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FUNGAL DISEASES

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Transgenic cucumber expressing an endogenous class III chitinase gene has reduced symptoms from *Botrytis cinerea*

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Abstract A class III chitinase gene (*CHI2*) is induced in cucumber plants (*Cucumis sativa* L.) in response to infection by pathogenic microorganisms. Infection of *Botrytis cinerea*, causal agent of gray mold disease on cucumber, also induces *CHI2* expression. To investigate whether CHI2 is involved in resistance to gray mold disease, transgenic cucumber plants were produced to overexpress the *CHI2* gene. One line was analyzed in detail in terms of disease resistance. The transgenic cucumber plant (CC2) constitutively expressed *CHI2* and reduced the symptoms of *B. cinerea* for 4 days after inoculation compared with nontransgenic plants. However, this inhibitory effect was not absolute, and CC2 eventually developed serious disease symptoms. Chitinase activity of the crude extract from CC2 leaves was higher than that from nontransgenic plants. A high-molecular-weight fraction containing CHI2 from CC2 leaves had fungistatic activity against *B. cinerea*. Interestingly, the low-molecular-weight fraction from CC2 leaves with CHI2 removed also had fungistatic activity against *B. cinerea*. Not only the introduced chitinase activity but also the endogenous defense reactions activated by overexpression of *CHI2* may be involved in the enhanced gray mold disease resistance in CC2.

Key words *Cucumis sativa* L. · Class III chitinase · Transgenic plants · *Botrytis cinerea* · Disease resistance · Antifungal substance

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Introduction

Chitinase catalyzes the hydrolysis of chitin, a linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc) residues. Plants respond to attack by pathogens such as fungi, bacteria, and viruses by expressing a large number of genes, many of which are believed to have a defensive role (Bol et al. 1990; McKenzie et al. 2002). Chitin is a common component of fungal cell walls and of the exoskeleton of arthropods (Bartnicki-Garcia 1968). Plant chitinases degrade chitin in fungal cell walls and can inhibit fungal growth (Brokaert et al. 1988; Schlumbaum et al. 1986). Some class I chitinases are localized in the vacuole, whereas other chitinases, including class III chitinases, are located outside the cell (Flemming et al. 1991; Neuhaus et al. 1991). Extracellular chitinases may directly block the growth of the hyphae invading intercellular spaces and possibly release fungal elicitors, which then induce additional chitinase biosynthesis and further defense reactions in the host (Barber et al. 1989; Mauch and Staehelin 1989).

CHI2 encodes cucumber class III extracellular chitinase, and its expression is induced by many factors, including fungal elicitors, salicylic acid, 2,6-dichloroisonicotinic acid, and acibenzolar-*S*-methyl (Narusaka et al. 1999). *CHI2* expression is commonly used as a marker of defense responses to pathogens (Kästner et al. 1998). Although CHI2 is likely to have multiple functions in cucumber plants because it is also induced in its aging leaves, specific parts of the flower, and the tendrils (Lawton et al. 1994), the actual function(s) of CHI2 are unknown. In the present study, we made transgenic cucumber plants that expressed cucumber class III chitinase gene (*CHI2*) to investigate whether the production of this enzyme is involved in resistance to gray mold disease caused by *Botrytis cinerea*. To our knowledge, this is the first report on class III chitinase overexpressed in transgenic plants that have reduced symptoms of fungal disease. In addition, we suggest that the *CHI2* overexpression unexpectedly induced defense responses, including the production of low-molecular-weight antifungal substance(s) and peroxidase activity.

Materials and methods

Northern hybridization

The total RNA of cucumber plants (cv. Shimoshirazu) was extracted as described by Verwoerd et al. (1989). RNA was extracted either from untreated plants in healthy condition or from sprayed plants with 5 ml of conidia suspension of *B. cinerea* (1×10^5 conidia/ml) and analyzed by Northern hybridization with *CHI2* DNA obtained as described later. *CHI2* DNA was labeled with peroxidase using an enhanced chemiluminescence (ECL) direct nucleic acid labeling and detection kit (Amersham Biosciences, Piscataway, NJ, USA). Hybridization signals were detected on X-ray film (Fujifilm, Tokyo, Japan).

