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Potentiation of Mycovirus Transmission by Zinc Compounds via Attenuation of Heterogenic Incompatibility in *Rosellinia necatrix*

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Heterogenic incompatibility is considered a defense mechanism against deleterious intruders such as mycovirus. *Rosellinia necatrix* shows strong heterogenic incompatibility. In the heterogenic incompatibility reaction, the approaching hyphae hardly anastomosed, a distinctive barrage line formed, and green fluorescent protein (GFP)-labeled hyphae quickly lost their fluorescence when encountering incompatible hyphae. In this study, transmission of a hypovirulence-conferring mycovirus to strains with different genetic backgrounds was attempted. Various chemical reagents considered to affect the programmed cell death pathway or cell wall modification were examined. Treatment with zinc compounds was shown to aid in transmission of mycoviruses to strains with different genetic backgrounds. In incompatible pairings, treatment with zinc compounds accelerated hyphal anastomosis; moreover, cytosolic GFP was transmitted to the newly joined hyphae. These results suggest that zinc compounds not only increase hyphal anastomosis but also attenuate heterogenic incompatibility.

The ascomycete fungus *Rosellinia necatrix* Prillieux causes severe root rot in fruit trees (1). Chemical disease control is generally used for disease management; however, this approach involves various problems, including effectiveness, cost, laborious treatment, and environmental contamination. "Virocontrol," which is a virological control mechanism based on a hypovirulence-conferring mycovirus, may reduce the pathogenicity of the fungal pathogen (2, 3). However, in most filamentous fungi, the heterogenic incompatibility system (i.e., the system that rejects genetically incompatible hyphae) prevents mycoviruses and malignant mitochondria from spreading among different fungal strains (4, 5). When the hyphal cell encounters nonself hyphal cells, programmed cell death (PCD) is triggered (4, 5). Therefore, for successful introduction of mycoviruses into a given fungal strain, suppression of the heterogenic incompatibility reaction is essential.

The strength of heterogenic incompatibility is variable in fungal species. When an individual mycelium encounters mycelia belonging to the same species, the mycelia attract each other (6) and try to fuse by anastomosis (5, 7). Although this phenomenon is commonplace in filamentous fungi, an absence of hyphal anastomosis has also been reported in some fungi (8, 9, 10, 11). For *R. necatrix*, an ultrastructural study revealed that heterogenic incompatibility occurred without anastomosis, and the mechanism involved was shown to be a novel vacuole-mediated type of PCD (12).

In *Cryphonectria parasitica*, mycovirus could be transmitted even in some incompatible combinations (13, 14, 15), which suggests that the timing or strength of PCD was different for different combinations of *vic* (vegetative incompatible) genes; therefore, mycoviruses may occasionally escape from the barrier system. However, this fungal system frequently anastomoses in incompatible combinations (13). In contrast, *R. necatrix* showed no opportunity for transmission of mycoviruses because the incompatible hyphae hardly anastomosed (12). Although heterogenic incompatibility genes in *R. necatrix* were not characterized, these genes seem to be variable because of the following observations. Numerous *R. necatrix* isolates belonging to different mycelial compatibility groups (MCGs) have been isolated previously (16). Moreover, ascospore isolates were obtained from a single ascus, and most ascospore isolates were shown to produce a barrage line paired with sibling ascospore isolates (17). Pyramiding with heterogenic incompatibility genes may produce a strong incompatibility reaction.

To accomplish virocontrol, a mycovirus transmission method that is functional even under conditions of strong incompatibility should be developed. Various techniques for artificial transmission of mycoviruses were used successfully, such as transformation with infectious cDNA clones of hypoviruses (18), transfection with synthetic transcripts derived from them (19), introduction of purified virus particles into protoplasts (20, 21, 22, 23), and protoplast fusion (24). These methods were not applicable for some fungi that were tolerant to cell wall digestive enzymes or for some mycoviruses that do not form virus particles. Attenuation of heterogenic incompatibility is promising as an alternative method to transmit mycovirus.

In this study, we screened diverse potential inhibitors of PCD and cell wall modification for suppressors of heterogenic incompatibility that lead to horizontal virus transmission. Furthermore, the effects of zinc compounds on heterogenic incompatibility were investigated by cytological analyses.

