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Transgenic cucumber plants harboring a rice chitinase gene exhibit enhanced resistance to gray mold (*Botrytis cinerea*)

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Abstract A rice chitinase cDNA (RCC2) driven by the CaMV 35S promoter was introduced into cucumber (*Cucumis sativus* L.) through *Agrobacterium* mediation. More than 200 putative transgenic shoots were regenerated and grown on MS medium supplemented with 100 mg/l kanamycin. Sixty elongated shoots were examined for the presence of the integrated RCC2 gene and subsequently confirmed to have it. Of these, 20 were tested for resistance against gray mold (*Botrytis cinerea*) by infection with the conidia: 15 strains out of the 20 independent shoots exhibited a higher resistance than the control (non-transgenic plants). Three transgenic cucumber strains (designated CR29, CR32 and CR33) showed the highest resistance against *B. cinerea*: the spread of disease was inhibited completely in these strains. Chitinase gene expression in highly resistant transgenic strains (CR32 and CR33) was compared to that of a susceptible transgenic strain (CR20) and a control. Different responses for disease resistance were observed among the highly resistant strains. CR33 inhibited appressoria formation and penetration of hyphae. Although CR32 permitted penetration of hyphae, invasion of the infection hyphae was restricted. Furthermore, progenies of CR32 showed a segregation ratio of 3:1 (resistant:susceptible). As the disease resistance against gray mold was confirmed to be inheritable, these highly resistant transgenic cucumber strains would serve as good breeding materials for disease resistance.

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Abbreviations *BA* 6-Benzyladenine · *ABA abscisis acid* · *cfu* colony forming unit

Introduction

Cucumber belongs to genus *Cucumis* and is widely cultivated in many areas of the world, especially Southeast Asia (Esquinas-Alcazar and Gulick 1983). Many cultivars of cucumber have already been bred, thereby enabling this genus to be cultivated under various conditions. The availability of highly disease resistant materials is essential in breeding programs of disease resistance. Because agricultural yield tends to be reduced constantly by various plant diseases which are generally caused by phytopathogenic fungi, bacteria and viruses, breeding for disease resistance becomes one of the most crucial objectives in cucumber cultivation.

Breeding materials which exhibit resistance to *Fusarium* wilt (Komada and Ezuka 1974), virus disease (Barnes 1961) and angular leaf spot (Shifriss et al. 1942) have been found and used to breed disease-resistant varieties of cucumber. However, it is very difficult to find desirable materials for the breeding of resistant cucumber against other diseases. Of particular importance is the disease caused by gray mold (*Botrytis cinerea*), which is considered to be one of the most serious diseases in cucumber cultivation. Gray mold infects many kinds of crops (Verhoelf 1980) and generated new isolates resistant to fungicides in the 1960s (Akutsu 1995). Due to the unavailability of breeding materials resistant against gray mold, to date disease resistance breeding has not progressed well. In this case, a transformation technique seems to be a useful method to produce novel breeding materials because this can make good use of isolated genes from a variety of species.

Disease resistance genes have recently been isolated (Martin et al. 1993; Jones et al. 1994; Mindrinos et al.

1994), and their functions analyzed using molecular biology technology. For example, one disease resistance gene, *Pto*, against *Pseudomonas syringae* pv *tomato* was introduced into tobacco, resulting in the improvement of disease resistance in this crop (Rommens et al. 1995). It is known that plants have defence systems which involve pathogenesis-related proteins, e.g. chitinase (Legrand et al. 1987; Nishizawa and Hibi 1991) and β-1,3-glucanase (Kombrink et al. 1988). Chitinase and β-1,3-glucanase catalyze the hydrolysis of β-1,4 linkages of the *N*-acetyl-D-glucosamine polymer, chitin and β-1,3-glucan, respectively. Several works on transgenic plants harboring these genes have already been published. The transgenic tobacco and canola which have been engineered with bean endochitinase gene were shown to exhibit resistance to *Rhizoctonia solani* (Broglie et al. 1991); the transgenic rice integrated with rice endochitinase driven by the 35S promoter also showed enhanced resistance to sheath blight (Lin et al. 1995); and the transgenic tobacco harboring rice endochitinase gene (Nishizawa et al. 1993) also possessed increased resistance against powdery mildew (*Erysiphe cichoracearum*) (Akutsu et al. 1997).