Introduction of *CHI2* into cucumber plants

Total DNA of the cucumber plants was extracted as described by Liu et al. (1995). *CHI2* was isolated from cucumber plants by the polymerase chain reaction (PCR) using two oligonucleotide primers, Chi2-1 (CTTTAAGCAATG GCTGCCCA) and Chi2-2 (GACTAAATGGAGAAGAT GAA), corresponding to the sequences adjacent to the coding region of the *CHI2*. PCR was performed using TaKaRa Ex *Taq* polymerase (TaKaRa Biomedicals, Shiga, Japan). The DNA sequence of this PCR product was determined with a DSQ-1000L autosequencer (Shimadzu, Kyoto, Japan) using a Thermo Sequenase cycle sequencing kit (Amersham Biosciences). An approximately 1-kb fragment containing an open reading frame (ORF) with a 9-bp 5′-flanking sequence and a 96-bp 3′-flanking sequence replaced the β-glucuronidase gene in pBI121. Cucumber cv. Shimoshirazu plants were transformed with the binary vector as previously described by Tabei et al. (1998).

Enzyme activity assay

Chitinase activity was evaluated as *N*-acetylglucosaminidase activity according to the method of Someya et al. (2000). Fresh leaves (1 g) from cucumber plants were frozen in liquid nitrogen immediately after collection and ground into a fine powder. The powder was dissolved in 9 ml of McIlvaine buffer (7.2 mM citric acid, 25.3 mM Na_2HPO_4, pH 6.0) and centrifuged at 6000 *g* for 60 min at 4°C. The supernatants (200 μl) were added to 0.01% ρ-nitrophenyl-*N*-acetyl-β-D-glucosamide and incubated for 30 min at 37°C. The reaction was stopped with 1 M Na_2CO_3, and the absorbance of the released ρ-nitrophenol (ρNP) (Sigma-Aldrich, Tokyo, Japan) was measured at 405 nm.

Phenylalanine ammonia-lyase (PAL) and peroxidase (POX) activity were assayed by the method of Matsuyama and Dimond (1973). For PAL, total protein extracts from 100 mg of cucumber leaves was incubated for 3 h at 30°C with 10 ml of 0.1 M Tris buffer (pH 8.0) containing 0.1% phenylalanine as a substance. The *t*-cinnamic acid produced was extracted with acidic ether. The ether was removed, and the residue was dissolved in 0.05 N NaOH. The absorbance of the released *t*-cinnamic acid was measured at 268 nm. For POX, fresh leaves (1 g) from cucumber plants were homogenized with 50 ml of cold 0.067 M phosphate buffer (pH 7.4), centrifuged at 13000 *g* for 10 min, and the proteins were precipitated by acetone. After the acetone was removed, the precipitates were dissolved in 1 ml distilled water. Each sample was incubated for 1 h at 25°C with 3 ml of 0.1 M Na acetate buffer (pH 4.7), 1 ml of 1% ρ-phenylenediamine, and 0.5 ml of 0.3% H_2O_2 solution. POX activity was measured at 485 nm.

For a detailed protocol of the lipoxygenase assay, refer to the method of Namai et al. (1990).

Confirmation of *NPT2* introduction

The integrated neomycin phosphotransferase gene (*NPT2*) was detected by PCR using two oligonucleotide primers, NPT-F2 (CATGATTGAACAAGATGGATTGCACGC AGGTTCTC) and NPT-R2 (CAGGAAGACCTCGTCA AGAAGGCGATAGAAGGCGAT), corresponding to the sequences of *NPT2*. PCR was performed using TaKaRa Ex *Taq* polymerase under the following conditions: initial denaturing at 92°C for 1 min, 92°C for 1 min, 55°C for 1 min, and 74°C for 2 min for 30 cycles, followed by a final 74°C extension for 5 min. Amplified DNA fragments were separated by electrophoresis on 1.5% agarose gel and detected by ethidium bromide staining.

Detection of resistance to gray mold

The scoring method used was described by Kishimoto et al. (2002). Cut leaves with petioles were inoculated with 1.2% agar disks (2 mm diameter) containing conidia of *B. cinerea* (1×10^5 conidia/ml), 2.5% glucose, and 1 mM inosine and incubated in controlled-environment chambers maintained at 20°C. Resistance to *B. cinerea* infection was evaluated in terms of the lesion type 4 days after inoculation.

