A THESIS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

Functional analysis of zoysiagrass chitinase genes

Ji-Nam Kang

(Supervised by professor Hyo-Yeon Lee)

Department of Biotechnology

GRADUATE SCHOOL

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1. Introduction

Zoysiagrass (*Zoysia japonica* Steud.) is one of the most important plants used in golf courses, athletic fields, home lawns, etc. The economic value of zoysiagrass has increased recently in the USA and Far East Asia, including Korea (Poeaim *et al.* 2005; Toyama *et al.* 2003). Demand for zoysiagrass in Korea increased roughly threefold between 2003 and 2012. Because of this increased demand for zoysiagrass, various agricultural chemicals including herbicides, insecticides and germicides are used for management of turf grasses annually. In particular, germicides are applied for maintenance of turf grasses on golf courses (Tae 2008). Grasses on golf courses can be damaged by fungal diseases, such as brown patch, dollar spot, *pythium* blight and large patch. Among them, large patch is an important disease of zoysiagrass fields caused by *Rhizoctonia solani* AG2-2 (IV) (Jung *et al.* 2008). Large patch occurs mainly in late spring and develops more rapidly than other fungal diseases. Also, control of large patch is difficult because of its scale and large lesions. Therefore, development of disease-resistant zoysiagrass by genetic engineering would be beneficial.

Chitinases are pathogen-related (PR) proteins that catalyze the hydrolysis of chitin. Chitin is a linear polymer of β-1,4-linked N-acetylglucosamine residues and is an important structural component of the cell walls of various pathogenic fungi, including *Rhizoctonia solani* (Khan and Shih 2004). Chitinases can be divided into endochitinases and exochitinases according to their mechanism of action. Endochitinases hydrolyze internal β-1, 4-glycoside bonds, while exochitinases catalyze reactions only at the non-reducing end of the chitin chain (Kasprzewska 2003). Also, chitinases are categorized into seven classes according to their primary structure. Class I chitinases have an N-terminal cysteine-rich domain (chitin binding domain, CBD), which

comprises 40 highly conserved amino acids, and can be divided into two subclasses, Ia and Ib, according to the presence of a C-terminal extension. Class II chitinases have high amino acid sequence similarity to class I chitinases but lack the N-terminal cysteine-rich domain. Class III chitinases have no sequence similarity with class I and class II chitinases. Class IV chitinases resemble with class I chitinases including cysteine-rich domain on N-terminal region but are smaller due to four deletions in catalytic domain (CD). Most known chitinases belong to these four classes (Collingel *et al.* 1993).

Chitinases are considered to be defense-related proteins in higher plants. Class I chitinases have antifungal activity against various pathogenic fungi. The class I barley chitinase clone 6N showed broad-range *in vitro* antifungal activity against *Botrytis cinerea*, *Pestalotia theae*, *Bipolaris oryzae*, *Alternaria* sp., *Curvularia lunata*, and *R. solani* (Kirubakaran and Sakthivel 2007). Class I vacuolar chitinase from *Nicotiana tabacum* cv. Samsun NN caused lysis of the hyphal tip and growth inhibition of *Fusarium solani*, and purified class I chitinase from pea pod inhibited mycelium growth of *Trichoderma viride*. Transgenic plants overexpressing class I chitinase have been demonstrated to inhibit fungal growth. Transgenic tobacco seedlings constitutively expressing a class I been chitinase gene showed enhanced antifungal activity in soil infested with *Rhizoctonia solani* compared with wild-type plants (Broglie *et al.* 1991). Transgenic indica rice cultivars introduced PR-3 rice class I chitinase gene (RC7) indicated increased resistance when challenged with *Rhizoctonia solani* (Datta *et al.* 2001). Also, transgenic grapevine and Italian ryegrass plants expressing rice class I chitinase (RCC2) exhibited enhanced resistance against *Elisinoe ampelina* and *Puccinia coronata*, respectively (Yamamoto *et al.* 2000; Takahashi *et al.* 2004).

In comparison, class II chitinases from these plants showed no antifungal activity *in vitro* (Mauch *et al.* 1988; Sela-Buurlage *et al.* 1993). Also, rye

seed chitinase-a (RSC-a, class I) showed more strongly antifungal activity on the re-extension of the hyphae, containing mainly mature cells, than chitinase-c did (RSC-c, class II) in the *in vitro* condition (Taira *et al*. 2002). However, transgenic tobacco and wheat plants introduced a barley class II chitinase exhibited enhanced resistance against *R. solani* and *Erysiphe graminis*, *Puccinia reconidita*, respectively (Jach *et al*. 1995; H. Oldach *et al*. 2001).

In addition, a cysteine-rich domain called the chitin-binding domain (CBD) in the N-terminal region of class I chitinases is related to allergic reactions. In comparison, class II chitinases lack the CBD and are not involved in allergic reactions (Diaz-Perales *et al*. 1988; Posch *et al*. 1999). Therefore, the *Zjchi2* gene might be useful for generating disease-resistant transgenic zoysiagrass.

In this study, we isolated two full-length chitinase genes, class I chitinase (*Zjchi1*) and class II chitinase (*Zjchi2*) from zoysiagrass. Structural property and expression analysis of these genes was performed. Furthermore, the two chitinases were overexpressed in *Escherichia coli* and purified for assessment of their *in vitro* antifungal activity. Also, we generated transgenic zoysiagrass that overexpressed the class II chitinase gene (*Zjchi2*) via *Agrobacterium*-mediated transformation from zoysiagrass calluses, although its monocot and plant regeneration is normally difficult (Toyama *et al*. 2003; Poeaim *et al*. 2005). Southern blot hybridization and polymerase chain reaction (PCR) were performed to confirm the transgenes insertion in the transgenic zoysiagrass. Furthermore, the antifungal activity was investigated to examine the disease resistance of transgenic zoysiagrass to *R. solani* AG2-2 (IV). To our knowledge, this study is the first to verify the antifungal activity of a class II chitinase in transgenic zoysiagrass.

2. Material and methods

2.1. Plant materials and cloning of chitinase genes

Mature seeds of zoysiagrass (*Zoysia japonica* Steud. cv Duckchang) were coat-removed and surface-washed with 100% ethanol by vigorous shaking for 1 min. Subsequently, the seeds were surface-sterilized using a mixture of sterilized water, Tween 20 and 2% sodium hypochlorite by gentle shaking for 15 min, and then washed three times in sterilized water. After drying on filter paper, the seeds were placed on half-length Murashige and Skoog (MS) agar medium in a sterilized glass bottle. All steps were carried out on a clean bench. To generate plants, seeds were cultivated in a growth chamber under a 16 h light/8 h dark cycle at 25 °C. After 2 months, the zoysiagrass seedlings were transferred to pots containing soil and cultivated in a green house. After 5-6 months, the mature zoysiagrass was harvested and stored at -80 °C for cloning and expression analysis of chitinase genes.

Total RNA was extracted from root of zoysiagrass using TRIzol reagent (MRC) according to the manufacturer''s instructions. Subsequently, total RNA was treated with DNase I using a DNA-free kit (Ambion) to inactivate contaminating DNA, and then immediately stored at -80 °C or used for cDNA synthesis. cDNA was synthesized using 1 μg of total RNA extracted from roots of zoysiagrass using an Access RT-PCR system (Promega). Two full-length chitinase genes were obtained from a 20 μL reaction mixture including 1 μL of cDNA, 0.5 μL (10 pmol) of *Zjchi1* (forward, 5′-CCCACACCAACACCAGAGCA GATT-3′; reverse, 5′-CTGCAGAGTAATAATTAAGCGTCAA-3′) or *Zjchi2* (forward,5′-ACACATTTGCAAGGCGAAGCG-3′; reverse,5′-CAGCGCACATC TTATTCGCCA-3′) specific primer and 10 μL of EmeraldAmp PCR Master Mix (TaKaRa) using PCR Thermal Cycler Dice (TaKaRa) under the following

conditions. Initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 65 °C for 30 s and extension at 72 °C for 30 s.

2.2. Expression analysis of chitinase genes

To test the mRNA accumulation of chitinase genes challenged to *R. solani* infection, pre-germinated zoysiagrass seedlings on half-length MS agar medium were transferred to fresh medium in sterilized glass bottles. *R. solani* AG2-2 (IV) inoculum (mycelium plug) was placed at the center of the medium. After 3 days, *R. solani* AG2-2 (IV) mycelium reached zoysiagrass seedlings, which were harvested at 24, 48 and 72 h for assay of chitinase gene expression. *Zjchi1* and *Zjchi2* expression was assayed by real-time PCR using a Thermal Cycler Dice, Real Time System Lite (TaKaRa). A 20 μL reaction mixture comprising 1 μL of cDNA (10×dilution), 0.5 μL (10 pmol) of *Zjchi1* (forward, 5′-GTACGGCGTCAT CACCAACAT-3′; reverse, 5′-CTCTGGCTGTAGCAGTCCAAGTT-3′) or *Zjchi2* (forward, 5′-CGTCATCACAAACATCATCAAC-3′; reverse, 5′-GAAC TTCCTCTGGCTGTAGCAGT-3′) specific primer, and 10 μL of iQ™ SYBR® Green Supermix (Bio-Rad) was subjected to real-time PCR amplification using the following conditions. An initial denaturation at 95 °C for 30 s was followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s. The zoysiagrass *β-actin* gene was also amplified as a reference. *Zjchi1* and *Zjchi2* expression was quantified by normalization to that of *β-actin*; their relative expression level was then calculated using the delta - delta CT method.

2.3. Vector construction for recombinant (r) chitinases

Two mature chitinase genes lacking signal peptide sequences were obtained by PCR amplification. The mature form of Zjchi1 comprising 299 amino acids

was amplified using the following primers: forward, 5′-GGATCCGAGCAGTGC GGGTCGCGGGCC-3′; and reverse, 5′-CTCGA GTCAGGCGCCGAATGGCCT CTG-3′. The mature form of Zjchi2 comprising 231 amino acids was amplified using the following primers: forward, 5′-GGATCCCAGCAGGGCGTGGGCTCG ATC-3′; and reverse, 5′-CTCGAGTCAGCCGAACTTCCTCTGGC-3′. Underlined sequences are *Bam*HI and *Xho*I restriction enzyme sites, respectively. PCR was performed at 94 °C for 5 min (first denaturation), followed by 30 cycles comprising 94 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR products were confirmed in 1% agarose gels and purified using an Expin™ Gel Extraction Kit (GeneAll). PCR error was analyzed using a pGEM T-Easy vector (Promega) by Macrogen, Korea. *Zjchi1* and *Zjchi2* were double-digested using *Bam*HI and *Xho*I and then ligated into *Bam*HI-*Xho*I digested pET 28a+ (Novagen) and pGEX 4T-1 (GE Healthcare Life Sciences) vectors, respectively.

2.4. Overexpression in *E. coli* and purification of r-chitinases

Escherichia coli BL21 (DE3) was used for overexpression of pET-Zjchi1 and pGEX-Zjchi2 according to Kirubakaran and Sakthivel (2007). *E. coli* BL21 (DE3) harboring pET-Zjchi1 or pGEX-Zjchi2 was inoculated into Luria-bertani (LB) medium containing kanamycin (50 μg/mL) or ampicillin (100 μg/mL), respectively, and incubated at 37 °C for 12 h with vigorous shaking. One milliliter of culture was inoculated into 100 mL of fresh LB medium containing the corresponding antibiotics, followed by incubation at 37 °C with vigorous shaking until an optical density (OD) 600 of 0.5; 0.5 mM isopropyl β-D-thiogalactoside (IPTG) was then added. After incubation at 18 °C for 4 h, cultures were harvested by centrifugation at 3000 rpm at 4 °C for 15 min and the supernatants were decanted. Pellets were resuspended in lysis buffer (50 mM Tris-Cl, 150 mM NaCl; pH 7.5) by vortexing and then immediately frozen

at -80 °C. Overexpression of r-chitinases was confirmed by sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE).