In the study reported here, we tried to produce novel resistant cucumber against gray mold (*Botrytis cinerea)* by introducing the cDNA of rice endochitinase gene RCC2 (Nishizawa et al. 1993) into cucumber. Disease resistance of transgenic cucumber against gray mold was examined by infection with *B. cinerea*, and the inheritance of the resistance against gray mold to progenies was also confirmed. Finally, we discuss these transgenic cucumber plants as novel breeding materials for disease resistance.

Materials and methods

Plant materials and vector

Cucumber cv 'Shimoshirazu' (from Genebank, MAFF, Japan; Accession no. 35976) was multiplied by artificial self-pollination and used in this experiment. Peeled cucumber seeds were sterilized for 15 min with sodium hypochlorite solution containing 1% active chlorine and then rinsed three times with sterile distilled water. These seeds were sown on phytohormone-free MS medium (Murashige and Skoog 1962) containing 30 g/l sucrose and 2.5 g/l gelrite (Wako, Japan), and cultured for 1 day at 25°C under dark conditions. Cotyledons from partially germinated seeds were separated, and cut into four pieces and used as explants.

A binary vector, pBI121-RCC2, was constructed to replace the GUS gene of pBI121 (Clontech, USA) with the cDNA (RCC2) of the rice chitinase gene (Nishizawa 1993). pBI121-RCC2 was integrated into *Agrobacterium tumefaciens* LBA4404 (Clontech, USA) by tri-parental mating and used as a vector.

Transformation and selection of transformants

Cotyledon explants were dipped in a tenfold diluted suspension of *Agrobacterium* overnight culture for 10 min. These explants were placed onto shoot induction medium (MS medium containing 2 mg/l BA, 1 mg/l ABA, 30 g/l sucrose and 2.5 g/l gelrite) and cultured for 3 days at 25°C under dark conditions as co-culture. After co-culture, explants were rinsed three times with sterile distilled water and cultured on shoot induction medium containing 200 mg/l claforan (Pfizer, Germany) for 7 days to eliminate *Agrobacterium* and promote shoot organogenesis. The explants were then transferred to shoot regeneration medium (MS medium containing 2 mg/l BA, 1 mg/l ABA, 30 g/l sucrose, 2.5 g/l gelrite, 25 mg/l kanamycin and 200 mg/l claforan). Regenerated shoots were separated from explants and cultured in vitro on phytohormone-free MS medium containing 100 mg/l kanamycin and 200 mg/l claforan to select transgenic cucumber plantlets. Elongated shoots were propagated by cutting and maintained on phytohormone-free MS medium containing 100 mg/l kanamycin and 200 mg/l claforan.

Confirmation of RCC2 gene introduction

The integrated RCC2 gene was detected from kanamycin-resistant shoots by polymerase chain reaction (PCR)-Southern hybridization and was also confirmed to be present in plantlets that exhibited resistance to gray mold. Total DNA of transgenic and control plants was extracted as follows. A small piece of stem (ca. 100 mg) was frozen using liquid nitrogen and ground using a mortar and pestle. The resulting cucumber powder was suspended in a mixture of 200 µl TE buffer (10 m*M* TRIS-HCl and 1 m*M* EDTA, pH 8.0) and 200 µl saturated phenol solution and shaken vigorously for 1 min. Following centrifugation (15,000 *g*, 3 min, 4°C), 200 µl of supernatant was transferred to a new microtube, and added with 200 µl saturated phenol and 200 µl chloroform were added. After centrifugation (15,000 *g*, 3 min, 4°C), the isolated DNA was purified by ethanol precipitation. Precipitated DNA was dissolved in 50 µl sterile distilled water, and 1 µl was used for PCR.