MATERIALS AND METHODS

Fungal isolates. The *R. necatrix* strains used were w97 (MCG 80; MAFF [Ministry of Agriculture, Forestry and Fisheries] gene bank no. 625116),

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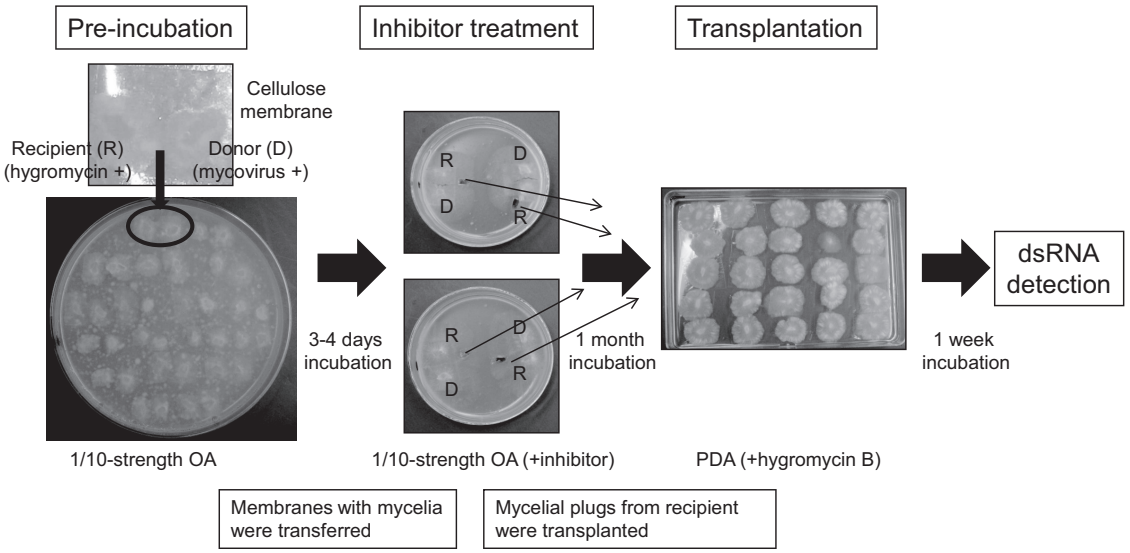

FIG 1 Experimental scheme for mycovirus transmission using inhibitors of heterogenic incompatibility. Donor and recipient strains were paired on a cellulose membrane and incubated for 3 to 4 days just before the mycelia contacted. Membranes with mycelia were then transferred into inhibitor-containing media and incubated for 1 month. Finally, mycelial plugs from the recipient strain that was the most adjacent to the donor strain were transplanted to hygromycin B-containing potato dextrose agar (PDA). Regrown colonies were tested for double-stranded RNA.

w370T1 (MCG 139; MAFF no. 645021), w779 (MCG 351; MAFF no. 328195), and w780 (MCG 351; same clone as w779). Hygromycin B-resistant strains transformed with pCPXHY1eGFP, w370T1-hyg, and w97-hyg were obtained from S. Kanematsu et al. (25). All isolates were self-compatible but were incompatible with the hyphae of other strains. Cultures were maintained in petri dishes on oatmeal agar (OMA) (26 g oatmeal, 5 g sucrose, and 15 g agar per liter) at 4°C until use.

Inhibitor treatment to transmit RnMBV1. Chemical reagents and enzymes used in this study are listed in Table S1 in the supplemental material. The concentration of each inhibitor was determined from the literature, and 10- and 100-fold dilutions were prepared. The experimental scheme is described in Fig. 1. We generated 1/10-strength oatmeal agar plates (2.6 g oatmeal, 5 g sucrose, and 15 g agar per liter) supplemented with different concentrations of the inhibitor. *R. necatrix* strains w779

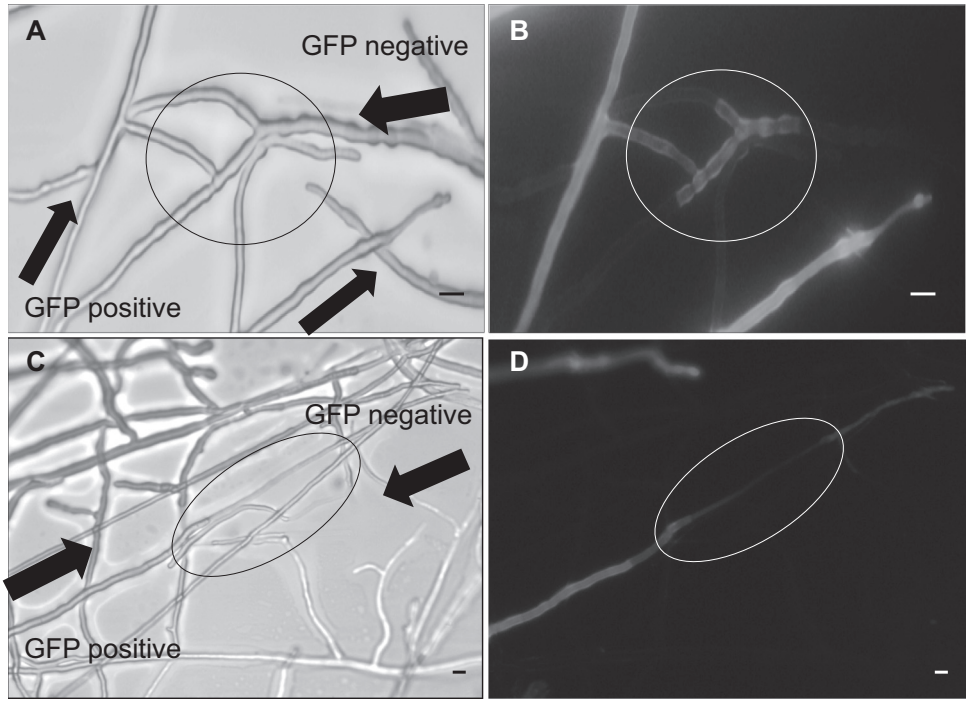

FIG 2 GFP fluorescence during hyphal contact of compatible and incompatible combinations. (A and B) Compatible combination (w97-hyg); (C and D) incompatible combination (w97-hyg versus w779). Shown are bright-field (A and C) and fluorescent-field (B and D) images. Circles designate hyphal contact areas. Bars = 10 μm.

