A Polysaccharide Derived from a *Trichosporon* sp. Culture Strongly Primes Plant Resistance to Viruses

Yi-Shu Chiu,¹ Pi-Yu Chen,¹ Tung Kuan,² Po-Chuan Wang,^{1,2} Ying-Ju Chen,^{1,2} Yu-Liang Yang,^{1,†} and Hsin-Hung Yeh^{1,2,†}

¹Agricultural Biotechnology Research Center, Academia Sinica, Taipei 11529, Taiwan; and ²Department of Plant Pathology and Microbiology, National Taiwan University, Taipei 10617, Taiwan

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Plant viruses cause devastating diseases in plants, yet no effective viricide is available for agricultural application. We screened cultured filtrates derived from various soil microorganisms cultured in vegetable broth that enhanced plant viral resistance. A cultured filtrate, designated F8 culture filtrate, derived from a fungus belonging to the genus *Trichosporon*, induced strong resistance to various viruses on different plants. Our inoculation assay found the infection rate of *Tobacco mosaic virus* (TMV)-inoculated *Nicotiana benthamiana* with F8 culture filtrate pretreatment may decrease to 0%, whereas salicylic acid (SA)-pretreated *N. benthamiana* attenuated TMV-caused symptoms but remained 100% infected. Tracking *Tobacco mosaic virus* tagged with green fluorescence protein in plants revealed pretreatment with F8 culture filtrate affected the initial establishment of the virus infection. From F8 culture filtrate, we identified a previously unknown polysaccharide composed of D-mannose, D-galactose, and D-glucose in the ratio 1.0:1.2:10.0 with a α -D-1,4-glucan linkage to be responsible for the induction of plant resistance against viruses through priming of SA-governed immune-responsive genes. Notably, F8 culture filtrate only triggered local defense but was much more effective than conventional SA-mediated systematic acquired resistance. Our finding revealed that microbial cultured metabolites provided a rich source for identification of potent elicitors in plant defense.

Viruses cause serious diseases in crops, and the lost yield is estimated to total more than \$30 billion annually (Sastry and Zitter 2014). However, because viruses are intracellular parasites, cost-effectively eliminating viruses from infected plants is difficult. Currently, no curative viricides are available for plant viral disease management. Thus, unlike other pests, if viruses can evade preventive control measures and infect plants, roguing

is the main option for farmers to prevent further spread of viral disease. Traditional resistance breeding, use of pesticides to control viral vectors, and transgenic approaches to control viral diseases in crops often come at the cost of time, compromised flavor, hazardous concerns to the environment and health, as well as issues associated with genetically modified foods (Herring 2008; Galvez et al. 2014). One way to alleviate this problem is to induce a plant immune response to defend against virus infection.

Plants protect against pest attack by structural barriers or preexisting pest-toxic chemicals (Dangl and Jones 2001). In addition, plants have evolved a complex immune response that can ward off attack by most microbes. Plants can recognize microbe- and pathogen-conserved molecular structures termed microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) and induce an immune response (Chisholm et al. 2006; Göhre and Robatzek 2008; Jones and Dangl 2006). An example of MAMPs/PAMPs are bacterial flagellin, chitin, or different glucans that constitute fungal and oomycete cell walls (Boller and Felix 2009; Newman et al. 2013). In addition to MAMPs/PAMPs, host-derived molecules may be generated during attacks by microbes or pathogens through lytic enzymes or mechanical forces. Some of these molecules may serve as elicitors of a plant defense response (Boller and Felix 2009; Dodds and Rathjen 2010). These molecules are termed danger- or damage-associated molecular patterns (DAMPs) (Boller and Felix 2009; Dodds and Rathjen 2010). MAMPs and DAMPs are perceived by membrane-localized pattern recognition receptors and induce a diverse array of defense responses commonly referred to as pattern-triggered immunity (PTI) (Boller and Felix 2009; Jones and Dangl 2006). PTI can defend against most pathogen infection; however, some plant pathogens have evolved effectors that are injected into the host cells to compromise PTI (Jones and Dangl 2006). To defend against pathogens armed with an effector, plants have evolved resistance proteins to detect these effectors and induce a stronger defense response, termed effector-triggered immunity (ETI) (Jones and Dangl 2006). ETI usually triggers programmed cell death at the site of infection to prevent further spreading of invasive pathogens. Both PTI and ETI are coordinated by phytohormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (Glazebrook 2005; Pieterse et al. 2012). The coordination between these hormones activates a specific immune response under PTI and ETI to protect against a particular pathogen (Robert-Seilanian et al. 2011; Pieterse et al. 2012). In general, the SA- and JA/ET-dependent immune responses are effective against biotrophic and necrotrophic pathogens, respectively (Glazebrook 2005).

In plants, local infection may also induce a systemic plant immune response. Two types of induced systemic immune

Yi-Shu Chiu and Pi-Yu Chen contributed equally to this work.

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[†]Corresponding authors: Hsin-Hung Yeh; E-mail: hyeh@sinica.edu.tw and Yu-Liang Yang; E-mail: ylyang@gate.sinica.edu.tw

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responses, systemic acquired response (SAR) and induced systemic response (ISR), have been reported in plants (Pieterse et al. 2014). ISR is induced by plant growth-promoting rhizobacteria and depends on JA and ET (Conrath 2011; Pieterse et al. 2014). The onset of SAR depends on SA and SAR is efficient against a broad spectrum of pathogens, including viruses (Gozzo and Faoro 2013). SAR is usually induced by pathogens that cause a necrotic lesion resulting from a hypersensitive response during an incompatible interaction or cell death with a compatible interaction (Durrant and Dong 2004; Hammerschmidt 1999).