The integrated RCC2 gene was detected by PCR using two oligonucleotide primers, RCG2SPE1 (5′-TGGATCCAGCGGCTCGT CGGTTG-3′) and NOS-3′ (5′-GTATAATTGCGGGACTCTAAT-3′), corresponding to the sequences of the RCC2 and NOS terminators, respectively. The standard PCR solution (20 µl) was composed of 1 µl template DNA, 1 µl of each primer (20 µ*M*), 1 µl dNTP (5 m*M*) and 1 unit of *Tth* polymerase (Toyobo, Japan) in the buffer recommended by the supplier. PCR was carried out in a thermal sequencer (TSR-300, Iwaki, Japan) under the following conditions: 92°C denaturing for 1 min as preheating, then 92°C denaturing for 1 min, 60°C annealing for 1 min and 74°C extension for 1 min for 40 cycles, and another 72°C extension for 5 min. Amplified DNA fragments were electrophoresed on 1.5% agarose gel (Agarose II, Sigma, USA) and detected by ethidium bromide staining. In order to confirm the presence of the RCC2 gene, we transformed the PCR product onto a nylon membrane (Boehringer Mannheim, cat. no. 1252365) and then hybridized it with labelled RCC2 gene isolated from plasmid. The procedures for labelling of RCC2 gene, Southern hybridization and visualization by the color substrate reaction were carried out according to the protocols given in the DIG DNA Labeling and Detection Kit (Boehringer Mannheim, cat. no. 1093657).

Detection of the RCC2 protein by ELISA

Total soluble proteins were extracted from leaves of the T_0 lines, CR20, CR32, and CR33 and the non-transformant by homogenization in 0.1 *M* sodium citrate buffer (pH 5.0) and centrifuged at 12,000 *g* for 7 min at 4°C. The supernatants from all samples were diluted to 1:100 in PBST (8 g NaCl, 0.2 g KCl, 2.9 g $Na_2HPO_4 \cdot 12H_2O$, 0.2 g KH_2PO_4, 0.2 g NaN_3 and 0.5 ml Tween 20 in 1 l distilled water). ELISA microtiter wells (96 well, Nunc) were then coated with 200 µl of the supernatant for 2 h at 37°C. After resin washing with PBST, 200 µl of polyclonal antibody against the RCC2 protein was added in serial dilutions to the wells and coupled to the antiserum overnight at 4°C. Rabbit antiserum was produced using RCC2 protein translated in *E. coli* as antigen. After resin washing again with PBST, 200 µl of an alkaline phosphatase-conjugated Goat anti-Rabbit IgG (TAGOIMMUNOLOGICALS^{TM}) was added to the wells and incubated for 4 h at 37°C. After resin washing, 200 µl of the 0.1 m*M* nitrophenyl phosphate eluted by diethanolamine, pH 9.8, was added to the wells and incubated for 50 min at room temperature. After the addition of 12% NaOH, the absorbance was measured

at 405 nm in a microplate autoreader (IMMUNO-MINI, InterMed) (Akutsu et al. submitted). The absorbance was compared to that of the control after the absorbance of the buffer was subtracted.

Detection of resistance against gray mold in transgenic cucumber plants

Non-transgenic cucumber variety, cv 'Shimoshirazu', was used as control. The leaves were inoculated with 1.2% agar disks (2 mm in diameter) containing conidia of *B. cinerea* (1×10^5 cfu/ml), 2.5% glucose and 1 m*M* inosine, and incubated in controlled environmental chambers maintained at 20°C. Susceptibility to gray mold infection was evaluated in terms of lesion type, which was measured at 4 days after inoculation in three independent trials. Observations on the behavior of the infection hyphae was continued until 10 days after inoculation. Susceptibility to gray mold infection was classified into three types based on the rate that the disease symptoms spread. These were as follows: non-spreading, disease symptoms were restricted completely; weak-spreading, rate of spreading of the disease symptoms was slower than control; and spreading, rate of spreading of the disease symptoms was the same as control.

To investigate the infection behavior of *B. cinerea* on transgenic cucumber plants, we inoculated the leaves of transgenic or control cucumber plants with 5 µl of conidial suspension (1×10^5 cfu/ml) of *B. cinerea* containing 2.5% glucose and 1 m*M* inosine. The leaves were incubated under the same conditions as described above. Conidia germination, formation of the secondary appressoria and penetration into leaves were examined 24 h after inoculation.

Light microscopy of B. cineria infection behavior

Leaves were collected from each CR32 and non-transformed cucumber plant at 1 day after inoculation. From these leaves, discs (diameter of 10 mm) were cut out, fixed and discolored in FAA solution (formaline:ethanol:acetic acid=1:1:1). After staining with methyl blue solution, the leaf discs were observed under a light microscope.

Inheritance of resistance against *B. cinerea*

In order to confirm the inheritance of the RCC2 gene and to demonstrate resistance against gray mold in progenies, we artificially self-pollinated two transgenic strains (CR32 and CR33) that are highly resistant against gray mold and examined their progenies. The RCC2 gene was detected by PCR-Southern hybridization, and resistance against gray mold was also investigated by artificially induced infection under the same conditions as described above.