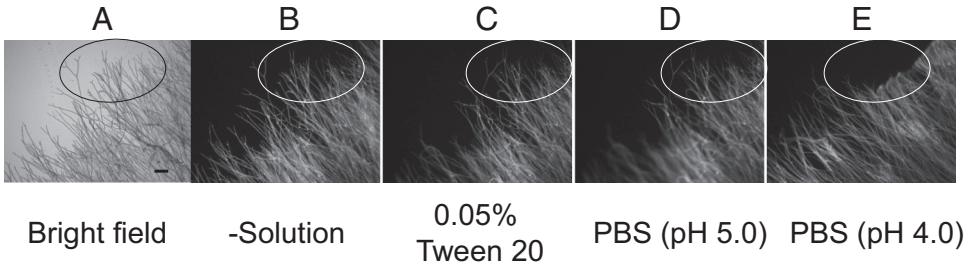

FIG 3 Effect of pH on cytosolic GFP fluorescence in *Rosellinia necatrix*. Each solution was dropped onto the circle, and the solution was exchanged with another solution (the exchange order was control [no solution], 0.05% [vol/vol] Tween 20, PBS [pH 5.0], and PBS [pH 4.0]). (A) Bright field; (B to E) fluorescence field. Circles designate areas where solutions were dropped. Bars = 50 μm.

(carrying *R. necatrix* megabirnavirus 1 [RnMBV1], which is a hypovirulence-conferring factor) (26) and w780 (carrying partitivirus) were used as donor strains, and hygromycin B-resistant strains w370T1-hyg and w97-hyg, which had a different MCG from that of the donor strains, were used as recipient strains. Mycelium plugs (2-mm square) of donor and recipient strains were planted onto cellulose membranes (1.5-cm square) placed 1.0 cm apart on the 1/10-strength oatmeal agar plate. When the donor and recipient strains were close to contact after 3 to 4 days of incubation, the membranes with mycelia were transferred onto the inhibitor-containing 1/10-strength oatmeal agar plate. After 3 to 4 weeks of additional incubation, the recipient mycelial plugs (2-mm square) that were closest to the donor colony were transplanted onto cellulose membranes (1.5-cm square) spread onto hygromycin B (100 μg/ml)-containing potato dextrose agar plates to detect double-stranded RNA (dsRNA).

Detection of dsRNA. Successful transmission of mycovirus was confirmed by detection of dsRNA. The mycelium on the cellulose membrane was placed into an extraction tube set (lysing matrix set A; MP Biomedicals, Santa Ana, CA) with 1 ml of nucleic acid extraction buffer (17), and dsRNA was extracted by using a FastPrep 24 instrument (MP Biomedicals). The extract was centrifuged (10,000 × *g*), and the supernatant was transferred. The supernatant was supplemented with an equal volume of 2-propanol and centrifuged (12,000 × *g*). The supernatant was discarded, and the pellet was washed with 70% (vol/vol) ethanol, dried, and dissolved in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The extracts were separated by electrophoresis on a 1.0% agarose gel in TAE buffer (40 mM Tris-acetate [pH 8.0], 1 mM EDTA). Using this protocol, all types of nucleic acid were extracted. To confirm the distinct dsRNA bands indicating transmission of the mycovirus, the obtained extracts were verified with the donor extracts.

Genomic DNA extraction and genotype identification. Genomic DNA was extracted according to the method of Ikeda et al. (17). To exclude contamination with donor mycelia during dsRNA extraction, universally primed PCR (UP-PCR) was performed to detect DNA polymorphisms (17, 27–29) by using primer AS4 (5′-TGTGGGCGCTCGACAG). PCR products were separated by electrophoresis on a 1.0% agarose gel in TAE buffer.