The r-chitinases were purified by affinity column chromatography. Cells harboring pET-Zjchi1 or pGEX-Zjchi2 were freeze-thawed twice on ice and then homogenized by sonication with a duty cycle of 70% for 5 min with 50 s intervals using a Sonics instrument (Vivra-cell™). The disrupted cells were centrifuged at 13,000 rpm at 4 °C for 20 min to remove unbroken cells and cell debris. The supernatant containing pET-Zjchi1 bound to Ni^{2+} Sepharose resin (GE Healthcare) and was washed using five volumes of wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole; pH 7.4). pET-Zjchi1 was eluted using elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole; pH 7.4). pGEX-Zjchi2 was purified using Glutathione Sepharose™ 4 Fast Flow resin (GE Healthcare). The supernatant containing pGEX-Zjchi2 bound to Glutathione Sepharose resin was washed using wash buffer (50 mM Tris - HCl, 150 mM NaCl; pH7.5) and then eluted with elution buffer (50 mM Tris - HCl, 10 mM reduced glutathione; pH 8.0). Subsequently, glutathione S-transferase (GST) was excised from the purified pGEX-Zjchi2 using bovine thrombin (Sigma). The purified r-chitinases were dialyzed against phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4; pH 7.4) and GST was removed by repeated GST affinity column chromatography. All steps were carried out at 4 °C. Purified r-chitinases were quantified using a UV spectrophotometer (Optizen POP Nano Bio) at 280 nm.

2.5. Antifungal activity of purified r-chitinases

To assay the antifungal activity of the purified r-chitinases, the following fungal pathogens were obtained from the Rural Development Administration Gene-bank Information Center, Republic of Korea: *Rhizoctonia solani* AG2-2 (IV), *Rhizoctonia solani* AG1 (IA), *Rhizoctonia cerealis*, *Sclerotinia sclerotiorum*,

Botrytis cinerea, *Fusarium culmorum*, *Fusarium graminearum*, *Pythium* sp., *Sclerotinia minor* and *Trichoderma reesei*. Mycelia of these fungi were grown on potato dextrose agar (PDA) at 25 °C for 10 days in darkness and then used for antifungal assays. Disk-diffusion tests were performed according to Ye and Ng (2005). Fungal plugs were prepared from mycelia of the above-mentioned fungi and placed at the center of fresh PDA plates. Cotton disks containing 300 μg/mL of the purified r-chitinases were placed close to the mycelium plugs. PBS buffer containing thrombin solution was used as a negative control. After incubation at 25 °C for 5 days, clear zones of inhibition of mycelium extension were examined. Three independent experiments were performed.

Microscopic analysis of inhibition of mycelium extension was performed using *T. reesei* and purified r-Zjchi2. Purified r-Zjchi2 at 125, 250, 500 and 100 μg/mL was added to test tubes containing 5 mL potato dextrose broth (PDB). The control tube contained PBS buffer with thrombin. Mycelia from *T. reesei* were inoculated onto fresh half-length PDB medium and incubated at 25 °C for 3 days with gentle shaking and then homogenized using an Ultra Turrax T-20 homogenizer (SS Motor). The dispersed *T. reesei* mycelia suspension (100 μL) was immediately inoculated into a test tube containing PBS buffer or purified r-Zjchi2 and then incubated at 25 °C for 3 days with gentle shaking. Inhibition of mycelium extension was evaluated by light microscopy (9100) (Zeiss).

2.6. Preparation of the binary expression vector

For vector modification, the binary vector pCAMBIA3301 was double-digested using the restriction enzymes *Hin*dIII and *Eco*RI. Then, the cassette containing the maize *ubiquitin* promoter and *Arbcs* transcription terminator containing a multi-cloning site consisting of *Bam*HI, *Sma*I, and *Sac*I restriction sites was

integrated into the space generated by the *Hin*dIII and *Eco*RI treatment. The open reading frame (ORF) of the *Zjchi2* gene (774 bp) was obtained from zoysiagrass cDNA by RT-PCR using the forward primer (5′-ggatccATGGCGAG AGTGGCGGGTTCG-3′) and reverse primer (5′-gagctcTCAGCCGAACTTCCTC TGGCT-3′). The small letters in the primer sequences indicate the *Bam*HI and *Sac*I restriction sites, respectively. The PCR product was inserted into a pGEM® T-easy vector (Promega) for sequencing; then, the pGEM® T-easy vector containing the *Zjchi2* gene was double-digested with *Bam*HI and *Sac*I. Finally, this product was inserted into the modified pC3301 binary vector, which we named IG2 vector. The construct contained the *β-glucuronidase* gene as a reporter gene and the *bar* gene as a selectable marker for herbicide resistance. The completed construct, named IG2-Zjchi2, was transformed into *Agrobacterium* strain EHA 105.

2.7. Development of transgenic zoysiagrass

To generate transgenic zoysiagrass, calluses from zoysiagrass seeds were prepared, and genetic transformation was carried out using the method of Toyama *et al.* (2003), with modification. The selected calluses were infected using the *Agrobacterium* disarmed strain EHA 105 containing IG2-Zjchi2, and were then cultured at 25 ℃ with gentle shaking (120 rpm). After infection for 24 h, the calluses were air-dried on sterilized filter paper and co-cultured in the dark for 3 d. Transgenic calluses were selected using 5 mg/L phosphinothricin (MB cells), and shoots and roots were induced from the putative transgenic calluses. The phosphinothricin acetyltransferase (PAT) strip test was used to examine whether these plants were transgenic (Sun *et al.* 2010). Subsequently, the plants were grown to maturity in half-strength Murashige & Skoog (MS) agar containing 1 mg/L of phosphinothricin and 250 mg/L of cefotaxime.

2.8. PCR and Southern blot analysis of the transgenic zoysiagrass

To verify the T-DNA insertion in the zoysiagrass genome, the *bar* and *Zjchi2* genes were amplified from genomic DNA extracted from the leaves of transgenic lines and wild-type plants using PCR performed with a 20-μL reaction mixture, including 1 μL of genomic DNA, 0.5 μL (10 pmol) of primers for *bar* (forward, 5′-GTCTGCACCATCGTCAACCACTA-3′; reverse, 5′-AAGTC CAGCTGCCAGAAACCCAC-3′) or Ubi-*Zjchi2* (forward, 5′-CGGGTCATCTTTT CATGCTT-3′; reverse, 5′-GAACTTCCTCTGGCT GTAGCAGT-3′) and 10 μL of EmeraldAmp® PCR Master Mix (TaKaRa). PCR was performed in a Thermal Cycler Dice (TaKaRa) under the following conditions: an initial denaturation at 94 ℃ for 2 min, followed by 30 cycles of 94 ℃ for 30 s, 58 ℃ for 30 s, and 72 ℃ for 30 s. The PCR products were verified by electrophoresis on 1% agarose gels.

For Southern blot analysis, 10 μg of genomic DNA was isolated from young leaves of transgenic and wild-type zoysiagrass using a G-spin™ IIp Genomic DNA Extraction Kit for plants (iNtRON) and were digested with *Bam*HI at 37 ℃ for 12 h. Post experiments were carried out according to Sun *et al.* (2010). The digested DNA was separated on 1% agarose gels and then transferred to a Hybond-N^+ nylon membrane (GE Healthcare). Digoxigenin-labeled specific probes were obtained by PCR using specific primers for the *Zjchi2* gene (forward, 5′-CATACGACGCTTTCATCACC-3′; reverse, 5′-GAACTTCCTCTGGC TGTAGCAGT-3′) and *bar* gene (forward, 5′-GTCTGCACCATCGTCAACCACT A-3′; reverse, 5′-AAGTCCAGCTGCCAGAAACCCAC-3′), according to the manufacturer's method. The probes were then hybridized to nylon membranes in high-sodium dodecyl sulfate (SDS) hybridization buffer (5× saline-sodium citrate, 50% formamide, 50 mM sodium phosphate, 2% blocking solution, 0.1% N-lauroylsarcosine, and 7% SDS) at 60 ℃ for 20 h; then the membranes were washed with washing buffer (0.2× saline sodium citrate and 0.1% SDS) at 60 ℃

for 15 min, twice. The signals were detected by chemiluminescence using CDP-Star® (Roche) and visually verified by LAS-4000 (FUJIFILM).

2.9. Analysis of transgenic zoysiagrass gene expression

Total RNA was extracted from young leaves of transgenic and wild-type zoysiagrass using TRIzol™ reagent (MRC), according to the manufacturer's instructions. The total RNA was treated with DNase I (Ambion) to remove contaminating DNA, and then 1 μg of total RNA was synthesized using the Access RT-PCR System (Promega). To verify the level of *Zjchi2* mRNA expression in transgenic and wild-type zoysiagrass, quantitative real-time PCR was carried out with a 20-μL reaction mixture containing 1 μL of cDNA, 0.5 μL (10 pmol) of *Zjchi2*-specific primers (forward, 5′-CGTCATCACAAACATCATC AAC-3′; reverse, 5′-GAACTTCCTCTGGCTGTAGCAGT-3′), and 10 μL of iQ™ SYBR® Green SuperMix (Bio-Rad) using Thermal Cycler Dice, Real Time System Lite (TaKaRa). The PCR amplification comprised an initial denaturation at 95 ℃ for 30 s, followed by 40 cycles at 95 ℃ for 5 s and 60 ℃ for 30 s. The zoysiagrass *β-actin* gene was amplified to normalize the *Zjchi2* mRNA expression in transgenic and wild-type zoysiagrass to that of *β-actin*.

2.10. Phenotype analysis of transgenic zoysiagrass

The growth and morphology of wild-type and transgenic zoysiagrass cultivated in greenhouse conditions were compared, according to a previous method (Bae *et al.* 2008). The plant height, length of the blade, width, and angle of the plant leaf were determined using the third-youngest leaf from the stolon of each plant to measure the phenotype variation of the wild-type and transgenic zoysiagrass.