Results and discussion

Production of transgenic cucumber harboring RCC2

Multiple shoots were induced from both explants infected by *Agrobacterium* and control explants (uninfected with *Agrobacterium*) on shoot induction medium supplemented with 25 mg/l kanamycin and 200 mg/l claforan. Explants with multiple shoots were transferred onto MS medium containing 100 mg/l kanamycin and 200 mg/l claforan to eliminate non-transgenic cucumber shoots. The selective antibiotic gradually slowed the growth of adventitious shoots from the control explants and also bleached them. Many adventitious shoots (more than 200 shoots) from explants infected by *Agrobacterium* maintained their green color and grew normally on MS medium containing 100 mg/l kanamycin and 200 mg/l claforan. These kanamycin-resistant shoots were assumed to have neomycin phosphotransferase (NPT II). From these, 60 elongated green shoots were selected randomly and examined by PCR-Southern hybridization for the presence of an integrated RCC2 gene. All kanamycin-resistant shoots examined had the RCC2 gene and were designated as CR strains. Transgenic cucumber plantlets were propagated by cutting in vitro, then acclimatized in a closed greenhouse. After acclimatization, transgenic cucumber plants grew normally, and there was no difference between transgenic and non-transgenic cucumber plants from viewpoints of growth and morphological characters.

Resistance of T_0 generation against gray mold

Acclimatized plantlets grown in a closed greenhouse were used to examine resistance against gray mold. The results of our evaluation of susceptibility to gray mold infection of 20 transgenic cucumber strains are shown in Table 1. Three CR strains (CR29, CR32 and CR33) exhibited high resistance against *B. cinerea*. One of the strains (CR33) which showed the highest resistance is shown in Fig. 1. However, there were some differences between CR33, and CR29 and CR32. CR33 was classified as non-infected, charactarized by the complete inhibition of appressoria formation and penetration of hyphae into leaves. On the other hand, CR29 and CR32 were classified as a non-spreading type, which permitted penetration of hyphae, they restricted invasion of the infection hyphae within the first two cells of the leaves, thus, completely preventing the spread of disease. Nine CR strains (CR3, -4, -6, -17, -18, -31, -34, -38 and -56) were weak-spreading types, meaning a gradual proliferation of penetrating hyphae into the leaf. These CR strains exhibited a higher resistance than the control. The remaining 8 CR strains (CR1, -8, -15, -20,

Table 1 Susceptibility of transgenic cucumber plants bearing the rice chitinase cDNA (RCC2) to *Botrytis cinerea* infection[a]

Resistance[b]	Transgenic cucumber strain (T_0 generation)
Highest resistance group	
Non-infected	CR33
Non-spreading	CR29, CR32
Intermediate resistance	CR3, CR4, CR6, CR17, CR18, CR31, CR34, CR38, CR56
Susceptible	CR1, CR8, CR15, CR20, CR37, CR42, CR55, CR57, Shimoshirazu[c]

[a] The leaves were inoculated with 1.2% agar discs (2 mm) containing the conidia of *Botrytis cinerea* (1×10^5 cfu/ml), 2.5% glucose and 1 m*M* inosine, and incubated in controlled environmental chambers maintained at 20°C. Susceptibility to infection was evaluated in terms of resistance in three independent trials 4 days after inoculation

[b] Non-infected, non-spreading: disease symptoms were restricted completely at 10 days after inoculation; intermediate resistance: rate of spreading speed of disease symptom was slower than control, susceptible: rate of spreading speed of disease symptoms was the same as control