Light and fluorescence microscopy. The observation of hyphal contact was described previously (12). *R. necatrix* strains were paired on cellulose membranes (1.5-cm square) laid onto 1/10-strength OMA plates with or without zinc compounds (0.5 to 1 mM $ZnCl_2$ and $ZnSO_4$) in 50-mm-diameter petri dishes (AS One, Osaka, Japan). One mycelial plug was placed on one side, and the confronting mycelial plug was placed on the opposite side; the plugs were separated by 5 to 10 mm. After incubation for 3 days at 25°C, the cellulose membranes were transferred into glass-bottomed culture dishes (diameter, 50 mm; MatTek, Ashland, MA, USA) that contained no growth medium; the hyphal contact zones were observed by using a fluorescence microscope (Biorevo BZ-9000; Keyence, Osaka, Japan). To evaluate the nature of the hyphal contact, hyphae that were in contact and for which both the tips were visible in the field of view of a 40× objective lens were identified. Hyphal fusion was evaluated on the basis of whether the cell walls had merged. Furthermore, the hyphae were tracked backwards from the zone of contact to identify the origin of

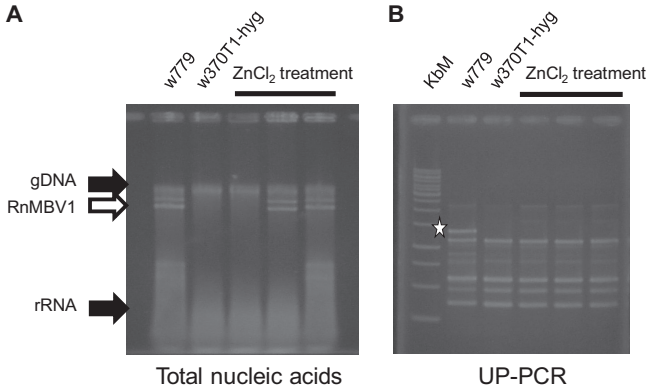

FIG 4 Zinc chloride treatment permitted transmission of *Rosellinia necatrix* megabirnavirus 1 (RnMBV1). (A) Total nucleic acids of *Rosellinia necatrix* isolates w779 (donor isolate) and w370T1-hyg (recipient) and zinc chloride-treated isolates. Common ethidium bromide-positive signals can be observed (black arrows), which represent genomic DNA (gDNA) and rRNA. Two fragments of the genome of RnMBV1 were detected in donor and zinc chloride-treated isolates (open arrow). (B) Universally primed PCR (UP-PCR) to detect DNA polymorphisms with primer AS4. The star designates the donor-specific fragment. KbM, kilobase marker (GeneRuler DNA ladder).

TABLE 1 Transmission rates of mycoviruses following treatment with zinc compounds[a]

Recipient strain and treatment	Concn (mM)	Mycovirus transmission rate (no. of transmissions/no. of trials) for indicated donor strain	
		w779 (RnMBV1)	w780 (partitivirus)
w370T1-hyg			
Control	0	0/30	0/15
$ZnCl_2$	0.1	0/15	0/13
	0.25	0/14	0/15
	0.5	1/10	2/12
	0.75	3/21	9/21
	1.5	5/29	10/12
$ZnSO_4$	1	4/24	NT
w97-hyg			
Control	0	NT	0/15
$ZnCl_2$	1	NT	8/39

[a] Mycovirus transmission rates were determined by the number of successful mycovirus transmissions per mycelial pairing. NT, not tested.

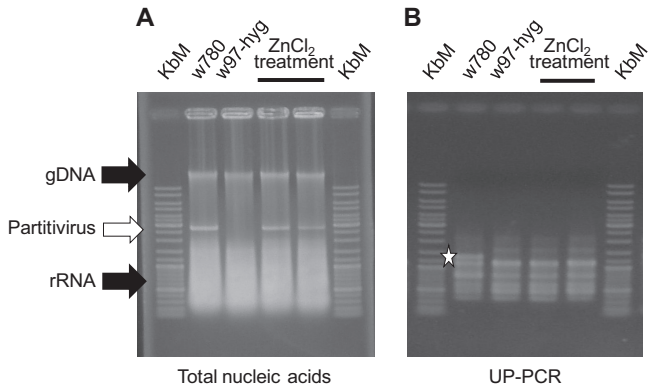

FIG 5 Zinc chloride treatment permitted transmission of *Rosellinia* partitivirus-like dsRNA. (A) Total nucleic acids of *Rosellinia necatrix* w780 (donor strain), w97-hyg (recipient strain), and zinc chloride-treated isolates. Common ethidium bromide-positive signals can be observed (black arrows), which represent genomic DNA (gDNA) and rRNA. The genome of *Rosellinia* partitivirus-like dsRNA was detected in donor and zinc chloride-treated isolates (open arrow). (B) UP-PCR to detect DNA polymorphisms with primer AS4. The star designates the donor-specific fragment. KbM, kilobase marker (GeneRuler DNA ladder).

each hypha (i.e., the isolate that produced the hypha) to confirm that the hyphae represented reciprocal pairs rather than self-pairs. Because some hyphae also extended vertically, hyphal crossings in which one hypha passed over another hypha without hyphal contact were excluded; such hyphal crossings were judged to occur when it was not possible to view both hyphae simultaneously in the same focal plane. The proportions of hyphal contact patterns were calculated by using observations of more than 50 hyphal contact zones in triplicate.