2.11. *In vitro*, *R. solani* inoculation and detached leaf analysis

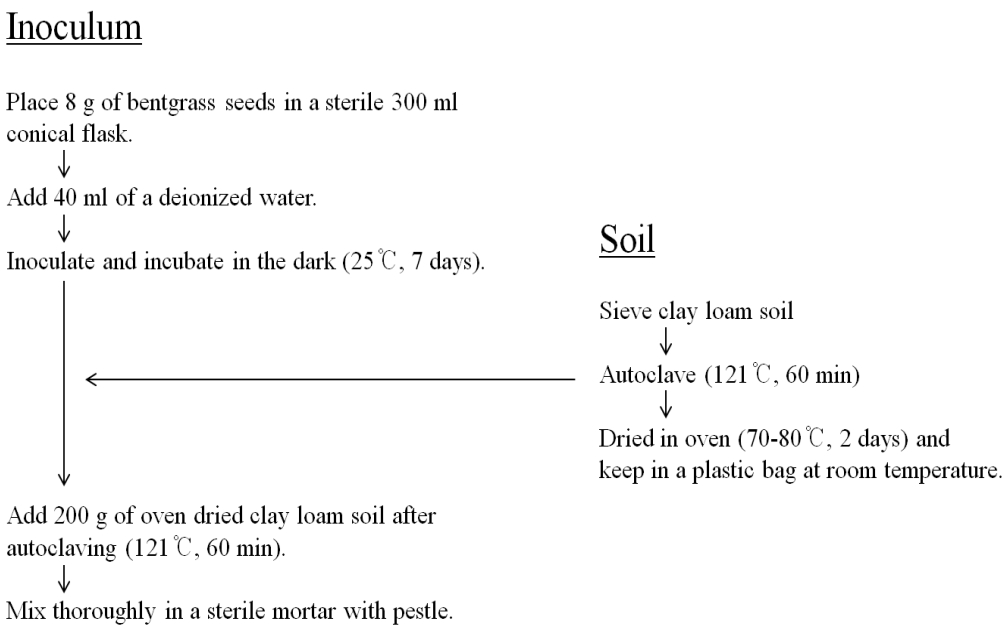
Transgenic and wild-type zoysiagrass was challenged with *R. solani* AG2-2 (IV), which caused a large patch. Detached leaves were analyzed according to the method of Zhou and Hu (2011). The *R. solani* was cultured on potato dextrose agar (PDA) at 25 ℃ for 7 d in the dark. Then, an 8-mm-diameter agar plug infested with mycelia of *R. solani* was placed in the center of 1.5% agar in a Petri dish (120 × 20 mm). Six young leaves from the stolons of transgenic and wild-type lines were cut and washed with 70% ethanol and sterile water. Then, the leaves were placed on the Petri dishes in contact with the agar plug infested with *R. solani*. The Petri dishes were placed in a growth chamber under a 16/8 h day/night cycle. After 11 d, the symptoms of large patch disease were quantified by calculating [(infected leaf area)/(total leaf area) × 100] using ImageJ (https://imageJ.nih.gov) and rated from 1 to 6 using a disease index representing 1 - 15, 16 - 30, 31 - 45, 46 - 60, 61 - 75, and 76 - 100% symptoms, respectively. The disease severity was estimated as [(A × 1) + (B × 2) + (C × 3) + (D × 4) + (E × 5) + (F × 6) × 100]/(total number of leaves used × 6) (Aoyagi *et al*. 1998; Kumar *et al*. 2009), where A to F were the numbers of leaves with a disease index of 1 to 6, respectively.

2.12. Soil-based infection analysis

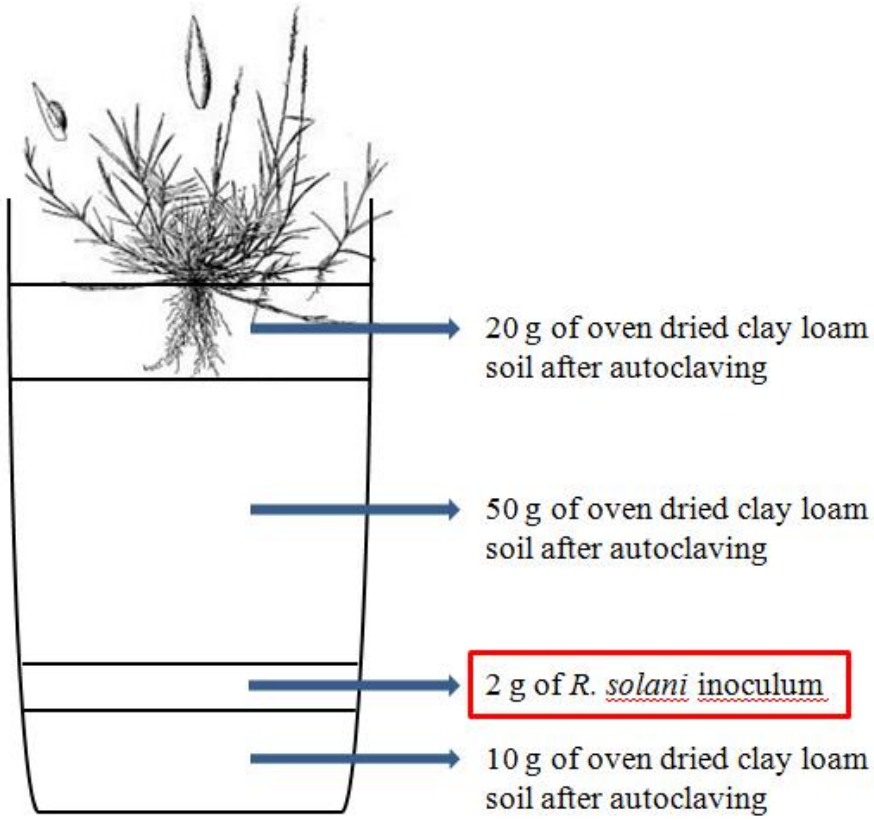
An inoculum of soil infected with *R. solani* was prepared with modification of a previous method (Tojo *et al*. 1993; Aoyagi *et al*. 1998). A PDA plug infested with *R. solani* mycelia was inoculated in a 300-mL flask containing 8 g of autoclaved bentgrass seeds and 40 mL distilled water. After 7 days at 25 ℃, the seeds were added to 200 g of autoclaved oven-dried clay loam soil and were then mixed using a mortar and pestle. Then, 2 g of the soil-based inoculum was added to a plant box (diameter, 65 mm; depth, 98 mm; SPL) containing

autoclaved oven-dried clay loam soil. Young plants from the stolons of wild-type and transgenic zoysiagrass were transferred in plastic pots and grown in a greenhouse for 1 month. The mature plants were transferred to the abovementioned plant box containing the *R. solani* inoculum after gently removing the soil from the roots, and were then cultivated in a growth chamber at 25 ℃, 65% relative humidity. Disease development was quantified 1 month after infection as the percentage of symptomatic leaves out of the total number of leaves on each plant (Dong *et al*. 2007).

(A)



(B)



(C)

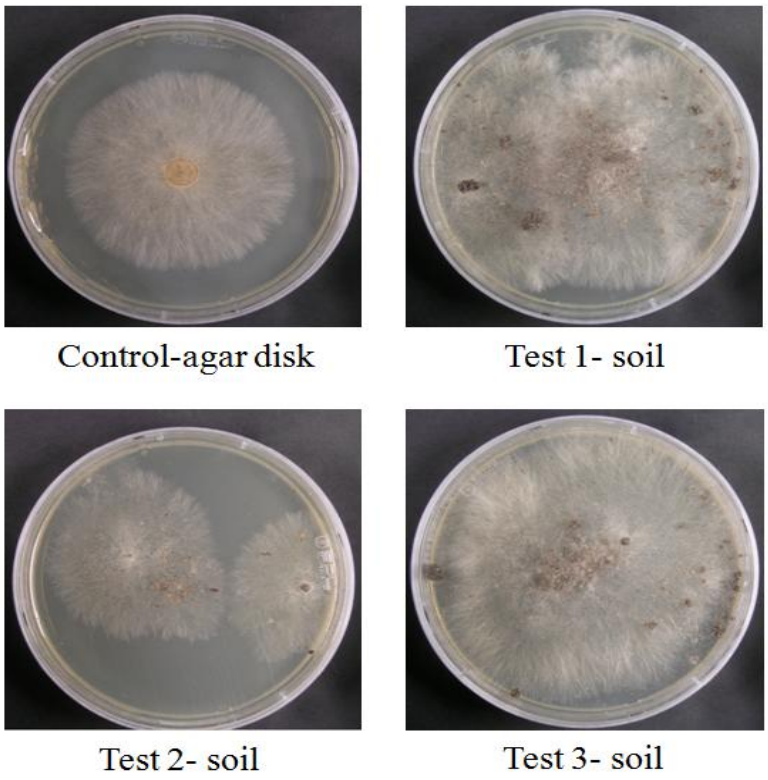


Figure 1. Procedure of soil-based infection analysis using bentgrass seeds. Preparation of soil-based *R. solani* inoculum (A) and pots (B) was carried out in clean bench to minimize contamination. (C) Mycelial growth test of soil-based *R. solani* inoculum on PDA.

3. Results

3.1. Sequence and structure of zoysiagrass chitinase genes

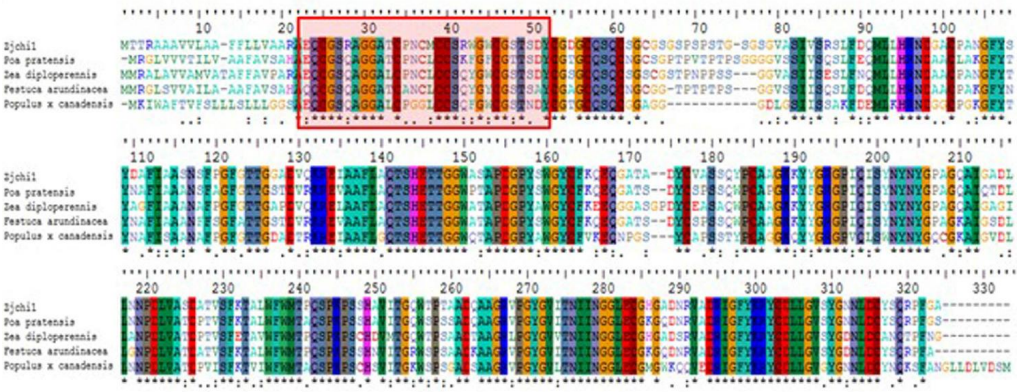
Full-length sequences of two zoysiagrass chitinase genes (*Zjchi1* and *Zjchi2*) were obtained from cDNA of zoysiagrass. The open reading frame (ORF) sequence of *Zjchi1* gene encoded 320 amino acids. SignalP and Protparam analysis using ExPASy indicated that the signal sequences comprised 21 amino acids and the predicted mature protein had a molecular mass of 31.3 kDa and a p*I* of 5.77. The native molecular mass and p*I* including the N-terminal signal sequence were 33.4 kDa and 6.39, respectively. Multiple alignment analysis of Zjchi1 using NCBI BLAST showed 80% similarity with the chitinases from *Poa pratensis* (AAF04454) and *Festuca arundinacea* (ACJ23248). In contrast, chitinases from *Zea diploperennis* (AAT40029) and *Populus x canadenis* (AEZ67300) showed 78 and 66% homology, respectively, with Zjchi1. Also, an alignment analysis verified the presence of a CBD in the N-terminal region of these two proteins (Fig. 2A).

The 258 amino acids encoded by the 774 bp ORF indicated a predicted molecular mass of 27.6 kDa and a p*I* of 9.33. A signal peptide of 27 amino acids was present among the 258 amino acids encoded by the ORF, and the predicted mature protein had a molecular mass of 25 kDa and a p*I* of 9.30. A leucine/valine-rich signal peptide and conserved catalytic domain including a putative sugar-binding site were present, but no CBD was found in the N-terminal region. Homology analysis using multiple alignments showed 73, 69, 68, and 65% similarity with the chitinases of *Oryza sativa* (BAA31997), *Hordeum vulgare* (ACJ68105), *Secale cereal* (AAG53610), and *Populus x canadensis* (AEZ67300), respectively. However, a CBD was present only in *Polulus x canadensis* class I chitinase (Fig. 2B). Comparison of the amino acid

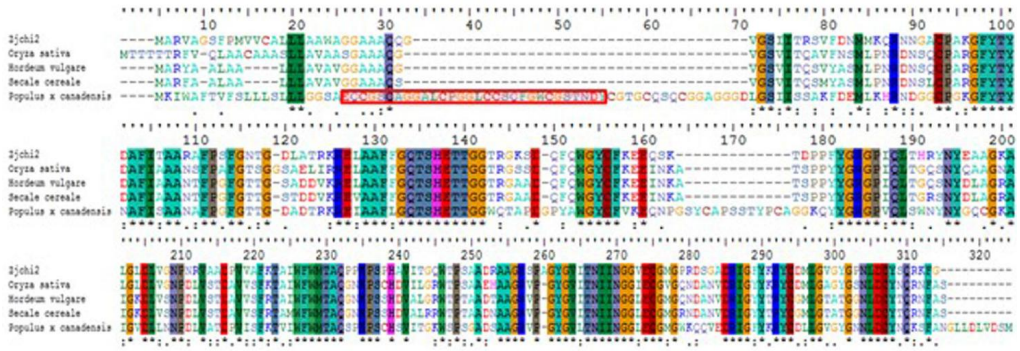
sequences of Zjchi1 and Zjchi2 suggested 63% homology. Also, the N-terminal region of Zjchi2 lacked a CBD (Fig. 2C).