[c] Control (non-transgenic) cucumber

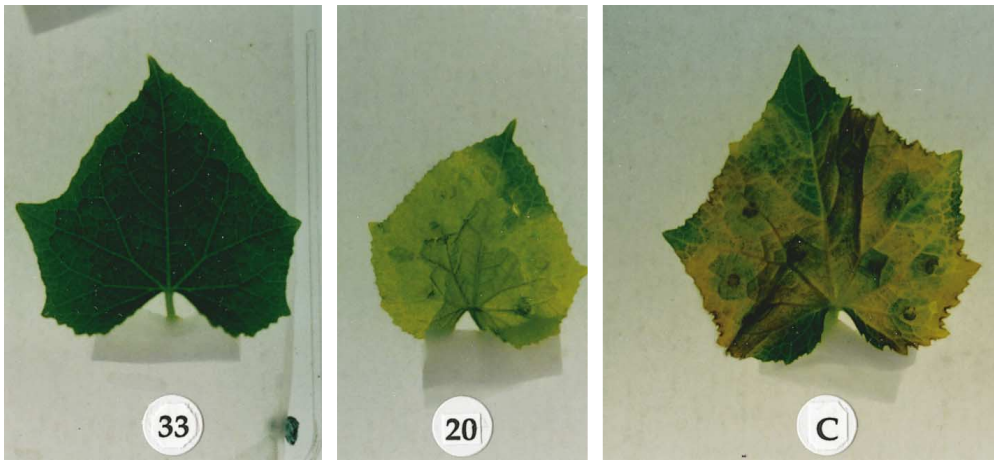

Fig. 1 Comparison of resistance against gray mold (*Botrytis cinerea*) between transgenic and non-transgenic plants at 4 days after artificial infection. ***C*** is non-transgenic cucumber plants of var 'Shimoshirazu'; *33* and *20* indicate transgenic cucumber plants harboring cDNA of rice chitinase gene RCC2–CR33 (non-spreading type) and CR20 (spreading type), respectively. Infection was done by inoculating conidia (1×10^5 cfu/ml) of *B. cinerea* onto the leaves of test plants

-37, -42, -55 and -57) were classified as the spreading type, and their level of resistance did not differ from that of the control. As the incubation period was extended, disease symptoms on the transgenic cucumber plants of the weak-spreading type gradually spread, while CR33 was not infected by *B. cinerea* and 2 strains (CR29 and CR32) of the non-spreading type restricted the spread of disease symptoms completely.

Table 2 Detection of RCC2 protein[a] produced in transgenic cucumber plants by ELISA analysis

	CR33	CR32	CR20	Control[a]
MV[b]	1.12	0.83	0.58	0.36
RV[c]	(3.1)	(2.3)	(1.6)	(1.0)

[a] Total soluble proteins were extracted from leaves of the T_0 lines, and polyclonal antibody against RCC2 protein was added in serial dilutions to the wells and coupled to the antiserum overnight at 4°C
[b] Non-transgenic cucumber var 'Shimoshirazu'
[c] Absorbance measured at 405 nm
[d] Relative value of RCC2 protein compared to control

Comparison of gene products and infection behavior in transgenic cucumber

The amounts of RCC2 products from 3 CR strains (CR20, CR32 and CR33) and the control were compared by ELISA analysis (Table 2). The level of gene expression of 2 resistant strains, CR32 and CR33, was 2.3 and 3.1 times higher than that of control, respectively. On the other hand, that of the susceptible strain, CR20, was only 1.6 times higher.

Infection behavior was compared among 3 CR strains (CR20, CR32 and CR33) and control variety cv 'Shimoshirazu' (Table 3). More than 95% of inoculated conidia germinated on the leaves of all 3 strains as well as on the control. Interestingly, the frequency of appressoria formation was different between the highest resistant strains, CR32 and CR33. Restricted formation of appressoria and no penetration of hyphae were observed in CR33. Most of the infection hyphae in the leaves of CR32 was restricted within an epidermal cell, and resistance against gray mold was observed (Table 3 and Fig. 2A, B). On the other hand, elongation of infection hyphae on the leaves of CR20 and

Table 3 Infection behaviour of *Botrytis cinerea* on transgenic cucumber plants (T_0) bearing the rice chitinase cDNA (RCC2)[a]

Transgenic cucumber strain (T_0)	Germination conidia[b] (%)	Formation of appressoria[c] (%)	Penetration (%)	Percentage of invasion of infection hyphae into leaves		
				1 cell[e]	2 cells[f]	>3 cells[g]
CR33	97.2	0.9	0	0	0	0
CR32	100	36.3	54.7	81.3	18.7	0
CR20	100	46.3	48.7	0	9.1	90.1
Control[h]	95.5	13.5	26.5	0	0	100