The systemic immune response is usually associated with a primed state, whereby plants respond either faster, more strongly, or both in activating defense responses when subsequently challenged by pathogens (Conrath et al. 2015; Mauch-Mani et al. 2017). Elevated levels of pattern recognition receptors, dormant signaling enzymes, transcription factors, and alterations in chromatin state have been suggested to provide plants with a memory to establish a defense primed state (Conrath et al. 2015; Mauch-Mani et al. 2017). The immune priming allows plants to better defend against pathogen invasion with lower resistance-associated fitness cost (Conrath et al. 2015; Mauch-Mani et al. 2017; van Hulten et al. 2006; Wang et al. 2015).

Viruses usually trigger the SA-related plant defense response, and exogenous application of SA or its analogs, such as benzo-thiadiazol-7-carbothioic acid-*S*-methyl ester (BTH) and acibenzolar-*S*-methyl (a derivative of BTH), can trigger the SAR against virus infection (Bektas and Eulgem 2015). However, effectiveness and phytotoxicity hamper the application of SA or its analogs in the field (Alexandersson et al. 2016; Gozzo and Faoro 2013).

In addition to synthetic chemicals, natural PAMPs and DAMPs have been reported to trigger immune responses against virus infection (Wiesel et al. 2014). Of note, each PAMP or DAMP mounts a different plant immune response (Wiesel et al. 2014). Simultaneous application of two typical bacterial PAMPs, flag 22 and lipopolysaccharide, to *Arabidopsis*

thaliana induced a typical SAR response but without necrotic lesions (Mishina and Zeier 2007). Therefore, application of combined PAMPs may provide increased protection with less damage to plants.

In view of the diversity of microorganisms and plants, many PAMPs and DAMPs may still be unidentified. In this study, we screened culture filtrates derived from soil microorganisms (may contain PAMPs) cultured with medium made from weeds and readily obtainable vegetables (may contain DAMPs) to induce effective plant defense against viruses. We obtained a culture filtrate derived from a *Trichosporon* sp. (F8 culture filtrate) that is much better than SA in inducing plant resistance to different viruses but without a fitness cost. We identified the specific mode of action of the F8 culture filtrate, which induced an unusual priming of SA-governed response genes, as well as the active component of the F8 culture filtrate. Our finding revealed an effective way to induce plant resistance against viruses. Furthermore, our detailed analysis uncovered several unusual features that provide important knowledge for further application of induced resistance against virus infection.

RESULTS

Screening of culture filtrates derived from soil microorganisms for inducing plant resistance against Tobacco mosaic virus (TMV).

To identify PAMPs and DAMPs that can provide plant defense against viruses, we first collected soil samples at different locations and followed a previously reported method for microorganism isolation (Ko et al. 2010). We isolated 199 microorganisms, including 77 actinomycetes, 68 bacteria, and 54 fungi.

We selected TMV and its local lesion host, *Nicotiana glutinosa*, as a system for the initial screening. TMV can induce local lesions on *N. glutinosa*. The number and size of lesions

Fig. 1. Lesion number and symptoms induced by Tobacco mosaic virus (TMV) with F2 and F8 culture filtrate pretreatment. **A**, *Nicotiana glutinosa* was pretreated with H₂O, 5 mM salicylic acid (SA), vegetable broth (VB), or F2 and F8 culture filtrate three times, with a 24-h interval between each spray. Pretreated plants were inoculated with TMV at 24 h posttreatment (hpt) and untreated plants were inoculated with buffer (mock) for a control. Photos were taken at 5 days postinoculation (dpi). **B**, Lesion numbers induced by TMV recorded at 5 dpi. Data are means ± standard deviation from three plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance (ANOVA) with Tukey's posttest. Experiments were repeated three times with similar results. **C**, *N. benthamiana* was pretreated with H₂O, 5 mM SA, VB, F2, or F8 culture filtrate for a total of three times, with a 24-h interval between each spray. All pretreated plants were inoculated with TMV at 24 hpt. Photos of TMV-inoculated plants were taken at 7 dpi. **D**, Disease index levels induced by TMV were recorded at 7 dpi. Box plots represent medians, 25 to 75 percentiles (boxes), and minimum and maximum values (whiskers) from six plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way ANOVA with Tukey's posttest. Experiments were repeated three times with similar results.

reflects the degree of resistance to TMV (Loebenstein 2009). The soil microorganisms were cultured in vegetable broth (VB) for 14 days, then, culture filtrates were directly sprayed on *N. glutinosa* (0.4 ml per plant) for a total of three times, with a 24-h interval between each spray. Then, TMV inoculum was rubbed onto leaves of *N. glutinosa*. The lesion number was counted at 5 days postinoculation (dpi). Because VB may contain DAMPs that trigger plant immunity, the results are represented as percent lesion number induced by TMV on plants pretreated with culture filtrates from isolated microorganisms relative to VB-pretreated plants. Plants pretreated with culture filtrates from two fungi (F2 and F8) had fewer lesions than did VB-pretreated plants (Supplementary Fig. S1).

To verify the ability of F2- and F8 culture filtrate to induce resistance in *N. glutinosa*, we treated plants with H₂O, SA, VB, or F2 or F8 culture filtrate followed by TMV inoculation. All experiments were repeated at least three times, and the results were consistent, showing much-reduced lesion numbers on *N. glutinosa* pretreated with F2 and F8 culture filtrate (Fig. 1A and B).

F8 culture filtrate induces strong resistance in *N. benthamiana*.