To determine the phylogenetic relationship of Zjchi1 and Zjchi2, consensus sequences of other plant chitinases were obtained from Levorson and Chlan (1997). Phylogenetic analysis of chitinases of the PR-3 group was carried out using the MEGA 7 software and the tree was constructed using the neighbor-joining method. As shown in Fig. 2D, chitinases from *Nicotiana tabacum* (CAA01530), *Solanum tuberosum* (CAA33517), *Solanum lycopersicum* (CAA78845), *Vitis vinifera* (CAA90970), *Gossypium hirsutum* (CAA92277), *Phaseolus vulgaris* (AAA33756), *Zea mays* (AAA62421), *Hordeum vulgare* (AAA56787), *Oryza sativa* (BAA03750) and *Arabidopsis thaliana* (AAA32769) belong to the class I chitinase family, as did Zjchi1. Zjchi2 was categorized as a class II chitinase, together with those of *Nicotiana tabacum* (AAA34106), *Solanum lycopersicum* (CAA78844), *Petunia x hybrid* (CAA35791), *Citrus sinensis* (CAA93847) and *Arachis hypogaea* (CAA57773).

(A)



(B)



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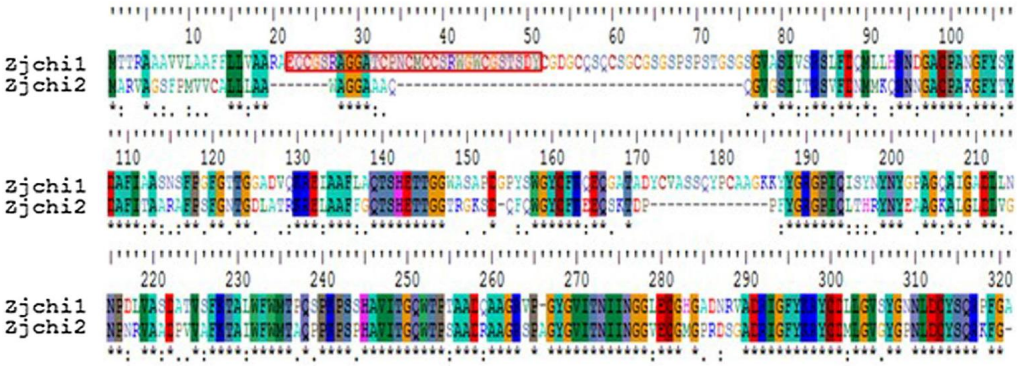


(D)

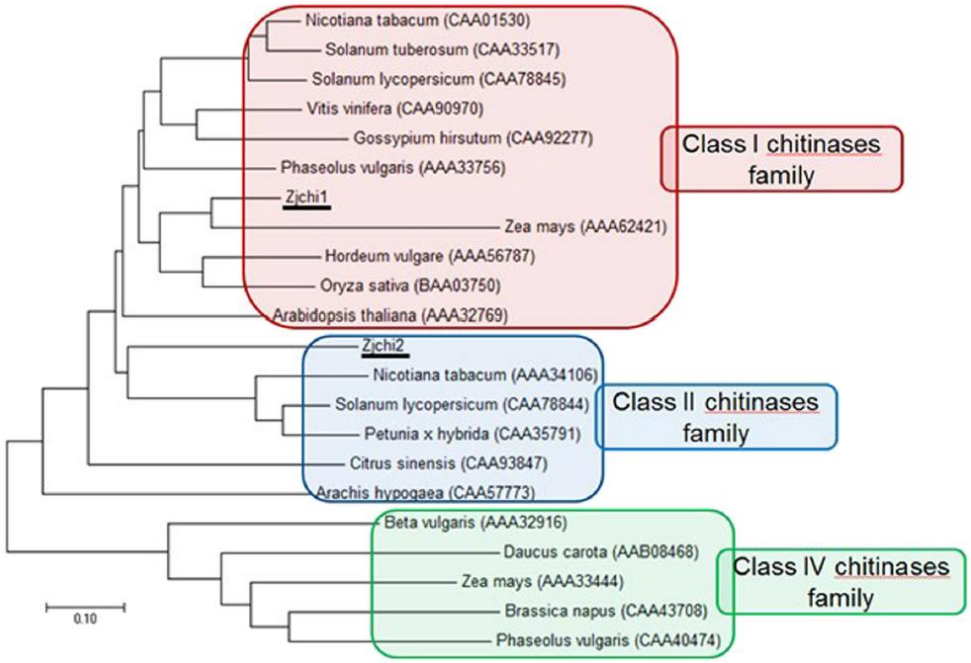


Figure 2. Comparison analysis of amino acid sequence of zoysiagrass chitinases. (A) Zjchi1 was compared to chitinases from *Poa pratensis*, *Zea diploperennis*, *Festuca arundinacea* and *Populus x canadensis*. (B) Zjchi2 was compared to chitinases from *Oryza sativa*, *Hordeum vulgare*, *Secale cereal* and *Populus canadensis*. (C) Amino acid sequence of Zjchi1 and Zjchi2 from zoysiagrass. The CBD is outlined in a red box. (D) Phylogenetic analysis of Zjchi1 and Zjchi2. Chitinases of the PR-3 group were used in the sequence alignment. The MEGA 7 software was used to construct the phylogenetic tree based on the neighbor-joining method.

3.2. Expression analysis and antifungal activity of chitinases

The *Zjchi1* and *Zjchi2* transcript abundance upon infection by *R. solani* AG2-2 (IV) *in vitro* over time was examined using quantitative real-time PCR. The results showed that *Zjchi1* and *Zjchi2* were induced by *R. solani* AG2-2 (IV) infection (Fig. 3). The transcripts of both zoysiagrass chitinase genes began to accumulate at 2 days and their transcript levels peaked at 3 days. *R. solani* AG2-2 (IV) infection increased *Zjchi1* and *Zjchi2* expression at 3 days by 18.86- and 96.66-fold compared with that in control plants.

To determine *in vitro* antifungal activity, *Zjchi1* and *Zjchi2* were sub-cloned into pET 28a+ and pGEX 4T-1 for overexpression in *E. coli* BL21 (DE3). Weak induction of r-Zjchi1 (pET-Zjchi1, 32 kDa) was achieved by addition of 0.5 mM IPTG at 18 ℃ for 4 h in LB medium containing 50 μg/mL kanamycin (Fig. 4A, lanes 1 - 2). Soluble protein was detected by SDS-PAGE (Fig. 4A, lane 3). In contrast, strong expression of r-Zjchi2 (pGEX-Zjchi2, 51 kDa) and a large quantity of soluble protein was detected following induction by 0.5 mM IPTG at 18 ℃ for 4 h in LB medium containing 100 μg/mL ampicillin (Fig. 4B, lanes 1 - 3). The r-chitinases were purified using Ni^{2+} and glutathione affinity column chromatography. Single bands corresponding to histidine-tagged Zjchi1 and GST-Zjchi2 fusion proteins were confirmed by SDS-PAGE (Fig. 4A and B, lane 5). Furthermore, GST was removed from the GST-Zjchi2 fusion protein (data not shown).

To investigate the antifungal activity of the purified r-chitinases, disk diffusion susceptibility tests were carried out. The purified r-chitinases showed antifungal activity against *R. solani* AG2-2 (IV), *R. solani* AG1 (IA), *R. cerealis*, *B. cinerea*, *F. culmorum*, *F. graminearum* and *T. reesei* (Fig. 5a - c, e - g, j), but not *S. sclerotiorum*, *Pythium* sp. and *S. minor* (Fig. 5d, h, i). Purified r-Zjchi2 exhibited marked and rapid antifungal activity against *T. reesei*. Therefore, a mycelium-extension inhibition assay in half-length PDB with *T.*

reesei was performed using r-Zjchi2. As shown in Fig. 6a, *T. reesei* mycelia grew in PDB containing PBS (control) but not in PDB containing 125, 250, 500 and 1000 μg/mL of r-Zjchi2. Light microscopic observation supported this result. Light micrographs showed well-developed branches and mycelium in PDB containing PBS buffer (control); in contrast, mycelial branching and extension were inhibited in PDB containing 250 and 1000 μg/mL r-Zjchi2 (Fig. 6b).

(A)

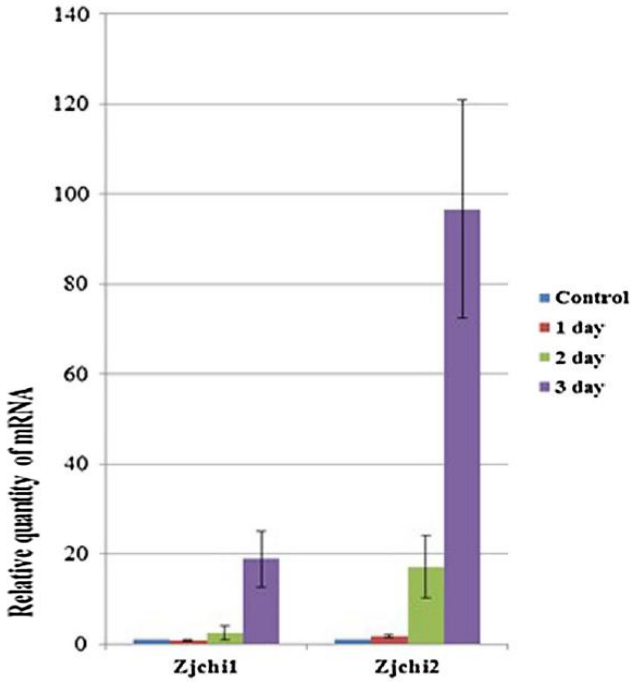


Figure 3. Expression of zoysiagrass chitinase genes in response to *R. solani* infection using real-time PCR. Transcript levels were measured using the delta-delta CT method. Error bars represent standard deviation (SD) from three replicates.

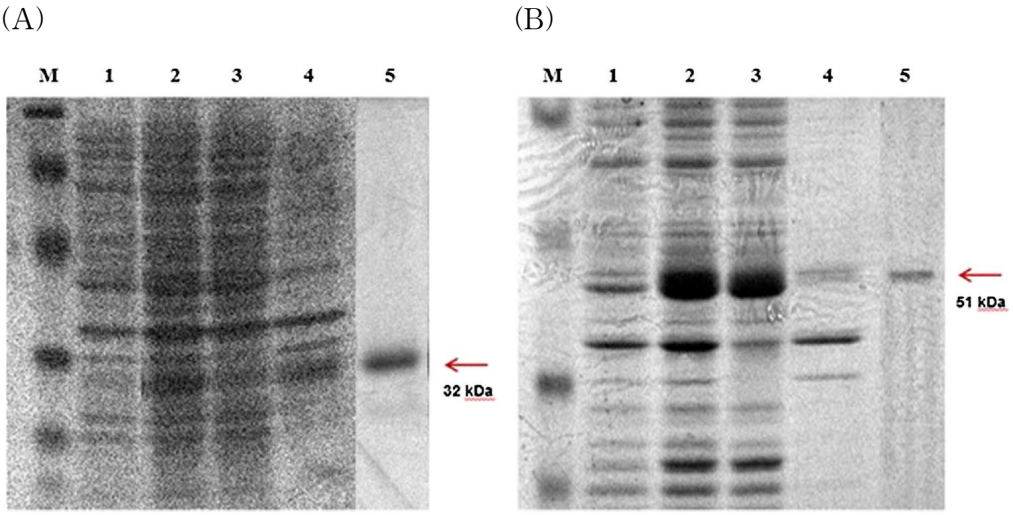


Figure 4. Overexpression in *E. coli* and purification of r-chitinases. (A) Histidine-tagged r-Zjchi1 and (B) GST-Zjchi2 fusion proteins were overexpressed in *E. coli* and confirmed by SDS-PAGE. The proteins were purified by affinity column chromatography. Lane M, protein molecular weight marker (Bio-Red); lane 1, total protein (-IPTG); lane 2, total protein (+IPTG); lane 3, supernatant (+IPTG); lane 4, pellet (+IPTG); and lane 5, purified r-chitinase.