TEM observation. *R. necatrix* strains (w97 and w779) were paired on a cellulose membrane (Rengo, Tokyo, Japan) laid onto 1/10-strength OMA medium for 3 days. Membranes with mycelia were transferred onto a glass slide overlaid with 1/10-strength OMA with or without zinc compounds and incubated for 1 day at 25°C in 90-mm-diameter petri dishes that contained agar medium (15 g/liter agar) to retain moisture. Specimen preparation and ultrathin sectioning for transmission electron microscopy (TEM) were performed according to the method of Inoue et al. (12). The sections were observed by using a JEM-1400 electron microscope (JEOL, Akishima, Japan) at an accelerating voltage of 80 kV. When collapse of some cell structures at the hyphal contact zones was observed, the parts of the cell contents that had collapsed in each interaction zone were scored. The proportions of the collapsed cell components were calculated by using observations of more than 30 hyphal contact zones in triplicate.

RESULTS

Visualization of cytosol during heterogenic incompatibility using a GFP-expressing strain of *R. necatrix*. Heterogenic incompatibility in *R. necatrix* results in a lack of anastomosis (12). We evaluated the dynamics of the cytosol during heterogenic incompatibility by using a green fluorescent protein (GFP)-expressing strain. In the compatible combination between the wild-type strain and the GFP-expressing strain, the contacted hyphae were anastomosed, and the cytosolic GFP was transmitted to the wild-type strain (Fig. 2A and B). On the contrary, in the incompatible combination, disappearance of cytosolic GFP fluorescence was observed at the hyphal contact zone (Fig. 2C and D). GFP fluorescence is sensitive to acidic conditions (30). Heterogenic incompatibility results from degeneration of the vacuolar membrane have been reported previously (12). Therefore, collapse of vacuoles was expected to lead to cytosolic acidification. Because it was difficult to determine the pH value in the cytosol, the effect of an exogenous pH shift on cytosolic GFP fluorescence in *R. necatrix* was investigated. Treatment with phosphate-buffered saline

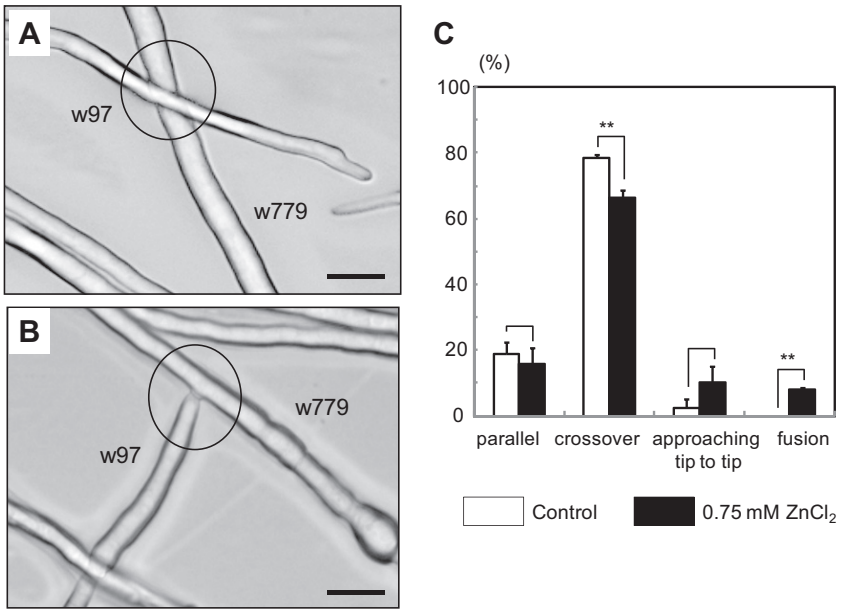

FIG 6 Effect of zinc chloride on hyphal contact in *Rosellinia necatrix*. (A) Hyphal contact for an incompatible combination (w97 versus w779) on 1/10-strength oatmeal agar medium. The circle indicates the hyphal contact zone (crossover). Bar = 10 μm. (B) Hyphal contact for an incompatible combination (w97 versus w779) on 1/10-strength oatmeal agar medium containing 0.75 mM zinc chloride. The circle indicates the hyphal contact zone (fusion). Bar = 10 μm. (C) Frequency of hyphal contact with or without zinc chloride. The nature of the hyphal contact was categorized into parallel, crossover, approaching tip to tip, and fusion (anastomosis). Error bars indicate standard deviations. **, significant difference ($P < 0.01$).