Besides using *N. glutinosa* for our initial screening, we also used *N. benthamiana*, a host that is highly susceptible to TMV, for a resistance assay. *N. benthamiana* was pretreated with H₂O, SA, VB, or F2 or F8 culture filtrate as for pretreatment of *N. glutinosa*. All treated plants were inoculated with TMV inoculum made from lyophilized leaves of TMV-infected *N. benthamiana*. Plants pretreated with H₂O, SA, VB, and F2 culture filtrate showed severe symptoms; however, most plants with F8 culture filtrate pretreatment remained healthy-looking (Fig. 1C and D). With reverse transcription-polymerase chain reaction (RT-PCR) to detect TMV on all treated plants, the infection rate was much reduced with F8 culture filtrate as compared with H₂O, SA, VB, or F2 culture filtrate pretreatment (Table 1). In our repeat experiments, the infection rate of TMV-inoculated plants with F8 culture filtrate pretreatment even decreased to 0% (Table 1). In addition, plants with F8 culture filtrate pretreatment and TMV infection showed no obvious symptoms (Fig. 1D). We prepared at least seven different batches of F8 culture filtrate, and the ability to induce resistance against TMV was consistent between different batches (Table 1). Of note, F2 culture filtrate did not induce resistance to TMV in *N. benthamiana* (Table 1; Fig. 1C and D), so our later experiments focused on F8 culture filtrate.

In addition to using inoculum made from lyophilized leaves of TMV-infected *N. benthamiana*, we also used different concentrations (1, 10, and 50 µg/ml) of purified TMV particles as inoculum to inoculate *N. glutinosa* and *N. benthamiana* pretreated with H₂O, SA, VB, or F8 culture filtrate (Figs. 2 and 3). The results are consistent with previous results, which showed that TMV particle-inoculated *N. glutinosa* and *N. benthamiana*

showed fewer lesions and less disease severity in F8 culture filtrate-treated *N. glutinosa* and *N. benthamiana*, respectively (Figs. 2 and 3). The results also showed that increased TMV particle concentration positively correlated with increased lesion number and disease severity on leaves of F8 culture filtrate-treated *N. glutinosa*, and *N. benthamiana*, respectively. The infection rate of TMV on *N. benthamiana* was also detected by use of quantitative (q)RT-PCR (Table 2). Reduced infection rate was only observed on F8 culture filtrate-treated *N. benthamiana* when TMV particles at the concentration of 1 µg/ml was used as inoculum (Table 2). Collectively, the data indicated that F8 culture filtrate can induce plant resistance to TMV infection, and an increased concentration of TMV inoculum will increase the infectivity.

More resistance with F8 culture filtrate than F8 culture pellets.

To investigate whether pellets of F8 culture also induce plant resistance against TMV, pellets of F8 culture were resuspended with H₂O (same volume as the original F8 culture). The resuspended pellet was squeezed twice in a high-pressure homogenizer to break down the fungi cells. The final F8 culture pellet suspension as well as H₂O, SA, VB, or F8 culture filtrate were used to pretreat *N. benthamiana*, followed by inoculation with TMV as previously described. F8-pellet-treated plants showed mild symptoms, with no obvious symptoms seen with F8 culture filtrate pretreatment (Supplementary Fig. S2A and B). Even when the concentration of F8 pellets was doubled, the TMV-induced symptoms were still more severe than with F8 culture filtrate pretreatment.

Treating local leaves with F8 culture filtrate does not protect against inoculation of the upper leaves.

To investigate whether F8 culture filtrate can protect plants systemically, we treated only the lower leaves (local) of *N. benthamiana* (method described above) with H₂O, SA, VB, or F8 culture filtrate and inoculated TMV in the upper untreated (systemic) leaves. As a control, we used F8 culture filtrate to treat whole plants followed by TMV inoculation. Whole plants pretreated with F8 culture filtrate showed much resistance to TMV (Fig. 4A and B); however, all plants with pretreatment of only local leaves showed severe viral symptoms (Fig. 4A and B). Therefore, F8 culture filtrate provides strong resistance in only treated leaves.

In another set of experiments, we pretreated local leaves of plants with H₂O, SA, VB, or F8 culture filtrate but did not inoculate them with TMV. We collected both the local and upper untreated (systemic) leaves at 24 h posttreatment (hpt) to analyze the expression of the *PR-1* gene (a SAR marker gene) (Zheng et al. 2015). *PR-1* gene expression was detected in SA- and VB- but not F8 culture filtrate-treated local leaves (Fig.

Table 1. The infection rate of Tobacco mosaic virus (TMV) on *Nicotiana benthamiana* with different pretreatments

Pretreatment ^a	Inoculum ^b	Experiment ^c								Total
		1	2	3	4	5	6	7	8	
–	Buffer	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/40
H ₂ O	TMV	5/5	5/5	5/5	5/5	5/5	5/5	5/5	5/5	40/40
SA	TMV	5/5	5/5	4/5	5/5	5/5	5/5	5/5	5/5	39/40
VB	TMV	5/5	5/5	5/5	4/5	5/5	5/5	5/5	5/5	39/40
F2	TMV	5/5	5/5	5/5	–	–	–	–	–	15/15
F8	TMV	1/5	0/5	0/5	1/5	0/5	1/5	0/5	0/5	3/40

^a Plants pretreated with H₂O, 5 mM salicylic acid (SA), vegetable broth (VB), and culture filtrate derived from F2 or F8 culture filtrate (F2 or F8).

^b Inoculums were made from lyophilized TMV-inoculated leaves.