Antifungal activity assay

Fresh leaves (5 g) from cucumber plants were frozen in liquid nitrogen immediately after collection and ground into a fine powder. The powder was dissolved in 5 ml of distilled water and centrifuged at 8000 *g* for 30 min at 4°C. The supernatants (4 ml) were dialyzed in distilled water (4 ml) overnight at 4°C. The solution outside the dialysis membrane was designated as the low-molecular-weight fraction (LF), which was expected to contain substances with a molecular weight below 12000. The inside solution was dialyzed twice with the same volume of distilled water to minimize the presence of low-molecular-weight substances. This solution was designated as the high-molecular-weight fraction (HF). The two fractions were heat-treated for 30 min at 70°C. These heat-treated samples were designated the heated LF (HLF) and the heated HF (HHF),

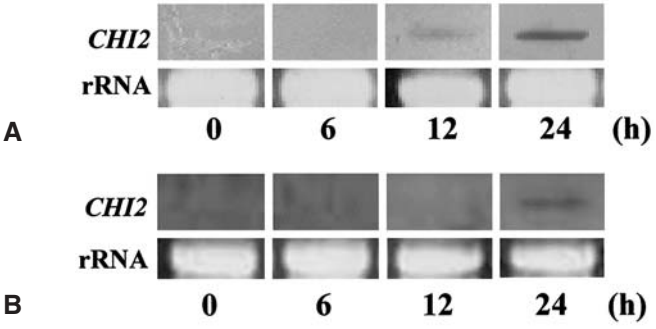

Fig. 1. Detection of class III chitinase gene (*CHI2*) expression by Northern hybridization. Total RNA was extracted from cucumber leaves sprayed with 5 ml of conidia suspension of *Botrytis cinerea* (1×10^5 conidia/ml) and hybridized with labeled *CHI2* DNA. **A** Results of the inoculated leaves. **B** Results of the upper leaves of the inoculated leaves

respectively. These samples (5 µl each) were then mixed with an equal volume of 5% glucose solution containing conidia of *B. cinerea* (1×10^5 conidia/ml) and dropped onto glass slides. The drops were covered with glass coverslips and incubated at 20°C. After incubation for 24 h, we observed the growth of the conidia with a microscope.

Results

Expression of *CHI2*

We investigated the induction of class III chitinase gene (*CHI2*) expression by *B. cinerea* in cucumber leaves by Northern analysis (Fig. 1). *CHI2* expression was not detected in the inoculated leaves at 0 or 6 h after inoculation. Induction of expression was evident after 12 h, and the amount of transcript continued to increase up to 24 h. Expression of *CHI2* was also induced in uninfected upper leaves of the inoculated plant (Fig. 1). However, this expression was slower than that of the inoculated leaves. These results indicated that *CHI2* is not expressed in an intact plant, but it is induced systemically within 24 h in response to infection by *B. cinerea*.

Resistance to *Botrytis cinerea* in CHI2-overexpressing cucumber plants

We investigated whether *CHI2* is involved in *B. cinerea* resistance. *CHI2* under the control of a CaMV 35S promoter was introduced into cucumber using *Agrobacterium tumefaciens*. Because *CHI2* is an endogenous cucumber gene, the presence of the transgene was detected by PCR to amplify a selection marker gene *NPT2* (Fig. 2). A transgenic line (CC2) was selected from numerous regenerated plants for its constitutive overexpression of the *CHI2* gene, confirmed by Northern analysis (Fig. 3). Chitinase activity of the crude extract from CC2 leaves was about three times that of nontransgenic plants (Fig. 4). When *B. cinerea* was used to inoculate the nontransgenic plants, the chitinase activity also increased. However, the increase was not as high as that in CC2 leaves. On the other hand, chitinase activity in CC2 decreased after the crude extract

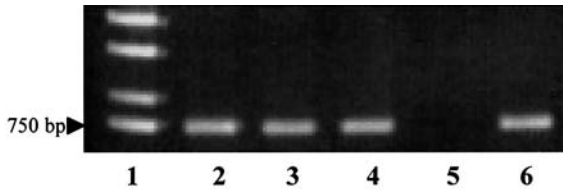

Fig. 2. Detection of the *NPT2* gene in the regenerated cucumber by polymerase chain reaction (PCR). Lane *1*, GeneRuler 1-kb DNA ladder marker; lane *2*, epistatic leaf of the regenerated cucumber plant; lane *3*, medium leaf; lane *4*, inferior leaf; lane *5*, a nontransgenic plant; lane *6*, positive control (pBI121)