[a] The leaves were inoculated with 5 µl of the conidia of *Botrytis cinerea* (1×10^5 cfu/ml) in 2.5% glucose and 1 m*M* inosine, and incubated in controlled environmental chambers maintained at 20°C. Conidia germination, formation of the secondary appressoria, and penetration were examined 24 h after inoculation
[b] Percentage of conidia germinated relative to the conidia inoculated
[c] Percentage of conidia forming secondary appressorium relative to the conidia germinated
[d] Percentage of hyphae penetrated into leaf
[e, f, g] Ratios of infection hyphae penetrated into one cell (e), two cells (f) and more than three cells (g) of cucumber leaves, respectively, to total infection hyphae counted
[h] Non-transformed cucumber var 'Shimoshirazu'

the control was not restricted, resulting in the spreading lesion type (Table 3 and Fig. 2C). Because more *B. cinerea* conidia were applied by artificial infection than by natural infection, clear symptoms of gray mold disease were observed at innoculation sites on the CR32 leaves (Fig. 2A). However, these symptoms did not spread out even if the incubation period was extended.

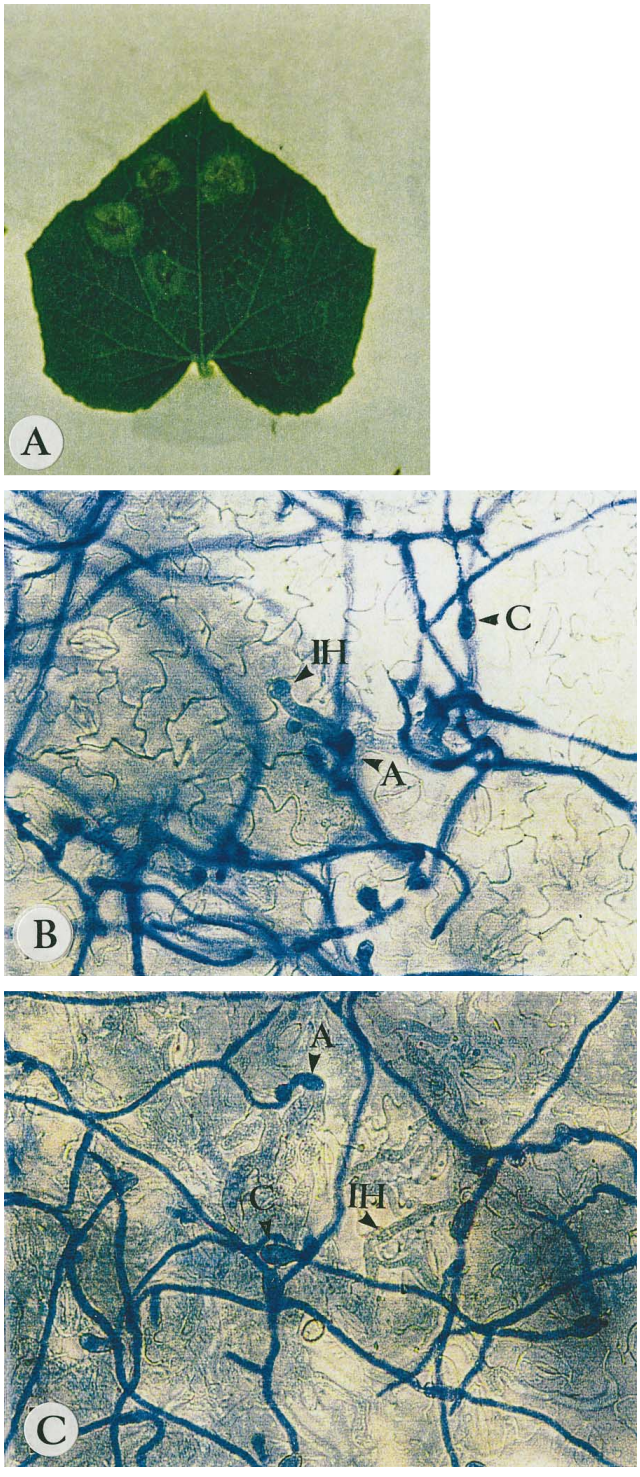

Fig. 2A–C Symptom on the transgenic cucumber plant (CR32) 4 days after inoculation of *B. cereria* (**A**) and infection behavior of *B. cinerea* on leaves of the CR32 (**B**) and non-transgenic cucumber plant (**C**). *Dark-blue lines* and *faint-blue lines* mean hyphae are elongated on the surface or the inside of the leaf, respectively. *IH* Infection hyphae; *A* appresorium; *C* conidia