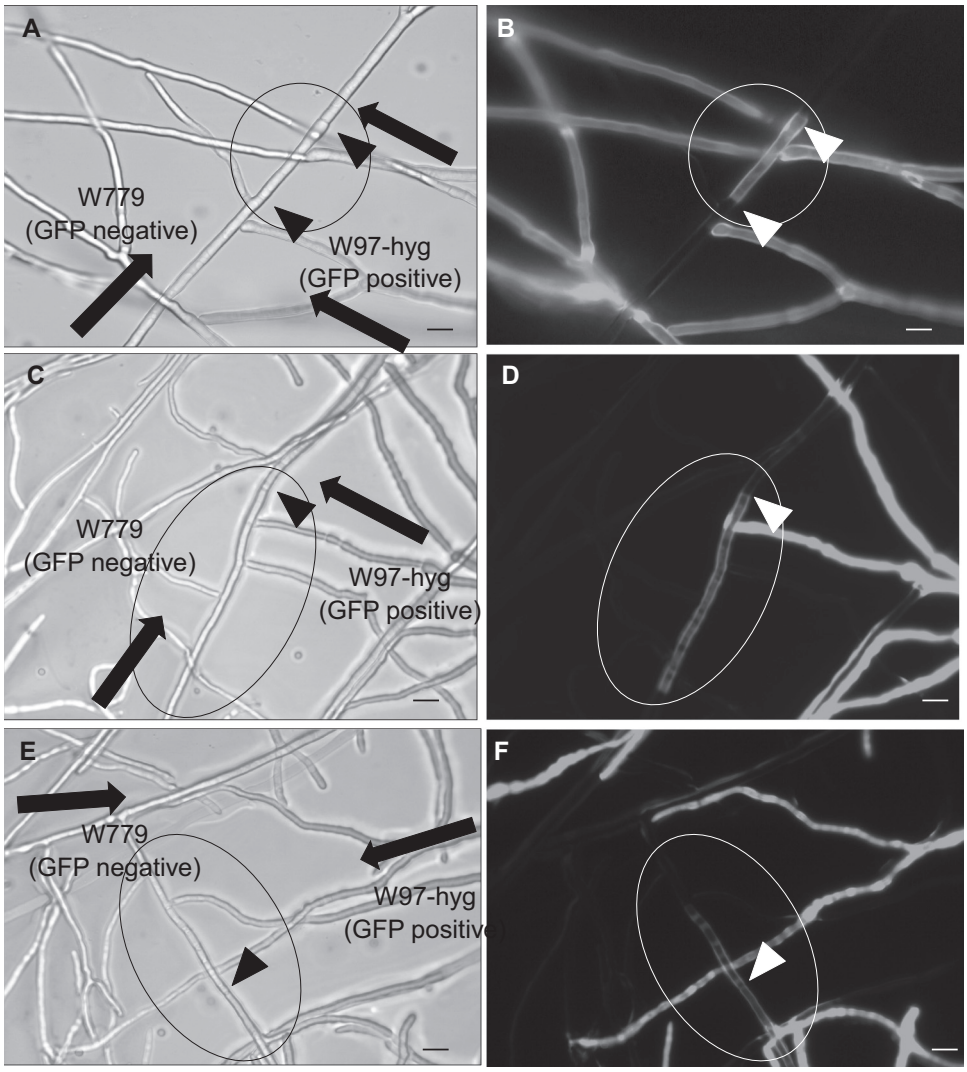

FIG 7 Transmission of cytosolic GFP to the opposite hyphae upon treatment with zinc chloride. (A, C, and E) Bright field. (B, D, and F) Fluorescence field. Circles indicate cytoplasmic GFP that was transmitted to the opposite hyphae. Arrowheads indicate hyphal septa. Bars = 10 μm.

(PBS) (pH 4.0) was found to abolish cytosolic GFP fluorescence (Fig. 3).

Inhibitor treatments to transmit RnMBV1. Eighty-seven types of inhibitors, listed in Table S1 in the supplemental material, were applied. For induction of anastomosis, cell wall-modifying reagents and cell wall-degrading enzymes were administered. For suppression of PCD, inhibitors involved in proteolytic pathways, phosphorylation signaling, and reactive oxygen species (ROS) signaling were used. Most of the inhibitors prevented transmission of RnMBV1. Only zinc chloride and pronase E treatments allowed transmission of RnMBV1 in the first screening. The zinc chloride and pronase E treatments were retested in a second screening. The effect of pronase E treatment (data not shown) could not be reproduced. However, in the case of treatment with 1.5 mM zinc chloride, RnMBV1 was found to be transmitted at a frequency of 15.1% (Fig. 4; see also Table S1 in the supplemental material). DNA polymorphism analysis revealed that the isolates containing the transmitted RnMBV1 were not contamination of the donor strain w779 (Fig. 4).

Application of zinc compounds for mycovirus transmission. The concentration of zinc chloride was evaluated. High concentrations (more than 2 mM zinc chloride) prevented growth on media (data not shown). Thus, the effective concentration of zinc chloride for RnMBV1 transmission ranged from 0.5 mM to 1.5 mM (Table 1).

Next, the efficacy of zinc as an ion or in a compound, i.e., in zinc chloride, was analyzed. Treatment with zinc vitriol aided in the transmission of RnMBV1 (Table 1), suggesting that zinc compounds are effective for mycovirus transmission.