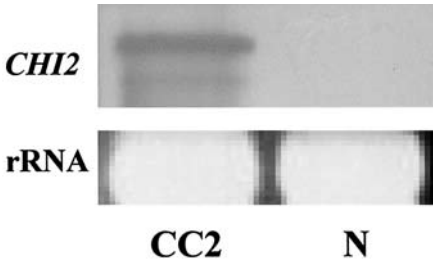

Fig. 3. Detection of the expression of the class III chitinase gene (*CHI2*) in transgenic cucumber by Northern hybridization. Total RNA was extracted from healthy cucumber leaves and hybridized with *CHI2* DNA. *CC2*, transgenic cucumber line expressing *CHI2*; *N*, a nontransgenic plant

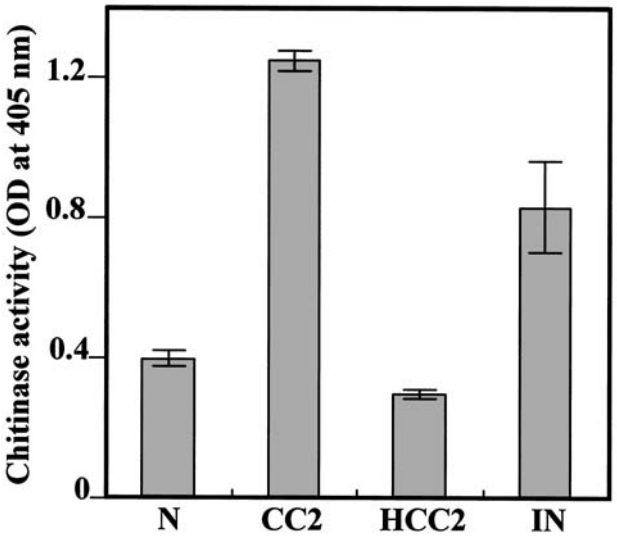

Fig. 4. *Chitinase activity* in class III chitinase gene (*CHI2*) expression in transgenic cucumber. The activities of the crude extract of leaves were measured as the optical density at 405 nm of ρ-nitrophenol (ρNP) released from ρNP-GlcNAc after 30 min of incubation at 37°C. *N*, nontransgenic plants; *CC2*, transgenic cucumber line; *HCC2*, crude extract of CC2 leaves after heating for 30 min at 70°C; *IN*, *Botrytis cinerea* (1×10^5 conidia/ml)-inoculated nontransgenic plants at 24 h after inoculation. Error bars are the standard error ($n = 3$)

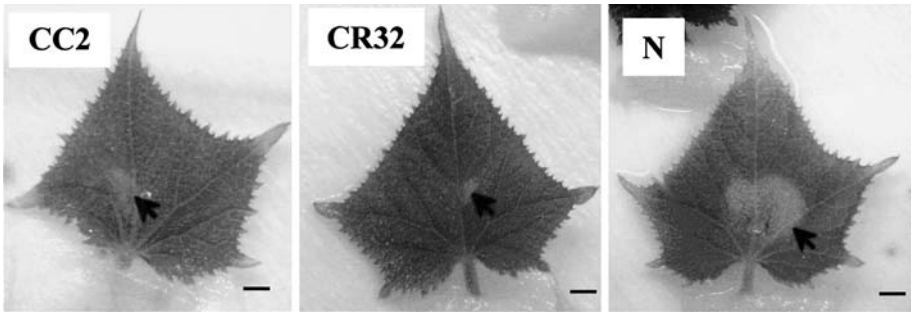

Fig. 5. Comparison of resistance to *Botrytis cinerea* between transgenic cucumber overexpressing a cucumber class III chitinase gene (*CHI2*) and a nontransgenic cucumber plant at 4 days after inoculation. *CC2*, transgenic cucumber line expressing *CHI2*; *CR32*, transgenic cucumber line expressing rice class I chitinase cDNA (*RCC2*); *N*, nontransgenic plant. The *arrow* shows symptoms of gray mold. *Bars* 5 mm