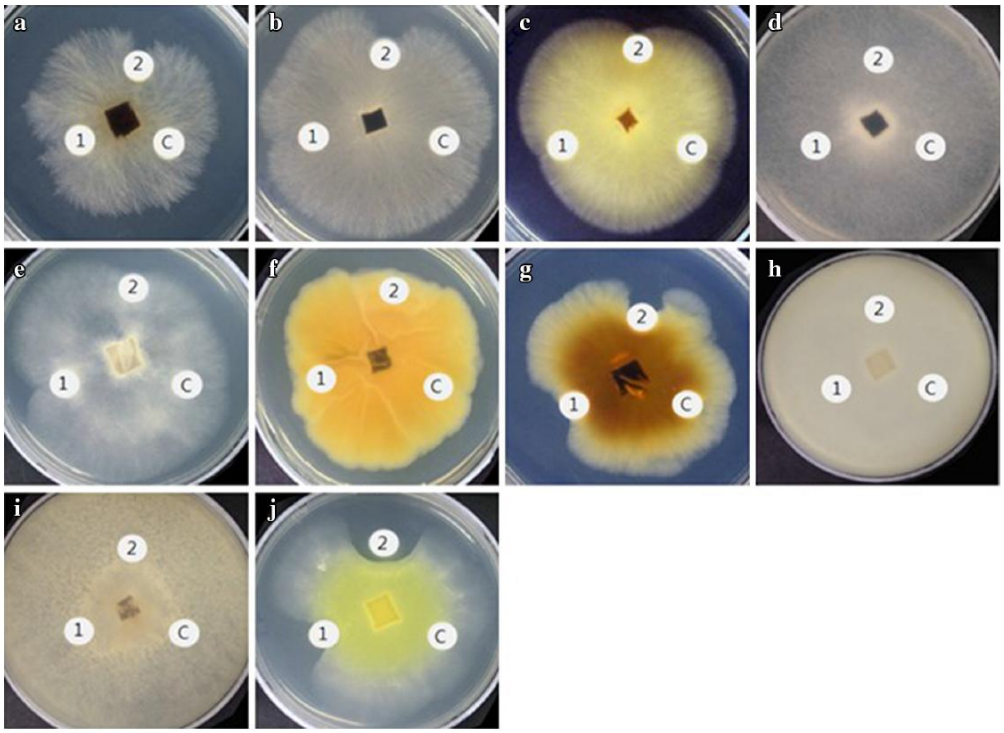


Figure 5. Disk diffusion susceptibility tests of purified r-chitinases on PDA. a *R. solani* AG2-2 (IV), b *R. solani* AG1 (IA), c *R. cerealis*, d *S. sclerotiorum*, e *B. cinerea*, f *F. culmorum*, g *F. graminearum*, h *Pythium* sp., i *S. minor* and j *T. reesei* were subjected to mycelium-extension inhibition analysis. The paper disks contained 40 μL of the following solutions: 1, 300 μg r-Zjchi1; 2, 300 μg r-Zjchi2; and 3, PBS containing thrombin.

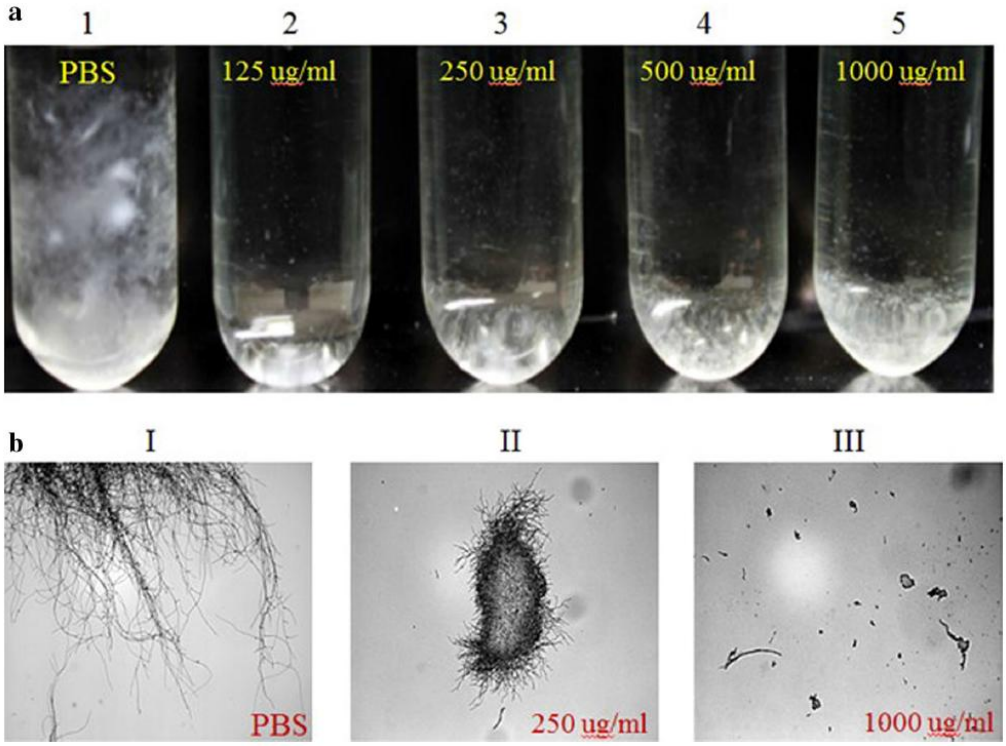


Figure 6. Inhibition of *T. reesei* mycelium growth by r-Zjchi2. a *T. reesei* was cultured on half-length PDB containing PBS buffer, or 125, 250, 500 or 1000 μg/mL r-Zjchi2 at 25 °C for 3 days in the dark with gentle shaking. b Light micrograph showing inhibition of *T. reesei* mycelial growth by PBS, and 250 and 1000 μg/mL r-Zjchi2.

3.3. Production and verification of transgenic zoysiagrass

In total, 14 putative T_0 transgenic zoysiagrass lines, including a vector control line (IG2), were generated by *Agrobacterium*-mediated transformation from calluses of zoysiagrass seeds. The putative transgenic and wild-type zoysiagrass lines were grown in half-strength MS agar containing 1 mg/L phosphinothricin and 250 mg/L cefotaxime. After 2 weeks, leaf tissue from these plants was harvested and crushed using a plastic pestle for the PAT protein strip test. All of the putative transgenic lines and line IG2 showed two violet bands within 1 min, whereas the wild-type control line showed only a single band (Fig. 7). This indicated that the PAT protein was expressed normally in the putative transgenic lines. Subsequently, all of the transgenic lines were transferred to plastic pots containing soil and grown to maturity in a greenhouse for further experiments.

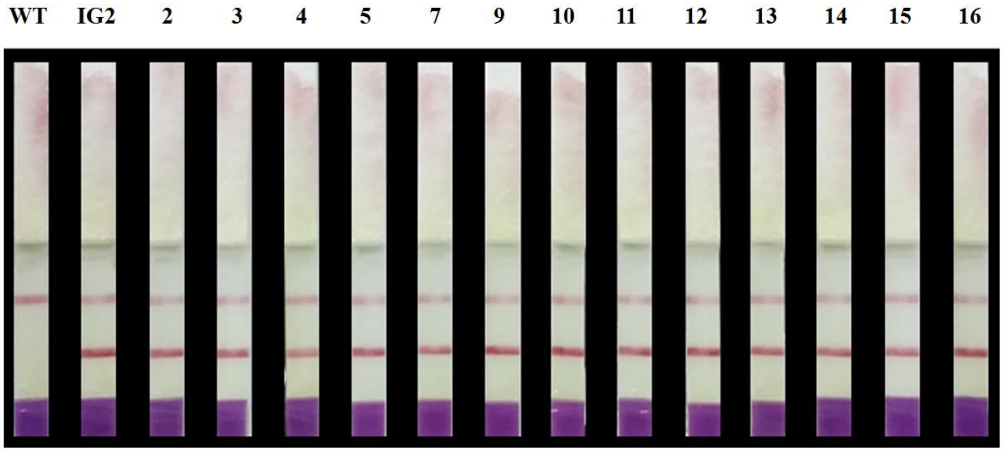


Figure 7. The phosphinothricin acetyltransferase (PAT) strip test in wild-type and putative transgenic lines. Leaf tissue powder from the plants was used in this study. The wild-type zoysiagrass showed only 1 band, whereas the transgenic lines (2 - 16), including the vector control line (IG2), showed 2 bands. The upper bands are the positive control and the bottom bands are the signal of specific antibodies coupled to a color reagent responsive to the PAT protein.

3.4. Molecular analysis of the transgenic zoysiagrass

To determine the T-DNA insertion, including the *bar* and *Zjchi2* genes, genomic DNA was extracted from the leaves of transgenic lines and wild-type zoysiagrass. Then, PCR and Southern blot analyses were performed using specifically designed probes (Fig. 8A). The PCR amplification of *Zjchi2* included the *ubiquitin* promoter region, which excluded the amplification of endogenous *Zjchi2* genes. As shown in Fig. 8B, *Zjchi2* was amplified in all transgenic lines, but not in the control lines (wild-type and IG2 vector control line). The *bar* gene was detected in all transgenic lines, including the IG2 vector control line, but not in the wild-type control line. This indicates that the T-DNA region was successfully integrated into the zoysiagrass genome.

Next, Southern blot analysis was performed using *bar*- and *Zjchi2*-specific probes (Fig. 8A). As shown in Fig. 8C, Southern hybridization using the *Zjchi2*-specific probe revealed two equal-size bands (about 9 and 5 kb) in all lines. These bands indicated that there were two copies of the endogenous *Zjchi2* gene in the zoysiagrass genome (Fig. 8C, left). In addition, single or multiple bands were observed on Southern hybridization with the *bar*-specific probe at different positions from the wild-type control line (Fig. 8C, right). The various hybridization patterns with the *bar*-specific probe confirmed that the transgenic zoysiagrass lines were from independent transformation events.

The expression of *Zjchi2* mRNA in the transgenic and control lines was analyzed by quantitative real-time PCR using *Zjchi2*-specific primers. This verified constitutive expression of the *Zjchi2* gene in transgenic zoysiagrass versus very weak expression of the *Zjchi2* gene detected in the control lines. The transgenic lines showed an increased mRNA level compared with the control lines, although the level of mRNA expression differed among the lines. There were more *Zjchi2* transcripts expressed in the single-copy lines (lines 2, 3, 9, 10, 11, 12, 14, and 16) than in the multi-copy lines (Fig. 9). In this study,

transgenic lines 5 and 7 were excluded because these lines grew very slowly compared with the other lines (data not shown).