^c Number of plants detected with TMV to total TMV-inoculated plants. All leaf samples were collected at 7 days postinoculation, and TMV was detected by reverse transcription-polymerase chain reaction. Experiments were repeated at least eight times. The F8 culture filtrate used in experiments 1 and 2 is from the same batch of preparation; F8 culture filtrate used in experiments 3 to 8 was prepared individually.

4C). However, only SA-treated plants (positive control) showed increased *PR-1* gene expression on systemic leaves (Fig. 4D). In addition, we collected leaves from plants with whole plants pretreated with H₂O, SA, VB, or F8 culture filtrate. Consistently,

PR-1 gene induction was detected in leaves of whole plants pretreated with SA and VB but not whole plants pretreated with F8 culture filtrate at 24 hpt (Fig. 4E). Thus, F8 culture filtrate does not induce canonical SAR.

Fig. 2. Lesion number on F8 culture filtrate-treated *Nicotiana glutinosa* inoculated with purified *Tobacco mosaic virus* (TMV) particles. **A**, *N. glutinosa* was pretreated with H₂O, 5 mM salicylic acid (SA), vegetable broth (VB), and F8 culture filtrate three times, with a 24-h interval between each spray. Pretreated plants were inoculated with 1-µg/ml, 10-µg/ml, or 50-µg/ml concentrations of TMV particles at 24 h posttreatment and untreated plants were inoculated with buffer (mock) for a control. Photos were taken at 5 days postinoculation (dpi). **B**, Lesion numbers induced by TMV particles was recorded at 5 dpi. UC = uncountable. Data are means $\pm$ standard deviation from three plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance, with Tukey's posttest.

To analyze whether F8 culture induces stronger resistance on TMV-inoculated leaves to infection, we inoculated TMV (1 $\mu\text{g}/\text{ml}$) on H_2O - or F8 culture filtrate-pretreated leaves of *N. benthamiana* and collected TMV-inoculated leaves at 24, 48, 72, and 96 h postinoculation (hpi) to analyze the TMV

accumulation and expression of the *PR-1* gene by qRT-PCR. The data showed that, in comparison with H_2O treated plants, decreased TMV accumulation and increased PR1 expression were observed on F8 culture filtrate-treated plants (Fig. 4F).

Fig. 3. Symptoms on F8 culture filtrate-treated *Nicotiana benthamiana* inoculated with purified *Tobacco mosaic virus* (TMV) particles. **A**, *N. benthamiana* was pretreated with H_2O , 5 mM salicylic acid (SA), vegetable broth (VB), or F8 culture filtrate (F8) three times at 24-h intervals. All plants were inoculated with 1- $\mu\text{g}/\text{ml}$, 10- $\mu\text{g}/\text{ml}$, or 50- $\mu\text{g}/\text{ml}$ concentrations of TMV particles at 24 h posttreatment. Photos were taken at 5 days postinoculation (dpi). **B**, Disease index levels induced by TMV were recorded at 5 dpi. Box plots represent medians, 25 to 75 percentiles (boxes), and minimum and maximum values (whiskers) from six plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance, with Tukey's posttest.

Treatment with F8 culture filtrate affects the establishment of initial TMV infection.

To analyze which virus infection steps are affected in F8 culture filtrate-treated plants, we used TMV tagged with green

fluorescence protein (GFP) to inoculate *N. benthamiana* and monitor the infection of TMV. Similar to our previous result, the infection rate of TMV-GFP was much reduced in *N. benthamiana* pretreated with F8 culture filtrate as compared with

Table 2. The infection rate of *Tobacco mosaic virus* (TMV) particles on *Nicotiana benthamiana* with different pretreatments^a

Pretreatment ^b	Inoculum	Experiment 1			Exp. 2	Exp. 3
		1 µg/ml	10 µg/ml	50 µg/ml	1 µg/ml	1 µg/ml
-	Buffer		0/5		0/3	0/3
H ₂ O	TMV particle	6/6	6/6	6/6	6/6	9/9
SA	TMV particle	6/6	6/6	6/6	6/6	15/15
VB	TMV particle	6/6	6/6	6/6	6/6	15/15
F8	TMV particle	4/6	6/6	6/6	3/6	6/15

^a Plants were inoculated with difference concentrations of TMV particles. Results are shown as number of plants detected with TMV to total TMV inoculated plants. All leaf samples were collected at 7 days postinoculation, and TMV was detected by reverse transcription-polymerase chain reaction.

^b Plants pretreated with H₂O, 5 mM salicylic acid (SA), vegetable broth (VB), and F8 culture filtrate (F8).

H₂O, SA, or VB (Supplementary Table S2). In addition, with TMV-GFP infection, the number of initial infection foci was reduced with both SA and F8 culture filtrate pretreatment (Fig. 5A and B). To compare TMV-GFP accumulation in each foci, we used a puncher to collect GFP foci under UV light and used qRT-PCR to quantify the TMV-GFP expression. The mean TMV accumulation per foci was similar with H₂O, SA, VB, and F8 culture filtrate pretreatment (Fig. 5C).

To understand whether the cell-to-cell movement of TMV was affected with F8 culture filtrate pretreatment, we measured the average size of TMV-GFP infection foci at 5 and 7 dpi. In TMV-GFP-infected plants, the mean size of infection foci did not differ between plants with different pretreatment at 5 and 7 dpi ($P < 0.05$) (Fig. 5A and D).

To analyze whether long-distance movement is affected by F8 culture filtrate pretreatment, we measured the average time for TMV-GFP to move to apical leaves. The mean time (days) for TMV to move to apical leaves was similar with different pretreatments (Fig. 5E and F).

F8 culture filtrate induces resistance to Turnip mosaic virus (TuMV) in *Brassica juncea*.