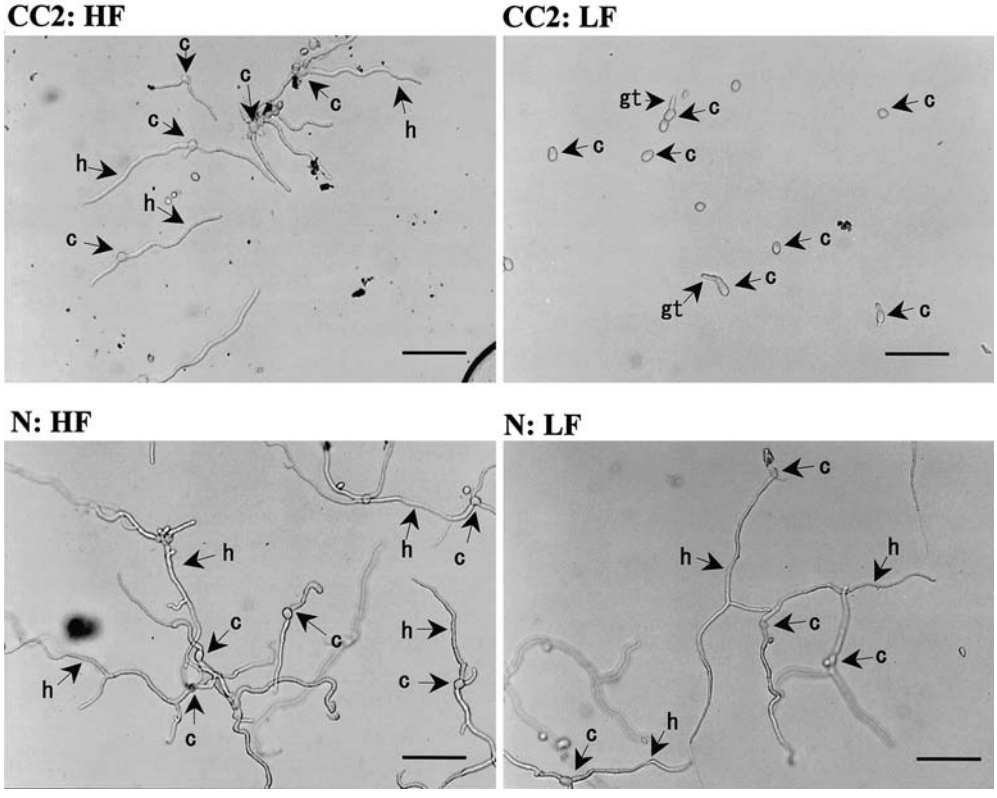

Fig. 6. Micrographs of antifungal activity of *Botrytis cinerea* of the high-molecular-weight fraction (*HF*) and the low-molecular-weight fraction (*LF*) of crude leaf extract from transgenic cucumber (*CC2*) expressing the cucumber class III chitinase gene (*CHI2*) in comparison to nontransgenic cucumber (*N*). The molecular weight of HF was higher than 12000, and LF was less than 12000. These fractions (5 µl) were each mixed with 5 µl of 5% glucose solution containing conidia of *B. cinerea* (1×10^5 conidia/ml) and dropped onto glass slides. The drops were covered with glass coverslips and incubated for 24 h at 20°C. *CC2*, transgenic cucumber line expressing *CHI2*; *N*, nontransgenic plant; *c*, conidia; *gt*, germ tube; *h*, hyphae. *Bars* 60 µm

was heated for 30 min at 70°C (Fig. 4). The chitinase activity of inoculated nontransgenic plants also decreased after heat treatment (data not shown).

We analyzed resistance to *B. cinerea* in CC2 using the conidia inoculation test. The spread of the symptoms in nontransgenic plants exceeded 15 mm 4 days after infection (Fig. 5). On the other hand, the diameter of the symptoms in CC2 was less than 10 mm. However, the effect of inhibition in CC2 was not complete, and at 7 days after infection leaves of CC2 were completely dead as a result of necrosis, as were the leaves of nontransgenic plants (data not shown). Other *CHI2*-overexpressing cucumber plants also gave similar results with CC2 (data not shown). We reported a transgenic cucumber plant (CR32) that expressed rice class I chitinase constitutively and had enhanced resistance to *Phytophthora nicotianae* var. *parasitica* (Kishimoto et al. 2003). Spread of the symptoms in CR32 was restricted to less than 5 mm diameter (Fig. 5). Thus, resistance against *B. cinerea* of CC2 is weaker than that conferred by CR32.