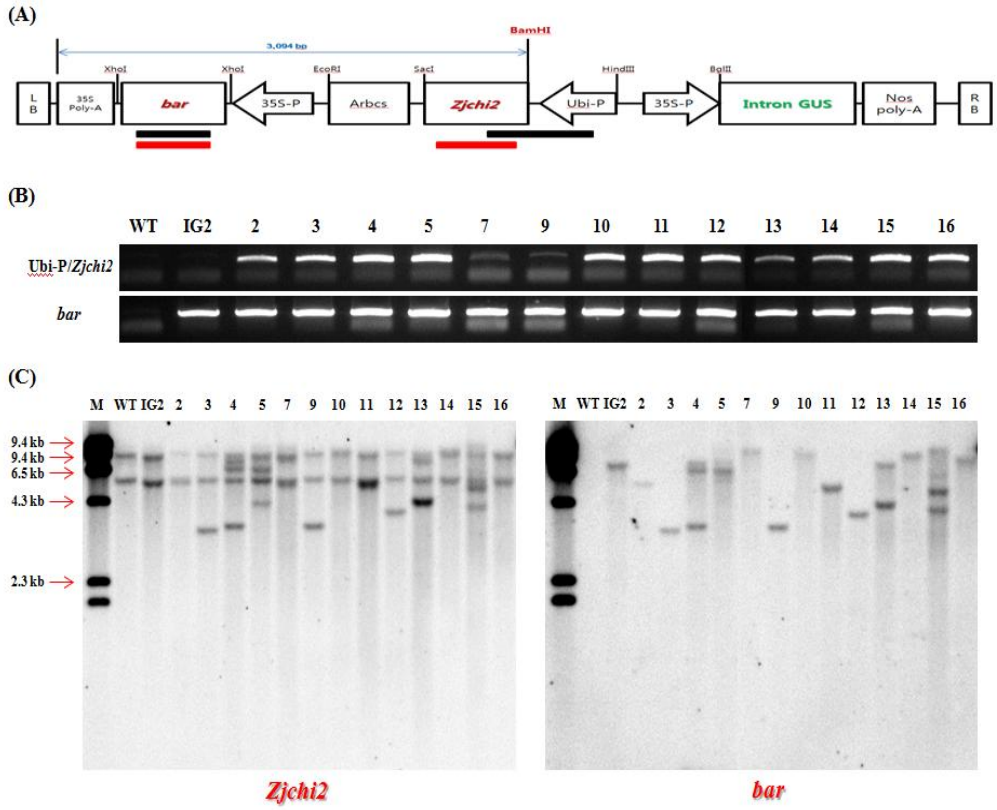


Figure 8. Molecular verification of the transgenes in transgenic zoysiagrass. (A) Diagram of the T-DNA region. The *Zjchi2* and *bar* genes were expressed constitutively by the maize *ubiquitin* promoter and *Cauliflower* mosaic virus (*CaMV*) 35S promoter, respectively. The black bars indicate the amplified region of *bar* and *Zjchi2* by polymerase chain reaction (PCR) and the red bars show the position of specific probes for Southern blot hybridization. (B) PCR analysis. Genomic DNA was extracted from young leaves of wild-type and transgenic lines. PCR was performed using specific primer sets (black bar in A) and then the PCR products were confirmed by electrophoresis on 1% agarose gels. (C) Southern blot hybridization. A total of 10 μg of genomic DNA was used for Southern blot analysis. Genomic DNA from transgenic plants was digested with *Bam*HI and then hybridized with the probes for the *bar* and *Zjchi2* genes (red bar in A). M, lambda *Hin*dIII molecular marker; WT, wild-type zoysiagrass; IG2, vector control; 2 - 16, transgenic zoysiagrass overexpressing the *Zjchi2* gene.

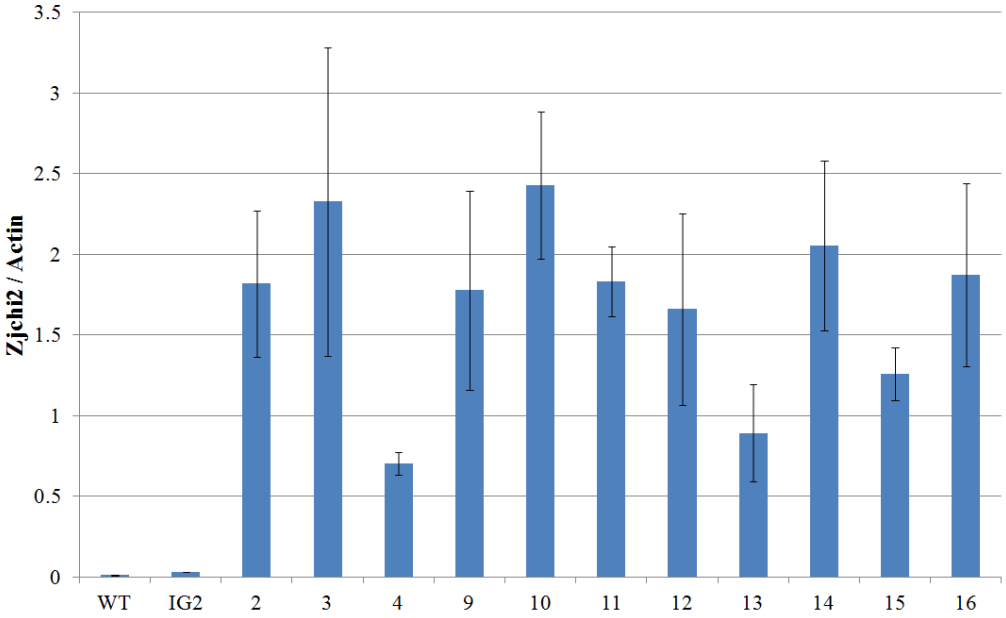


Figure 9. Expression analysis of transgenic zoysiagrass. Quantitative real-time PCR was used to analyze *Zjchi2* gene expression. A total of 1 μg of RNA was extracted from young leaves of all lines and synthesized for PCR. The accumulation of *Zjchi2* transcripts was higher in the transgenic zoysiagrass lines than in control lines (WT and IG2). Error bars represent the standard deviation (SD) of 3 replicates.

3.5. Morphology analysis of transgenic zoysiagrass

The morphology of zoysiagrass can be classified using Kitamura's horticultural classification method based on leaf width and length. We investigated the phenotypic variation among the wild-type and transgenic zoysiagrass lines (Table 1). There were no differences in plant height, length of the blade, width, or angle of the plant leaf among the wild-type and transgenic lines. The vector control line (IG2) and transgenic lines were essentially indistinguishable from wild-type plants (Fig. 10). Therefore, the vector control line (IG2) line and multi-copy lines (lines 4, 13, and 15) were excluded from further study.

Table 1. Morphological characterization of wild-type and transgenic zoysiagrass grown under greenhouse conditions.

		WT	IG2	T2	T3	T4	T9	T10	T11	T12	T13	T14	T15	T16
Plant height	Average	74.5	71.5	70.9	73.4	78.7	75.6	78.0	72.0	76.1	74.6	79.2	74.8	80.0
	SD[a]	14.6	13.0	17.7	10.9	13.7	14.8	13.0	12.5	17.1	17.1	15.1	16.1	17.1
	t-test	NS[b]	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3rd leaf length	Average	30.3	34.5	31.6	28.0	27.9	24.1	30.6	36.3	31.6	24.4	31.0	23.8	23.5
	SD	18.3	11.7	10.4	5.1	8.8	6.9	9.6	13.4	18.9	8.7	8.1	15.6	6.1
	t-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
3rd leaf width	Average	3.1	3.1	3.0	3.1	3.0	2.8	2.9	2.8	2.9	2.7	3.1	2.8	2.7
	SD	0.2	0.3	0.4	0.3	0.4	0.3	0.2	0.5	0.3	0.4	0.1	0.4	0.3
	t-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
Leaf angle	Average	65.0	64.6	64.9	63.6	67.7	64.4	58.0	59.4	61.7	66.9	58.4	62.0	64.3
	SD	6.9	6.2	8.8	7.8	12.2	6.9	6.8	4.2	6.9	6.7	10.1	14.3	12.0
	t-test	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a] Standard deviation

[b] NS:Not significant, considered statistically insignificant at 0.05 level at t-test between wild-type and transgenic lines

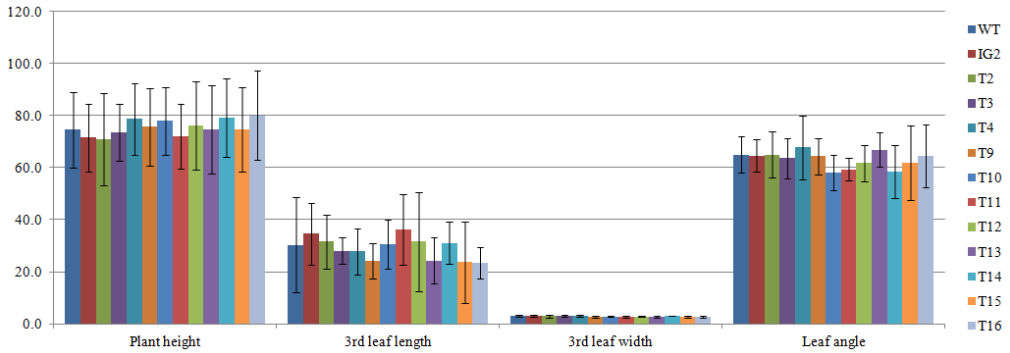


Figure 10. Analysis of phenotype variation between wild-type and transgenic zoysiagrass lines. Any differences among plant height, 3rd leaf length, 3rd leaf width, and angle of a plant leaf not indicated between wild-type and transgenic lines as $P > 0.05$ (Student's t-test). Error bars represent standard deviation (SD) from ten leaves of each plants.

3.6. Antifungal activity analysis of transgenic zoysiagrass

The wild-type and single-copy lines (lines 2, 3, 9, 10, 11, 12, 14, and 16) were grown to maturity in a greenhouse for detached leaf assays. Agar plugs infested with *R. solani* mycelia were put on 1.5% agar in Petri dishes, and then six young, detached leaves of transgenic and wild-type plants were placed on the agar. In the wild-type, approximately 45% of the leaf area was infected, whereas transgenic lines 2, 3, 10, 11, and 14 showed antifungal activity. Of note, transgenic lines 2, 3, and 10 showed significant resistance to *R. solani* infection, with approximately 18, 24, and 19% of the leaf area infected, respectively (Table 2; Fig. 11A). In comparison, transgenic lines 9, 12, and 16 showed no antifungal activity. A reddish-brown color on the leaves of zoysiagrass is the typical phenotype of large-patch disease (Toda *et al.* 2004). The disease symptoms were observed visually in wild-type and transgenic lines (Fig. 11B). Seven days after inoculation, the transgenic lines showed disease symptoms similar to the wild-type; however, the transgenic lines maintained antifungal activity for up to 11 d after inoculation compared with the wild-type. This indicates that the transgenic zoysiagrass lines 2, 3, and 10 overexpressed Zjchi2 and possessed significant antifungal activity against *R. solani* infection.

To determine the disease resistance of transgenic zoysiagrass against *R. solani* infection, young plants from the stolons of transgenic lines 2, 3, and 10 were transferred into plastic pots containing soil and then grown to maturity in a greenhouse. After 1 month, the mature plants were transferred to a plant box containing autoclaved, oven-dried clay loam soil containing *R. solani* inoculum. One month after infection, symptoms of large-patch disease were observed in the wild-type and transgenic lines. The disease severity was calculated as the percentage of symptomatic leaves out of the total number of leaves for each plant. As shown in Fig. 12, the wild-type plant had about 50% infected leaves, whereas transgenic lines 2, 3, and 10 showed a significant reduction in the

disease index, with about 10, 35, and 20% infected leaves, respectively (Table 3). These data are similar to the results of the detached leaf assay, which suggests that transgenic lines 2, 3, and 10 possess disease resistance against *R. solani* infection.

Table 2. Disease indices for wild-type and transgenic zoysiagrass used detached leaf assay.