Different mechanism for resistance against gray mold was postulated from our current results. In CR32, appressoria formation and penetration of hyphae into the leaves were observed; however, elongation of the penetrating hyphae was restricted to within the first two cells of the leaves. On the other hand, both appressoria formation and penetration of hyphae into leaves were restricted in CR33. This phenomenon has already been observed in transgenic tobacco plants in which the RCC2 gene has been introduced (Akutsu et al. submitted). The reason for this difference has not yet been clarified. The production level of RCC2 protein as detected by ELISA analysis was not remarkably different between CR32 and CR33. Antifungal substances, e.g. phytoalexin, which can be analyzed by thin-layer chromatography were not detected from both CR32 and CR33 strains under the present conditions (date not shown). Constitutive expression of chitinase might be responsible for the stimulation of the plant defense system. Although chitinase does not degrade the coat protein of virus, PR-3 protein (chitinase) can be induced by viral infection (Brederode et al. 1991).

Inheritance of the RCC2 gene and resistance against *B. cinerea* of the T_1 generation

In order to investigate the inheritance of resistance against *B. cinerea*, the progenies of the CR32 and CR33 strains were obtained by self-pollination. The progenies of CR32 were examined for the presence of the RCC2 gene by PCR-Southern hybridization and resistance against *B. cinerea* by artificial infection. It was confirmed that 50 of 68 plants exhibited resistance against gray mold. These progenies were found to harbor the RCC2 gene (Fig. 3). The segregation of disease resistance among the progenies was in accordance with the predicted Mendelian ratio of 3:1 (resistant:susceptible), as tested by χ^2 analysis ($\chi^2=0.078$, $P<1\%$). Moreover, the integrated RCC2 gene was also confirmed in 7 of 13 progenies of CR33, exhibited as resistance against *B. cinerea* (χ^2 analysis has not been done yet due to the insufficient number of progenies). These results proved that the RCC2 gene in transgenic cucumber plants,

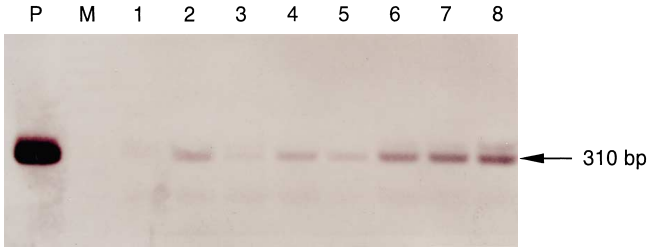

Fig. 3 Detection of the rice chitinase gene (RCC2) integrated in T_1 generation of CR32 by PCR-Southern hybridization. *Lanes 1–8* Progenies of CR32, *lane P* plasmid containing RCC2 gene, *lane M* X174/*Hae*III

CR32 and CR33 strains, was transmitted to their progenies together with the disease resistance against gray mold.

Efficient transformation systems for cucumber

We have produced many putative transgenic cucumber plantlets that kept their green color and grew normally on the MS medium containing a selective antibiotic (100 mg/l kanamycin). Our results demonstrated that all of the green adventitious shoots examined were transformants.

In this regeneration system, 1-day-old cotyledons as explants and a novel shoot induction medium (MS medium supplemented with 2 mg/l BA, 1 mg/l ABA, 30 g/l sucrose and 2.5 g/l gelrite) were used. We found that the addition of ABA into shoot induction medium increased the efficiency of shoot organogenesis and induced multiple shoots (Tabei, in preparation). We believe that this efficient shoot regeneration system will contribute to the production of many transgenic cucumber plants.

Previous reports on transformation of cucumber (Trulson et al. 1986; Chee 1990; Sarmento et al. 1992; Tabei et al. 1994) described only the introduction of an antibiotic resistant gene and/or a β-glucuronidase gene as marker genes. In this study, we have been successful in producing novel transgenic cucumber strains that integrated a useful gene for improving characteristics useful in agriculture.

Three transgenic cucumber strains, CR29, CR32 and CR33, harboring the rice chitinase gene (RCC2) exhibited enhanced resistance against gray mold. The resistance was stably inherited in the progenies of CR32 and CR33. Since breeding materials for disease resistance against gray mold have not been found in genetic resources of cucumber, these transgenic cucumber plants are expected to serve as new breeding materials for the disease resistance.

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