To analyze whether F8 culture filtrate induced resistance to different viruses in other plants, we treated plants from an economically important vegetable, *Brassica juncea*, with H₂O, VB, and F8 culture filtrate, using the method mentioned above, and inoculated the plants with TuMV. TuMV induced severe mosaic and stunting symptoms in plants without any treatment or with VB pretreatment or induced severe mosaic symptoms in plants with H₂O pretreatment (Fig. 6A); however, F8 culture filtrate pretreatment did not confer obvious symptoms (Fig. 6A and B).

F8 culture filtrate pretreatment does not cause resistance-associated fitness cost.

To analyze whether F8 culture filtrate pretreatment reduces resistance-associated fitness cost in plants, we analyzed the fresh weight and leaf area in untreated and F8 culture filtrate-treated *N. benthamiana* and *Brassica juncea*. Both F8 culture filtrate-treated and untreated *N. benthamiana* and *Brassica juncea* showed similar leaf area and fresh weight (Fig. 6C and D).

F8 fungi is most related to *Trichosporon scarabaeorum*.

To identify the F8 fungi, we designed primers (Supplementary Table S1) to amplify the D1/D2 domain and the intergenic

transcribed spacer (ITS) regions of the 26S rDNA (Scorzetti et al. 2002). Phylogenetic analysis was performed by neighbor-joining (NJ) and maximum likelihood (ML) methods. The results of both methods were similar and indicated that F8 fungi is related to *T. scarabaeorum* (Fig. 7A and B).

F8 is a yeast-like fungus. After culturing the fungi on PDA plate for 15 days, the colony diameter grew to 54 to 57 mm. The F8 colony is beige butter-like and wrinkled on PDA but silk-like and flat on V-8 agar plate (Fig. 7C). Under microscope observation, F8 generated arthroconidia. The arthroconidia were hyaline, short rod-shaped, and obovate (Fig. 7C). Together, molecular phylogenetic analysis of the D1/D2 domain, ITS, and morphological features showed that our identified F8 fungus belongs to the basidiomycetous yeast *T. scarabaeorum*.

A fraction of polysaccharide derived from F8 culture filtrate is the major active component for inducing plant virus resistance.

To identify one or more active functional compounds in F8 culture filtrate that are responsible for inducing plant resistance, we separated the F8 culture filtrate by size-exclusion chromatography. Eight fractions (F8-fr1 to F8-fr8) were eluted by H₂O with a Sephadex LH20 column. Each fraction was analyzed by matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS), and the activity in inducing resistance against TMV was initially analyzed in *N. glutinosa*. Our repeat experiments indicated that the second fraction (F8-fr2) induced the strongest antiviral activity in *N. glutinosa*. To determine whether F8-fr2 also induced strong resistance in *N. benthamiana*, we treated *N. benthamiana* with F8-fr1 to F8-fr4, then, inoculated plants with TMV (Supplementary Fig. S3). Consistently, F8-fr2 induced the strongest antiviral activity in *N. benthamiana* (Supplementary Table S3), although the F8 culture filtrate still induced the best resistance.

On MALDI-TOF MS, F8-fr2 showed a polysaccharide signal, with no obvious signals observed after F8-fr4. To purify F8-fr2 polysaccharides, we created three consecutive precipitations with increasing concentrations of ethanol (20, 50, and 80%). The H₂O-resuspended pellets derived from 80% ethanol precipitation (F8-fr2-80% EtOH ppt) exhibited the strongest antiviral activity (Fig. 8A and B). Results of MALDI-TOF MS analysis suggested that F8-fr2-80% EtOH ppt mainly

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Fig. 4. F8 culture filtrate does not confer systemic protection against *Tobacco mosaic virus* (TMV) or conical systemic acquired resistance (SAR) in *Nicotiana benthamiana*. The lower three leaves (local) of *N. benthamiana* were pretreated with H₂O (H₂O⁻), 5 mM salicylic acid (SA⁻), vegetable broth (VB⁻), or F8 culture filtrate (F8⁻) three times, with a 24-h interval between each spray. The three upper leaves (systemic) were inoculated with TMV at 2 days posttreatment. Whole plants treated with F8 culture filtrate (F8) three times, with a 24-h interval between each spray, was used as control. **A**, Photo taken at 7 days postinoculation and **B**, the level of disease induced by TMV was recorded. Box plots represent medians, 25 to 75 percentiles (boxes), and minimum and maximum values (whiskers) from five plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance, with Tukey's posttest. Experiments were repeated at least three times with similar results. For expression analysis of SAR marker gene *PR-1*, local leaves of plants were pretreated with H₂O, 5 mM SA, VB, or F8 culture filtrate three times, with a 24-h interval between each spray. After treatment, **C**, local leaves and **D**, systemic leaves were collected at 24 h posttreatment (hpt). Whole plants treated with H₂O, 5 mM SA, VB, or F8 culture filtrate were controls. The relative expression of *PR-1* was detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Data are means $\pm$ standard deviation (SD) from three plants. Student's *t* test was used to analyze the data between indicated treatment and the H₂O-pretreated control group. Asterisks indicate significant differences between H₂O- or F8-treated plants (one asterisk [*] indicates $P < 0.05$, two [**] $P < 0.01$). ns = not significant. Experiments were repeated at least three times with similar results. **E**, The whole plant was pretreated with H₂O, 5 mM SA, VB, or F8 three times, with a 24-h interval between each spray. Leaves were collected at 24 hpt. The relative expression of *PR-1* was detected by qRT-PCR. Data are means $\pm$ SD from three plants. Student's *t* test was used to analyze data between the indicated treatment and the H₂O-pretreated control group. Asterisks indicate significant differences between H₂O- or F8-treated plants (* indicates $P < 0.05$, ** $P < 0.01$). ns = not significant. Experiments were repeated at least three times with similar results. The whole plant was pretreated with H₂O or F8 three times, with a 24-h interval between each spray. Pretreated plants were inoculated with TMV at 24 hpt. Inoculated leaves were collected at 24, 48, 72, and 96 h postinoculation (hpi). The relative expression of *PR-1* was detected by qRT-PCR. Data are means $\pm$ SD from three to five plants. Asterisks indicate significant differences between H₂O- or F8-treated plants (* indicates $P < 0.05$, ** $P < 0.01$). ns = not significant. **F**, The whole plant was pretreated with H₂O or F8 three times, with a 24-h interval between each spray. Pretreated plants were inoculated with TMV at 24 hpt. Inoculated leaves were collected at 24, 48, 72, and 96 hpi. The relative expression of *PR-1* was detected by qRT-PCR. Data are means $\pm$ SD from three to five plants. Student's *t* test was used to analyze the data between F8 treatment and H₂O-pretreated control group. Asterisks indicate significant differences between H₂O- or F8-treated plants (* indicates $P < 0.05$, ** $P < 0.01$). ns = not significant.