Additionally, different combinations of mycovirus species and strains (different MCGs) were evaluated. Strain w780 was used as a donor that contained partitivirus-like dsRNA with a high level of similarity to RnPV3 (31) (ca. 70% in the RNA-dependent RNA polymerase [RdRP] nucleotide sequence). The partitivirus appeared to be frequently transmitted upon treatment with zinc chloride. Especially for the treatment with 1.5 mM zinc chloride, 83.3% of pairings demonstrated transmission of partitivirus (Fig. 5 and Table 1). Next, w97-hyg (MCG 80) was used as a

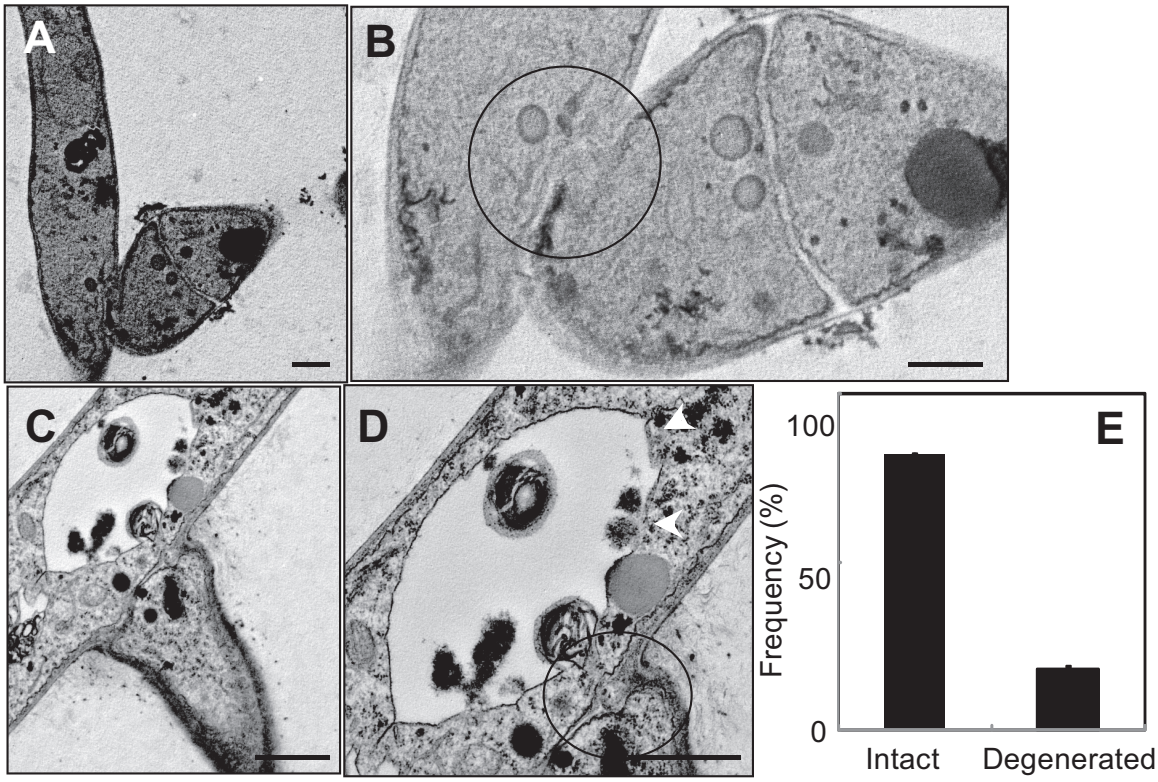

FIG 8 Ultrastructure of hyphal contact zone between w97 and w779 treated with zinc chloride. (A to D) The hyphae that came in contact were anastomosed (circles). Panels B and D show enlarged images of panels A and C, respectively. (A and B) Intact anastomosed hyphae; (C and D) anastomosed hyphae degenerating at the vacuolar membrane. Arrowheads indicate a degenerated vacuolar membrane. Bars = 0.5 μm. (E) Frequency of vacuolar membrane degeneration in the hyphal anastomosed area. Error bars indicate standard deviations from 3 independent observations.

recipient, and w780 was used as a donor. In this analysis, 20.5% of pairings transmitted partitivirus, which was a lower frequency than that observed for the pairing between w370T1-hyg and w780 (Table 1). The isolates that received transmission of RnMBV1 in the present study showed significantly reduced virulence (data not shown).

Transmission of cytosolic GFP to the opposite hyphae. Hyphal contact was observed in the incompatible combinations with or without 0.75 mM zinc chloride (Fig. 6). No anastomosis was observed in the hyphal contact zones in the incompatible combinations (w97 versus w779) (Fig. 6C). In contrast, hyphal anastomosis increased with treatment with zinc chloride (Fig. 6C). GFP fluorescence was detected in pairings of GFP-positive and GFP-negative strains. Cytosolic GFP was found to be transmitted to the opposite GFP-negative strain (Fig. 7). Although most GFP transmission was restricted to the first septa, ca. 10% ($n = 158$ fusion sites) of contacting hyphae extended the first septa (Fig. 7E and F).