Antifungal activity of crude leaf extracts of transgenic cucumber

We also investigated the antifungal activity in crude extracts of CC2 leaves. After leaf crude extracts were centrifuged to remove the residue, we fractionated these supernatants into a high-molecular-weight fraction (HF) and a low-molecular-weight fraction (LF) by dialysis. The HF contained proteins with a molecular weight of more than 12000 including CHI2. The LF had a molecular weight below 12000. Figure 6 shows the growth of *B. cinerea* conidia incubated in each fraction 24 h after treatment. The CC2 HF had more antifungal activity against *B. cinerea* than did the nontransgenic HF (Fig. 6). In a theoretical sense, the

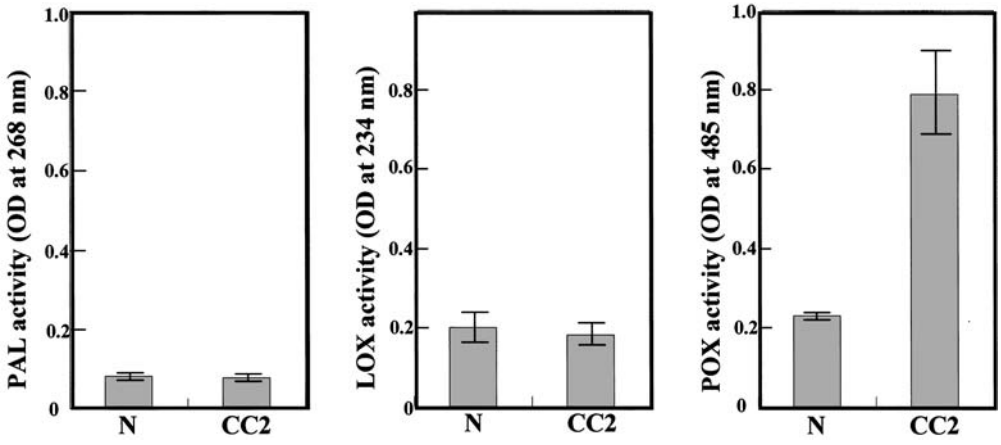

Fig. 7. Phenylalanine ammonia lyase (*PAL*), lipoxygenase (*LOX*), and peroxidase (*POX*) activity in class III chitinase gene (*CHI2*) expression in transgenic (*CC2*) and nontransgenic (*N*) cucumber. The detail of the enzyme assays are described in the text. Error bars are the standard error ($n = 3$)

CC2 LF did not contain CHI2. However, CC2 LF was also fungistatic against *B. cinerea*. The chitinase activity of CC2 leaf crude extract was attenuated by heat treatment. The fungistatic activity of CC2 HF was also eliminated by heating for 30 min at 70°C (data not shown). On the other hand, the fungistatic activity of CC2 LF was not influenced by heat treatment (data not shown). These results suggest that CC2 produces one or more heat-stable antibiotic substances of low molecular weight.

Some enzymatic activities relating to cucumber defense response

We investigated whether important enzymes of cucumber defense response were activated in CC2. Phenylalanine ammonia lyase (PAL) and lipoxygenase (LOX) are thought to be key enzymes of the defense response in cucumber plants (Martinez et al. 2001). Peroxidase (POX) is an enzyme of lignin synthesis. Accumulation of lignin is also an important defense system in cucumber (Yedidia et al. 1999). Figure 7 shows PAL, LOX, and POX activity in nontransgenic cucumber plants and CC2. PAL and LOX activities in CC2 were almost equal to those in nontransgenic plants, but POX activity in CC2 was higher than in the nontransgenic plant.