consists of polysaccharides (Fig. 8C). F8-fr2-80% EtOH ppt was further separated by high-performance liquid chromatography (HPLC) into three peaks (*1 to *3) (Fig. 8D). The *2 peak (designated F8-polysaccharide) showed the strongest antiviral activity (Fig. 8A and B). The sugar composition of F8-polysaccharide was analyzed by gas chromatography (GC)-MS, which revealed D-mannose, D-galactose, and D-glucose in the ratio 1.0:1.2:10.0. Linkage analysis with GC-MS and nuclear magnetic resonance (NMR) analysis suggested the F8-polysaccharide is mainly α -D-1,4-glucan (Fig. 8E and F). HPLC analysis revealed the molecular weight of the F8-polysaccharide as 4.2 to 12.6 kDa (Supplementary Fig. S4).

F8-polysaccharide induces priming of SA-governed immune response genes.

To analyze how F8-polysaccharide induces plant viral resistance, we measured the expression of SA-, JA-, and ET-responsive immune marker genes in H₂O- and F8-polysaccharide-treated plants (Cortes-Barco et al. 2010; Milling et al. 2011; Zhu et al. 2014). We first confirmed the induction of marker genes induced by

SA (*PR-1* and *PR-2*), JA (*PDF-1.2* and *PR-4*), and ET (*Osm* and *EREBP*) in *N. benthamiana* (Supplementary Fig. S5A). Plants were pretreated with H₂O and F8-polysaccharide three times, with a 24-h interval between each spray, as described previously, then, leaves were collected from one set of individual plants at 0, 12, 24, 36, 48, 72, and 96 hpt. In another set of plants with H₂O and F8-polysaccharide pretreatment, TMV was inoculated at 24 hpt and the inoculated leaves from individual plants were collected at 0, 12, 24, 48, and 72 hpi. We detected the accumulation of TMV and the expression of SA-, JA-, and ET-responsive immune marker genes (Fig. 9A to D). Consistently, TMV accumulation at 48 and 72 hpi was lower in F8-polysaccharide-pretreated than H₂O-pretreated plants in our three repeats. Inconsistent induction of *PR-4* (JA marker gene) and *Osm* (ET marker gene) was observed in the three repeats. Therefore, the expression of *PR-4* and *Osm* genes in *N. benthamiana* is not related to F8-polysaccharide treatment. The expression of *PR-1*, *PR-2*, *PDF-1.2*, and *EREBP* was consistent in our repeats (Fig. 9A to D). A comparison of H₂O- and F8-polysaccharide-pretreated plants over different times showed that the expression of *PR-1* was highly induced at 12 hpt

Fig. 5. *Nicotiana benthamiana* infected with Tobacco mosaic virus tagged with green fluorescence protein (TMV-GFP). **A**, *N. benthamiana* was pretreated with H₂O, 5 mM salicylic acid (SA), vegetable broth (VB), or F8 culture filtrate (F8) three times, with a 24-h interval between each spray. All plants were inoculated with TMV-GFP at 24 h posttreatment. Photos were taken under UV light at 5 and 7 days postinoculation (dpi). Scale bar = 2 cm. **B**, The mean number of TMV-GFP infection foci per plant at 5 dpi. Data are means $\pm$ standard deviation (SD) from two to five plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance (ANOVA) with Tukey's posttest. **C**, The relative accumulation of TMV-GFP per foci. TMV-GFP was quantified by quantitative reverse transcription-polymerase chain reaction, and β -actin was an internal control. Data are means $\pm$ SD from two to five plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way ANOVA with Tukey's posttest. **D**, The area (in square millimeters) of TMV-GFP infection foci was measured at 5 and 7 dpi. **E**, Photos of TMV-GFP-infected plants taken under white light or UV light at 13 dpi. **F**, The mean time (days) for TMV-GFP to move to apical leaves. Experiments were repeated at least three times with similar results. Data are means $\pm$ SD from two to five plants.

and gradually reduced (Fig. 9A). However, after TMV inoculation, the expression of *PR-1* was highly induced in plants treated with F8-polysaccharide at 48 and 72 hpi (Fig. 9B). The expression pattern of *PR-2* was similar to that of *PR-1* with or without TMV inoculation (Fig. 9C and D). The expression

of *PR-1* and *PR-2* show a typical priming expression pattern (Martinez-Medina et al. 2016).