DI*	%
1	0-15
2	16-30
3	31-45
4	46-60
5	61-75
6	76-100

	DS[a]	SD[b]	*t*-test
WT	46.2963	14.25445	-
T2	**18.51852**	**1.603751**	**0.002** **
T3	**24.53704**	**4.877617**	**0.041** *
T9	50.92593	14.25445	0.796
T10	**19.44444**	**4.811252**	**0.003** **
T11	31.94444	5.00771	0.179
T12	47.22222	2.77778	0.941
T14	26.38889	2.405626	0.061
T16	43.98148	12.13458	0.838

$$\text{DI}^{*}\text{: Disease index} = \frac{\text{Total leaf area}}{\text{Infected leaf area}} \text{X } 100$$

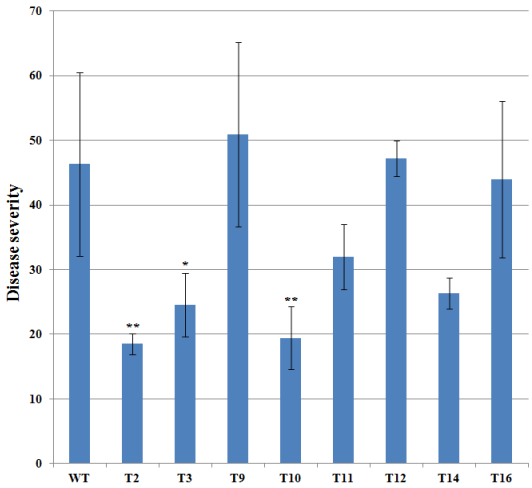
[a] $\text{Disease severity} = \dfrac{\textbf{Total number of leaf used x 6}}{(A\text{x}1)+(B\text{x}2)+(C\text{x}3)+(D\text{x}4)+(E\text{x}5)+(F\text{x}6)} \textbf{ x 100}$

[b] Standard deviation

* *P*-value < 0.05

** *P*-value < 0.005

(A)



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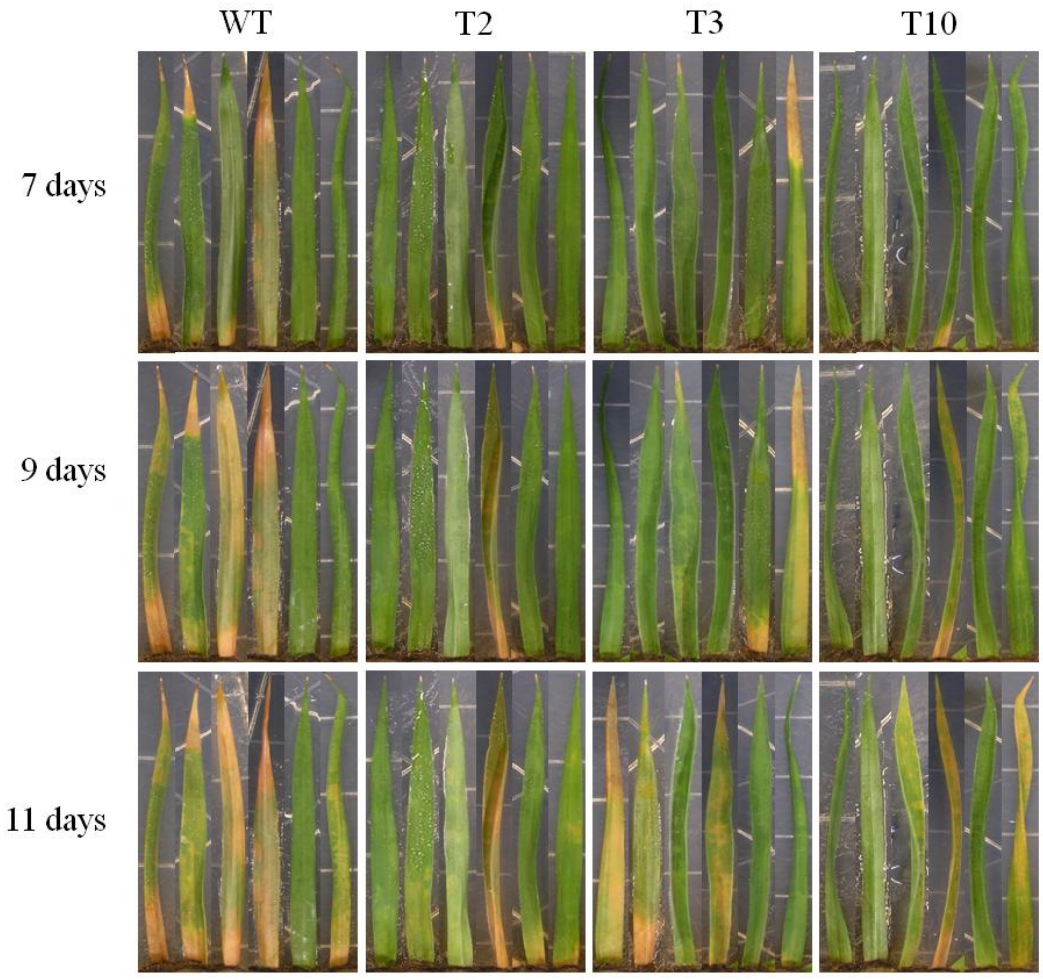

Figure 11. Antifungal activity of transgenic zoysiagrass against *R. solani* infection. (A) Disease severity in wild-type and transgenic zoysiagrass lines was calculated using a detached leaf assay 11 d after inoculation as [(A × 1) + (B × 2) +(C × 3) + (D × 4) + (E × 5) + (F × 6) × 100]/(total number of leaves used × 6)] based on the disease index. Error bars represent the SD of 3 independent experiments. The values for transgenic lines 2, 3, and 10 differed significantly compared with wild-type plants at $^{*}P < 0.05$ or $^{**}P < 0.005$ (Student's *t*-test). (B) Development of large-patch disease on detached leaves of transgenic zoysiagrass. Symptoms of *R. solani* infection on the detached leaves of transgenic zoysiagrass were observed 7, 9, and 11 d after inoculation. A reddish-brown color on the leaves is a typical symptom of large-patch disease.

Table 3. Disease indices for wild-type and transgenic zoysiagrass used soil-based infection assay.

	WT	T2	T3	T10
DS[a]	48.8	10.4	35.7	19.8
SD[b]	6.5	3.7	3.4	4.9
***t*-test**	-	**0.0009****	**0.0356***	**0.0034****

[a] Disease severity = $\frac{\text{Total number of leaves in plant}}{\text{Symptomatic leaves in plant}} \times 100$

[b] Standard deviation

* *P*-value < 0.05

** *P*-value < 0.005

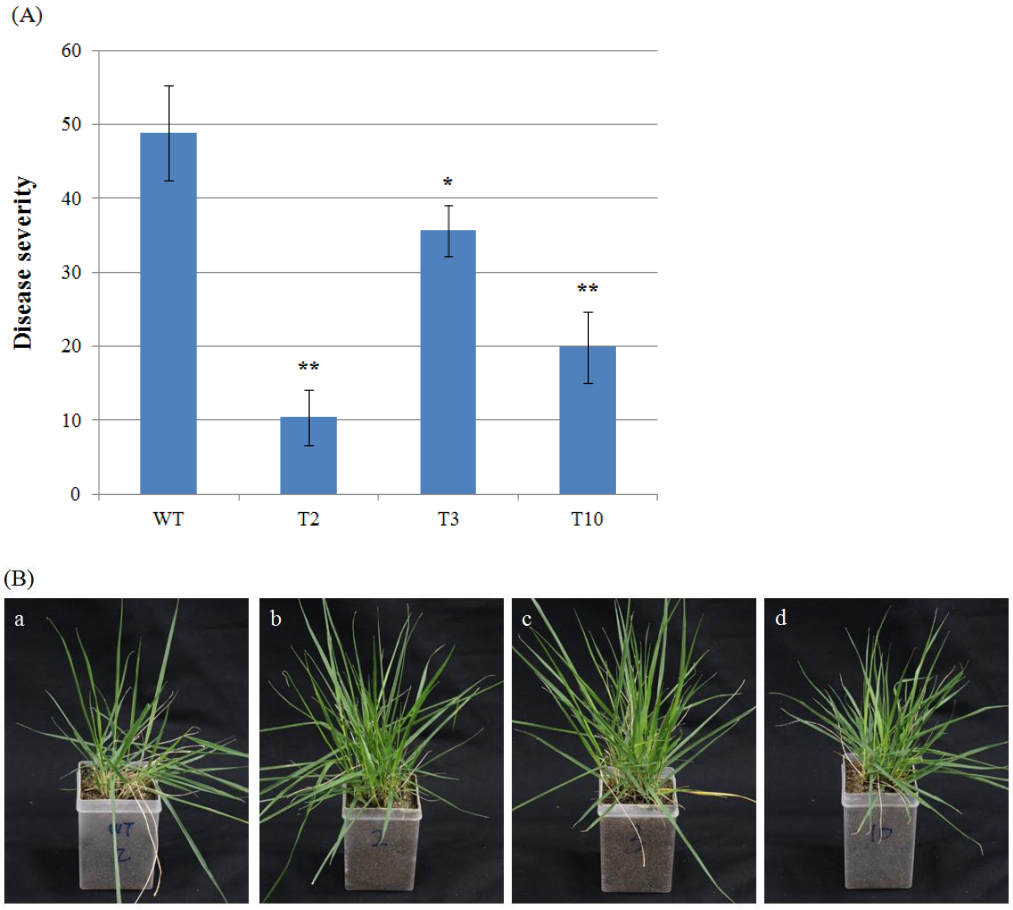

Figure 12. Whole plant assay based on soil containing *R. solani*. Disease severity in wild-type and transgenic zoysiagrass was measured as [(symptomatic leaves in plant)/(total number of leaves in plant) × 100]. The error bars represent the SD from 3 independent experiments. Significant differences in the transgenic lines compared with wild-type plants are indicated as $^{*}P < 0.05$ and $^{**}P < 0.005$ (Student's *t*-test).

4. Discussion

Zoysiagrass (*Zoysia japonica* Steud.) is an important turf grass used for golf courses, athletic fields and lawns in Far-East Asia, including Korea, Japan and China (Toyama *et al.* 2003). Zoysiagrass is damaged by large-patch disease caused by *Rhizoctonia solani* AG2-2 (IV). Large-patch disease commonly occurs in the spring and falls as small (10 cm) patches, which rapidly expand to 5 or 10 m and exhibit sheath blight and a reddish-brown color (Toda *et al.* 2004). Germicides are applied to prevent this disease during the zoysiagrass growth season, which has resulted in annually increasing economic and environmental losses (Jung *et al.* 2008). Therefore, development of disease-resistant zoysiagrass using genetic engineering would be beneficial.