Discussion

We demonstrated that transgenic cucumber plants (CC2) expressing an endogenous class III chitinase gene (*CHI2*) that reduced symptoms by *Botrytis cinerea*, and that CHI2 containing an HF from CC2 leaves had fungistatic activity. The fungistatic activity was eliminated by heat treatment (70°C, 30 min). The chitinase activity of CC2 leaf extract was also reduced by heat treatment (70°C, 30 min). Thus, we believe that accumulation of CHI2 in CC2 enhances resistance to *B. cinerea*. Some in vitro tests using chitinases exhibited lysis of fungal hyphae (Taira et al. 2002). However, dramatic lytic activities were not observed microscopically in CC2 HF (Fig. 6). Lysis of hyphae may require a higher concentration of chitinase. In the inoculation test, gray mold resistance of CR32, which overexpresses a rice class I chitinase (RCC2), was stronger than that of CC2. RCC2 has a vacuolar targeting signal and localizes intracellularly in cucumber plant (Kishimoto et al. 2002). On the other hand, CHI2 is a secretory enzyme (Neuhaus et al. 1991). *B. cinerea* invades both intracellular and intercellular space in plant tissues. Thus, *B. cinerea* encounters RCC2 or CHI2 in these transgenic plants. Possibly, chitinases accumulated in the vacuole inhibit the growth of the hyphae more efficiently than secretory chitinases.

In our analysis of chitinase activity, nontransgenic plants also have some chitinase activity. Siegrist et al. (1994) reported chitinase activity in cucumber plants treated with some elicitors. The result also showed a background as large as that in our results. Conceivably, the cucumber plant has a low constitutive *N*-acetylglucosaminidase activity, which is resistant to increased temperature. CHI2 has two isoforms: CHI1 and CHI3. These isoforms are not induced by various elicitors in cucumber plants (Lawton et al. 1994), and only *CHI2* expression is used as a molecular marker for the defense response (Kästner et al. 1998). Thus, increased activity in samples in Fig. 4 in mainly due to the induction of CHI2 expression by *B. cinerea.*

We also demonstrated that the LF of CC2, which did not contain CHI2, had fungistatic activity. In addition, the fungistatic activity of CC2 LF was not affected by the heat treatment. These results suggest that endogenous heat-stable antifungal substance(s) might be constantly produced in CC2. We previously reported that a rice class I chitinase-overexpressing transgenic cucumber accumulated low-molecular-weight antifungal substances (Kishimoto et al. 2003). As far as we know, no one has investigated whether an unexpected defense system may be activated in chitinase-overexpressing transgenic plants.

Fawe et al. (1998) reported a low-molecular-weight antifungal substance in cucumbers. The chemical nature and mechanism of induction of this substance were similar to those of flavonoid phytoalexins detected in other plants. Cucumber plants also have six-carbon (C_6) and nine-carbon

(C_9) aldehydes, which are (*E*)-2-hexenal, (*Z*)-3-hexenal, 3,6-(Z,Z)-nonadienal, and others (Matsui et al. 2000). These short-chain aldehydes are present in only small quantities in intact cucumber plants, but they are produced rapidly in response to wounding. Recently, numerous plant short-chain aldehydes have been reported to have antibiotic activity against pathogenic microbes and fungi (Nakamura and Hatanaka 2002; Wright et al. 2000). These low-molecular-weight antibiotic substances might be induced in CC2. We carried out a preliminary investigation on the activity of key enzymes for flavonoids or short-chain aldehyde synthesis in CC2 and nontransgenic plants. PAL is a key enzyme catalyzing the upstream step in the flavonoid pathway (Bate et al. 1994). LOX is an enzyme catalyzing fatty acid oxygenation, an essential step in the oxylipin pathway (Bell and Mullet 1993). However, PAL or LOX activity in CC2 were almost equal to those of nontransgenic plants. On the other hand, we found that POX was constitutively activated in CC2. This result indicates possible activation of a novel defense system in CC2. Perhaps, a synthetic pathway for lignin is activated in CC2. Some lignin precursors of low molecular weight (<12 000), which are ρ-coumaralaldehyde, ferulic acid, sinapaldehyde, and others, are known to have antifungal activity (Barber et al. 2000). Martinez et al. (2001). suggested that induction of peroxidase is dependent on a salicylic acid (SA)-, ethylene-, or jasmonic acid (JA)-signaling pathway in *Cucumis*. Perhaps this SA-, ethylene-, or JA- signaling pathway is activated in CC2.

Conclusions

We demonstrated that constitutive expression of CHI2 influences disease resistance to *B. cinerea* in cucumber plants. In addition, we suggested that the *CHI2* overexpression unexpectedly induced a defense response that included the production of low-molecular-weight antifungal substance(s) and POX activity. The substance(s) should be clarified in further studies.

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