PDF-1.2 was induced at 12 hpt and 12 hpi but with no significant difference between H₂O- and F8-polysaccharide-pretreated plants in our repeated experiments. No significant

Fig. 6. Symptoms induced by *Turnip mosaic virus* (TuMV) in *Brassica juncea* pretreated with F8 culture filtrate and fitness cost analysis of *Nicotiana benthamiana* and *Brassica juncea* with F8 culture filtrate pretreatment. **A**, *Brassica juncea* was pretreated with H₂O, vegetable broth (VB), or F8 culture filtrate (F8) three times, with a 24-h interval between each spray, then, were inoculated with TuMV at 24 h posttreatment. Photos were taken at 13 days postinoculation (dpi). Scale bar = 5 cm. **B**, The disease index induced by TuMV was recorded at 13 dpi. Box plots represent medians, 25 to 75 percentiles (boxes), and minimum and maximum values (whiskers) from six plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance, with Tukey's posttest. Experiments were repeated three times with similar results. **C**, Shoots of six *N. benthamiana* (15 days after seeding) or **D**, *Brassica juncea* (10 days after seeding) without treatment (WO) or with F8 pretreatment were used to measure fresh weight and leaf area at 20 or 25 days posttreatment, respectively. All leaves were photographed with identical illumination and exposure conditions. The average leaf area per plant was calculated.

Fig. 7. Identification of F8 fungi. **A**, Phylogenetic tree of F8 fungi based on the D1/D2 region and **B**, intergenic transcribed spacer region by neighbor-joining and maximum likelihood methods. The results of both analysis methods was similar, and only the neighbor-joining tree is shown here. **C**, F8 fungi were cultured on 10% V-8 agar (V-8) and potato dextrose agar (PDA) plates for 15 days at 25°C. Colony morphology was photographed and presented with front and back side. Microscopy depicting the morphology of arthroconidia and hyphae of F8 fungi grown on V-8 plate, 25°C, for 7 days. Scale bar = 20 µm.

induction of *EREBP* expression was observed in H₂O- and F8-polysaccharide-pretreated plants in our repeats.

DISCUSSION

Our results successfully demonstrate that a natural product can induce prominent plant immunity in highly susceptible hosts, to confer effective resistance to virus infection without cost to the fitness of the plant. We successfully identified a culture filtrate derived from a fungi (F8) belonging to the genus *Trichosporon* within class Basidiomycete that greatly enhanced virus resistance in different susceptible plants against different viruses without obvious resistance-associated fitness cost. The F8 culture filtrate induced an unusual type of defense priming that differs from but is more effective than SA-induced resistance. On further fractionating the F8 culture filtrate, we identified a fraction of polysaccharide (F8-polysaccharide) composed of D-mannose, D-galactose, and D-glucose, in the ratio 1.0:1.2:10.0, with a α -D-1,4-glucan linkage and an estimated molecular weight of 4.2 to 12.0 kDa, that is responsible for inducing plant resistance against viruses.

Several polysaccharides, including carrageenans (α -1, 3 and β -1, 4-glycosidic), fucans (α -1,4 and α -1,3 glycosidic), and laminarin (β -1,6-1,3 glucans) have been reported to induce plant resistance against TMV in other local lesion hosts, including *Nicotiana tabacum* cv. Xanthi and *N. tabacum* L. cv. Samsun H (Ghannam et al. 2013; Klarzynski et al. 2000, 2003;

Nagorskaia et al. 2008; Reunov et al. 2004; Vera et al. 2012). However, our data indicate that even though F2 and F8 culture filtrates induced similar resistance in *N. glutinosa* (Fig. 1A and B), only F8 culture filtrate induced prominent resistance in *N. benthamiana* (Fig. 1C and D). Therefore, substances that trigger resistance in local lesion hosts may not be able to trigger resistance in susceptible hosts and using one or more local lesion hosts alone for resistance analysis may not be sufficient for screening antiviral substances in susceptible plants.

An interesting phenomenon was that F8 culture filtrate did not induce typical SAR in *N. benthamiana*. F8 culture filtrate induced only local resistance and did not induce SAR. However, even though SA can induce SAR, SAR did not provide as effective protection against TMV as did F8 culture filtrate (Fig. 4). Although SAR can enhance plant resistance against a broad spectrum of pathogens, SAR induction may not be the best strategy for protecting plants against viruses.

Our tracking assay using TMV-GFP to inoculate *N. benthamiana* indicates that F8 culture filtrate mainly reduced the number of initial infection foci. Once TMV establishes the initial infection, TMV accumulation (per infection foci), and cell-to-cell and long-distance movement did not differ much between plants treated with H₂O or F8 culture filtrate. Our results are similar to previous reports showing that certain tobacco plants treated with mycolaminaran (a branched β -1,3-glucan purified from the cytoplasm of *Phytophthora megasperma*) also inhibited initial viral infection (Kopp et al. 1989; Zinnen et al. 1991).