In this study, we cloned and characterized two full-length chitinase genes (*Zjchi1* and *Zjchi2*) from zoysiagrass. Sequence analysis showed that *Zjchi1* was 1188 bp in length with 5′ and 3′ UTRs but did not contain an intron (data not shown). A homology search of Zjchi1 showed a structural similarity of 84% with FaChit1, which also lacks an intron, and 80% similarity with the amino acid sequence. Also, *FaChit1*, a class I chitinase gene of tall fescue turf grass, is induced by fungal elicitors, dehydration, ethylene and mechanical wounding (Wang *et al.* 2009). The presence of a CBD, HR and deficiency of C-terminal extension are characteristics of the class Ib chitinase family (Collingel *et al.* 1993). Therefore, these results suggest Zjchi1 to be a class Ib chitinase, which are activated by biotic and abiotic stresses. The full-length *Zjchi2* gene of 1318 bp contained two introns and three exons between the 5′ and 3′ UTRs. The location of introns in this gene is similar to that of other intron-containing plant chitinase genes. The two introns in *Zjchi2* were of 86 and 233 bp (data not shown), compared to 919 and 2436 bp for the peanut and sugar beet chitinase genes (Kellmann *et al.* 1996; Berglund *et al.* 1995). Short introns (125 - 69 bp)

were also detected in *FaChi2-1* and *FaChi2-2* from strawberry (Khan Anwar and Shih Ding 2004). Moreover, introns in *Mnchi1* from *Mikania micrantha* were 1171 and 621 bp in length (Li *et al.* 2010). These results indicated that the length of introns in plant chitinase genes varies considerably. Alignment of the amino acid sequence of Zjchi2 showed identities of 51, 59 and 69% with FaChi2-1 and FaChi2-2 from strawberry and the class II chitinase from winter rye (*Secale cereal*), respectively. *FaChi2-1* and *FaChi2-2* were induced by *Colletotrichum fragariae* and *Colletotrichum acutatum* (Khan Anwar and Shih Ding 2004) and class II chitinase from winter rye showed response to freezing stress (Yeh *et al.* 2000). Antifreeze proteins of 15 - 5 kDa were secreted into the apoplast of winter rye (Yeh *et al.* 2000), and have characteristics similar to those of PR proteins, including chitinases such as Zjchi2. The amino acid sequence of Zjchi2 indicated the absence of a CBD at the N-terminal, and the presence of an HR and C-terminal extension; moreover, the CD was similar to that of Zjchi1. These results suggested that Zjchi2 belongs to the class II chitinase family and is induced by fungal infection or freezing stress. Phylogenetic analysis supported these results. Thus, we concluded that Zjchi1 and Zjchi2 are class Ib and class II chitinases, respectively, and are induced by biotic and abiotic stresses.

Zjchi1 and *Zjchi2* expression in response to *R. solani* infection was examined *in vitro* by quantitative real-time PCR. A large quantity of transcripts of both genes accumulated in zoysiagrass seedlings at 3 days compared to control plants. Interestingly, the *Zjchi2* transcript level at 3 days was 96.66- and 5.11-fold higher than that in control plants and that of the *Zjchi1* gene, respectively. This result suggested that the two chitinase genes were induced by *R. solani* infection, and that *Zjchi2* plays a more important role than *Zjchi1* during *R. solani* AG2-2 (IV) infection. After 4 days of *R. solnai* infection, zoysiagrass seedlings were sufficiently damaged to make them unsuitable for use in the experiment (data not shown).

To investigate their biological functions, recombinant Zjchi1 and Zjchi2 were overexpressed in *E. coli* BL21 (DE3). The presence of the soluble forms of histidine-tagged Zchi1 and GST-Zjchi2 fusion proteins was verified by SDS-PAGE, and they were then purified using affinity column chromatography. The two purified recombinant chitinases showed *in vitro* antifungal activity against *R. solani* AG2-2 (IV), *R. solani* AG1 (IA), *R. cerealis*, *B. cinerea*, *F. culmorum*, *F. graminearum* and *T. reesei* but not
against *S. sclerotiorum*, *Pythium* sp. and *S. minor*. These fungi are known plant pathogens. *Rhizoctonia solani* AG2-2 (IV) causes large-patch disease of turf grasses, including zoysiagrass, and root rot and leaf blight in sugar beet (Aoyagi *et al*. 1998). *Rhizoctonia solani* AG1 (IA), which causes sheath blight of rice, an important disease in rice-growing regions, causes brown-patch disease in tall fescue (Tan *et al*. 2007; Martin and Lucas 1984). Sharp eyespot disease is caused primarily by *R. cerealis*, which is an important limiting factor in wheat production in China (Chen *et al*. 2008; Chen *et al*. 2009). The plant pathogen *B. cinerea* causes gray mold disease in diverse host plants, including *Arabidopsis thaliana* (Have *et al*. 1998). Most cultivated plants can be damaged by *Fusarium* species; e.g., *F. culmorum* causes *Fusarium* ear blight disease in wheat, barley, oats, rye and triticale; moreover, ear rot of maize caused by *F. graminearum* is a serious problem in North America (Parry *et al*. 1993). Also, *Fusarium* crown and root rot caused by *F. graminearum* is one of the main diseases of turf grass in central Saudi Arabia (Humaid *et al*. 2011).

Many class I chitinases exhibited antifungal activity *in vitro* and in transgenic plants. Purified 28 kDa chitinase A and chitinase B from maize seeds showed antifungal activity against *T. reesei*, *Alternaria solani* and *Fusarium oxysporum* (Huynh *et al*. 1992). Purified barley chitinase showed antifungal activity *in vitro* against *B. cinerea*, *Pestalotia theae*, *Alternaria* sp., *Curvularia lunata* and *R. solani* (Kirubakaran and Sakthivel 2007). *In vitro*, a class I chitinase from pea pods inhibited the growth of *Trichoderma viride* (Mauch *et*

al. 1988) and a tobacco class I chitinase inhibited *Fusarium solani* germlings (Sela-Buurlage *et al*. 1993). Basic class I chitinase from rye seed inhibited the extension of the hyphae of *Fusarium oxysporum*, *R. solani*, and *Trichoderma* sp. *in vitro* (Taira *et al*. 2002). Transgenic plants overexpressing class I chitinases have been shown to inhibit fungal growth. Transgenic tobacco seedlings constitutively expressing a class I bean chitinase gene showed enhanced antifungal activity in soil infested with *R. solani* compared with wild-type plants (Broglie *et al*. 1991). Transgenic Indica rice cultivars with an introduced PR-3 rice class I chitinase gene (RC7) showed increased resistance when challenged with *R. solani* (Datta *et al*. 2001). Transgenic grapevine and Italian ryegrass plants expressing a rice class I chitinase (RCC2) exhibited enhanced resistance against *Elsinoë ampelina* and *Puccinia coronata*, respectively (Yamamoto *et al*. 2000; Takahashi *et al*. 2004).

In comparison, class II chitinases from tobacco and chickpea showed no antifungal activity *in vitro* (Sela-Buurlage *et al*. 1993; Vogelsang and Barz 1992). A class II rye seed chitinase showed less antifungal activity than a class I rye seed chitinase, *in vitro* (Taira *et al*. 2002). However, transgenic tobacco and wheat plants containing a barley class II chitinase exhibited enhanced resistance against *R. solani* and *Erysiphe graminis*, and *Puccinia reconidita* (Jach *et al*. 1995; Oldach *et al*. 2001). These results show that not all chitinases show equal antifungal activity *in vitro*, which could affect the products of genetic engineering with chitinase-encoding genes (Punja and Zhang 1993).

In this study, we isolated class I (*Zjchi1*) and II (*Zjchi2*) chitinases from zoysiagrass. The purified class II chitinase showed significantly greater antifungal activity than the purified class I chitinase *in vitro*. These results suggest that the class II chitinase can be used to generate zoysiagrass resistant to *R. solani* AG2-2 (IV) instead of a class I chitinase. In addition, the CBD in class I chitinases is related to allergic reactions (Diaz-Perales *et al*. 1988; Posch *et al*. 1999). Therefore, we believe that the *Zjchi2* gene is better for generating

fungus-resistant transgenic zoysiagrass.

Therefore, we produced 14 T_0 transgenic zoysiagrass lines, including a vector control line (IG2), using *Agrobacterium*-mediated transformation (Toyama *et al.* 2003). The results of BASTA selection and the PAT protein strip test suggest that T-DNA was integrated into the genomes of the 14 T_0 transgenic zoysiagrass lines. These results were supported by the PCR and Southern blot analyses. The PCR result showed the presence of the *bar* gene and recombinant *Zjchi2* gene in the transgenic zoysiagrass lines. Integration of T-DNA into the genome of the transgenic zoysiagrass lines was confirmed by Southern blot hybridization. The two copies of the endogenous *Zjchi2* gene were verified by a *Zjchi2*-specific probe, whereas hybridization using the *bar*-specific probe indicated a single or multiple copies in the transgenic zoysiagrass lines. These results suggest that the recombinant *Zjchi2* gene was integrated as single and multiple copies into the zoysiagrass genome. The expression of the recombinant *Zjchi2* gene was analyzed using quantitative real-time PCR. The transgenic lines expressed different amounts of mRNA. In general, the transgenic lines with multiple copies (lines 4, 13, and 15) expressed less *Zjchi2* mRNA than did the single-copy lines (lines 2, 3, 9, 10, 11, 12, 14, and 16). Gene silencing and co-suppression by multiple gene copies have been reported (Vaucheret *et al.* 1998; Wilde *et al.* 2000). The expression level also differed significantly among the transgenic lines integrating a single gene copy. This can be explained as a position effect: the T-DNA insertion is random within the plant genome and the activity of the introduced genes may be influenced by adjacent plant DNA (Hobbs *et al.* 1990).

The wild-type and transgenic lines were grown to maturity in a greenhouse for real-time PCR and disease resistance tests. After 1 month, significant phenotype variation was observed among the transgenic lines. Transgenic lines 5 and 7 showed delayed development and growth compared with the other lines and then died. Genetic engineering depends on stable integration in terms of

expression level, predictable inheritance, and invariability of the phenotype of the introduced transgenes (Sun *et al*. 2006). In many cases, however, instability of the transgenes occurs in transgenic plants (Finnegan and McElroy 1994; Stam *et al*. 1997). Therefore, transgenic lines 5 and 7 were excluded from the real-time PCR and disease resistance tests.

To examine the resistance of transgenic zoysiagrass to *R. solani*, detached leaf and soil-based whole plant assays were performed. Transgenic lines 2, 3, and 10 showed significantly enhanced disease resistance compared with the wild-type. These results suggest that recombinant Zjchi2 worked normally in the transgenic zoysiagrass. However, transgenic lines 9, 12, and 16 showed similar disease symptoms as the wild-type. Increased protection does not always correspond to the level of enhanced enzymes in transgenic plants. Tobacco transgenic plants expressing barley chitinase gene that accumulated more enzyme showed limited antifungal activity in transgenic lines compared with other transgenic lines (Jach *et al*. 1995).

Large patch disease is the most important disease of zoysiagrass in Korea, Japan, and southern North America. Zoysiagrass is the main host of this disease caused by *R. solani* AG2-2 (IV) (Toda *et al*. 2003). Germicides and fungicides have been applied to control this disease in zoysiagrass fields, but cause serious environmental pollution. Therefore, the production of disease-resistant zoysiagrass by genetic engineering should be beneficial. In this study, we isolated two chitinase genes from zoysiagrass. Structural analysis showed that the isolated chitinases belong to the class Ib and class II families. Biological function analysis using purified r-chitinases showed broad-spectrum antifungal activity. Also, we generated transgenic zoysiagrass overexpressing the *Zjchi2* gene and examined its antifungal activity against *R. solani* AG2-2 (IV). The transgenic zoysiagrass showed significantly reduced disease symptoms compared with wild-type plants. To our knowledge, this report is the first to examine the antifungal activity of a class II chitinase in transgenic zoysiagrass. Since

zoysiagrass is a commercially important plant, our results will facilitate the generation of fungus-resistant turf grass and crop plants.

5. Conclusions

In this study, we isolated two chitinase genes from zoysiagrass. Structural analysis showed that the isolated chitinases belong to the class Ib and class II families.

Biological function analysis using purified r-chitinases showed broad-spectrum antifungal activity.

Further, we generated 14 T_0 transgenic zoysiagrass that overexpressed the class II chitinase gene (*Zjchi2*) via *Agrobacterium*-mediated transformation from zoysiagrass calluses.

Southern blot hybridization and polymerase chain reaction (PCR) were performed to confirm the transgenes insertion in the transgenic zoysiagrass.

The antifungal activity was investigated to examine the disease resistance of transgenic zoysiagrass to *R. solani* AG2-2 (IV). Transgenic lines 2, 3, and 10 showed significantly enhanced disease resistance compared with the wild-type.

To our knowledge, this study is the first to verify the antifungal activity of a class II chitinase in transgenic zoysiagrass.

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