubroot disease in *B. rapa* (Application No. 10-2021-0136091) were filed for Korean patent.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture12091416/s1, Table S1: List of primers used for qRT-PCR-based relative quantification of *Brassica rapa EXP* genes; Table S2: List of *cis*-regulatory elements presented in the putative promoters of EXP genes; Table S3: Pearson correlation statistics for Clubroot response *BrEXP* genes; Data S1: The putative promoter sequences of *BrEXPA34*, *BrEXPA20* and *BrEXPA21* genes.

Author Contributions: Conceptualization, S.I.L.; investigation, M.M., S.R.P., J.-I.P. and S.I.L.; writing—original draft preparation, M.M.; writing—review and editing, M.M. and S.I.L.; supervision, funding acquisition, S.I.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Rural Development Administration (Korea) through the Rural Program for Agricultural Science and Technology Development, grant number PJ01495701, and the New Breeding Technology Center, grant number PJ01653701.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: This manuscript includes the necessary data, either as figures or as Supplementary Data.

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

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