Ultrastructure of hyphal contact zone. TEM observation of the compatible combinations revealed that hyphal anastomosis, cell wall fusion, and reconstruction of septa had occurred and that Woronin bodies accumulated at the sites of hyphal anastomosis (12). In the incompatible combinations, disconnection of membranes (i.e., plasma membrane, tonoplast, nuclear membrane, and mitochondrial membrane) without hyphal anastomosis was observed (12). In the zinc chloride treatment in the incompatible combination, the hyphae were found to be fused with each other, and cytosolic contents seemed to be transmitted to the opposite hyphal cell (Fig. 8). Cells with fused hyphae were detected with the zinc chloride treatment. Furthermore, parts of vacuole membranes were found to be disconnected, which suggests that the anastomosis of hypha eventually results in death (Fig. 8D and E).

DISCUSSION

Heterogenic incompatibility is considered to prevent the spread of mycoviruses and malignant mitochondria among different fungal strains (4, 5). Some minor vegetative incompatibility genes are not involved in this mycovirus defense mechanism (13, 14, 15). In *R. necatrix*, the heterogenic incompatibility reaction was drastic, and therefore, hyphal anastomosis rarely occurred in the incompatible interaction. Therefore, transmission of the mycovirus to strains with a different genetic background was difficult.

In this study, suppression of PCD mediated by heterogenic incompatibility was attempted by using inhibitor treatments. If the heterogenic incompatibility in *R. necatrix* induced typical PCD mechanisms such as apoptosis or autophagy, well-known inhibitors could have been used to suppress the cell death. However, ultrastructural analysis revealed that the heterogenic incompatibility in *R. necatrix* was a novel type of PCD that involves vacuolar membrane degeneration (12). Therefore, various types of inhibitors were tested for the transmission of mycovirus.

Zinc chloride treatment was found to aid in transmission of the mycovirus. Zinc chloride was effective for different mycovirus species and different MCG combinations, suggesting that zinc chloride treatment can be widely applied for other fungus/myco-

virus systems. Moreover, RnMBV1 was also transmitted in the treatment with zinc vitriol, which suggests that the zinc ion was the active substance for mycovirus transmission.

Zinc ions are known to inhibit apoptosis by targeting caspase-3 activation in mammalian cells (32, 33). However, heterogenic incompatibility in *R. necatrix* did not show any hallmarks of apoptosis; i.e., genomic DNA fragmentation, mitochondrial swelling, or cytoplasmic vacuolization was not observed (12). Therefore, the effect of zinc chloride on heterogenic incompatibility was evaluated. Zinc chloride treatment was found to increase hyphal anastomosis and attenuate PCD. Hyphal anastomosis is essential for transmission of the mycovirus. However, it was unclear how zinc chloride treatment increased hyphal anastomosis. In *R. necatrix*, hyphal anastomosis frequently occurred in compatible combinations (12). Therefore, in incompatible combinations, some secretion substance(s) would suppress hyphal anastomosis. In addition, zinc chloride was found to potentiate transfer of cytosolic GFP to the opposite hypha via anastomosis. This finding was conclusive in demonstrating the success of mycovirus transmission. Under normal culture conditions (without zinc chloride), disappearance of cytosolic GFP exclusively at the hyphal contact zone in incompatible combinations was determined. This finding suggests that incompatible hyphae immediately underwent PCD without any opportunity to transmit the mycovirus to the opposite hypha. The disappearance of GFP may be explained by cytosolic acidification through vacuolar collapse. Degeneration of the vacuolar membrane was found to be critical for heterogenic incompatibility in *R. necatrix* (12). Thus, zinc ions may protect against vacuolar degeneration by inhibiting proteolytic enzymes.

Furthermore, the frequency of mycovirus transmission upon treatment with zinc chloride was found to vary among mycovirus species. A partitivirus was more efficiently transmitted to the recipient than RnMBV1. This difference was specific in the incompatible combination because the transmission efficiency of RnMBV1 was comparable to that of RnPV1 in the compatible combination (34). The partitivirus family has the smallest coat protein molecule among dsRNA viruses and shows a different protein organization from that of other dsRNA viruses (35). These structural differences of the virion may explain the differences in the frequency of mycovirus transmission.

The finding that most of the transmitted cytosolic GFPs were restricted to the first septa suggested that the hyphal cell was compartmentalized by septal plugging (36), and mycovirus transmission frequency would be decreased. In this study, however, cytosolic GFP was infrequently transmitted via extended septa. In this situation, the mycovirus would transmit via extended septa, and the recipient strain would establish as a mycovirus-infected strain.

The present results show that treatment with zinc ions increased anastomosis, suppressed heterogenic incompatibility, and subsequently permitted transmission of mycovirus. This is a novel approach to transmit mycovirus in fungal species. Whether the zinc chloride treatment targeted the common machinery for induction of anastomosis and suppression of heterogenic incompatibility remains undetermined. Future studies are required to determine the target of zinc ions in suppressing heterogenic incompatibility in filamentous fungi.

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