Fig. 8. Fractionation of F8 culture for antiviral activity analysis and identification, nuclear magnetic resonance, and linkage analysis of active functional compounds derived from F8 culture filtrate. *Nicotiana benthamiana* was pretreated with H₂O, 5 mM salicylic acid (SA), vegetable broth (VB), F8 culture filtrate (F8), F8-sephadex fraction 2 (F8-fr2), H₂O-resuspended pellets derived from F8-fr2 precipitated with 20, 50, and 80% ethanol (F8-fr2-20%, fr2-50, and fr2-80% EtOH ppt), and high-performance liquid chromatography (HPLC)-purified peaks 1 to 3 (*1 to *3) from H₂O-resuspended F8-fr2-80% EtOH ppt three times, with a 24-h interval between each spray. We define peak *2 as F8-polysaccharide. Pretreated plants were inoculated with *Tobacco mosaic virus* (TMV) at 24 h posttreatment. **A**, Photos of TMV-inoculated plants taken at 7 days postinoculation (dpi). **B**, The disease index induced by TMV was recorded at 7 dpi. Box plots represent medians, 25 to 75 percentiles (boxes), and minimum and maximum values (whiskers) from six plants. Different letters above the bars represent significant differences among groups, $P < 0.05$ by one-way analysis of variance, with Tukey's posttest. Experiments were repeated three times with similar results. **C**, Matrix-assisted laser desorption-ionization time of flight mass spectra of F8-sephadex fraction 2 (F8-fr2), H₂O-resuspended pellets derived F8-fr2-20% EtOH ppt, 50% EtOH ppt, and 80% EtOH ppt, and HPLC-purified peaks *1 to *3 from H₂O-resuspended F8-fr2-80% EtOH ppt at m/z 1,800 to 6,000, where the most peaks are. **D**, HPLC-evaporative light scattering detection profile of F8-fr2-80% EtOH ppt. Three major peaks, *1 to *3, were collected for matrix-assisted laser desorption-ionization time of flight mass spectra analysis shown in C. *2 was defined to be F8-polysaccharide. **E**, Heteronuclear single quantum coherence spectrum (¹H-¹³C HSQC, D₂O, 323K, 600 MHz) of F8-polysaccharide. Determination of the ¹J_{HC} of G1 (alpha configuration) was based on the satellite signals of G1 (d_H 5.63, d_C 99.9) in the heteronuclear multiple bond correlation spectrum (¹H-¹³C HMBC). **F**, Gas chromatography mass spectrometry (GC-MS) linkage analysis of F8-polysaccharide. 1,4-linked-D-glucopyranosyl residue was the main linkage composition.

Although we cannot rule out the possibility that F2 or F8 culture filtrate may inhibit virus infection, our data suggested that SA-governed immune responses are highly induced on F8 culture filtrate or F8-polysaccharide treated plants during TMV infection (Figs. 4F and 9B and D). The phenomenon we observed of low infection foci but similar virus accumulation per foci and similar cell-to-cell and long distance movement between plants treated with H₂O and F8 culture filtrate fits the proposed scheme for microbe-plant interactions during infection, in which microbe-encoded PAMPs can trigger the plant PTI to protect against pathogen infection and pathogens may encode one or more effectors to suppress PTI for successful infection (Jones and Dangl 2006). Recent reports indicated that plant viral double-stranded RNAs can act as PAMPs that trigger PTI responses against viral infection in *Arabidopsis* (Niehl et al. 2016). In addition, TMV replicase has been found to interact with and degrade a NAC domain transcription factor, ATAF2. Because ATAF2 can induce host defense responses, the accumulation of TMV replicase will cause gradual degradation of ATAF2, thus leading to an attenuated defense response during TMV infection (Wang et al. 2009). Our data suggest that F8 culture filtrate treatment greatly enhanced PTI.

Our analysis revealed that F8-polysaccharide is the major component in F8 culture filtrate for inducing plant resistance. F8-polysaccharide induces a typical priming expression pattern of SA-responsive genes (Fig. 9A to D). A previous report demonstrated

that priming involves fewer fitness costs (Conrath et al. 2015; Mauch-Mani et al. 2017; van Hulst et al. 2006; Wang et al. 2015), which may explain, in part, why F8 culture filtrate treatment did not confer any observable growth defect (Fig. 6C and D).

The origin of F8-polysaccharide is intriguing. The composition and structure have not been previously revealed. The F8-polysaccharide is obviously not directly derived from original VB culture medium because VB did not induce effective resistance in plants as compared with F8 culture filtrate treatment (Figs. 1, 2, 3, and 6B). Also, F8 pellets (contain mostly F8 fungi cell debris) do not induce as effective a resistance as F8 culture filtrate on *N. benthamiana*. F8-polysaccharide may come from the fermentation process between VB and F8 fungi, but its origin remains elusive. Although F8-polysaccharide is the major compound identified from F8 culture filtrate, plant resistance induced by F8-polysaccharide is not as effective as F8 culture filtrate (Fig. 8; Supplementary Table S3). This suggests that substances other than F8-polysaccharide in the F8 culture filtrate may also play roles in enhancing plant resistance.

Our analysis revealed that F8-polysaccharide is the major component in F8 culture filtrate for inducing plant resistance. F8-polysaccharide induces a typical priming expression pattern of SA-responsive genes (Fig. 9A to D). A previous report demonstrated that priming involves fewer fitness costs (Conrath et al. 2015; Mauch-Mani et al. 2017; van Hulst et al. 2006; Wang et al. 2015), which may explain, in part, why F8 culture

Fig. 9. The relative expression of salicylic acid (SA) associated immune marker genes. **A**, The relative expression of SA-responsive genes *PR-1* and **C**, *PR-2* detected by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Plants were pretreated with H₂O or F8-polysaccharide for total of three times, with a 24-h interval between each spray, then, samples collected at 0, 12, 24, 36, 48, 72, 96 h posttreatment (hpt). **B** and **D**, *Tobacco mosaic virus* (TMV) inoculation or mock treatment was conducted on different indicated 24-hpt plants. Leaf samples were collected from TMV-infected plants at 1, 36, 48, and 72 h post-TMV inoculation. The relative expression of *PR-1* (**B**) and *PR-2* (**D**) was detected by qRT-PCR. Data are means $\pm$ standard deviation from three to five plants. All lettering indicates statistical significance ($P < 0.05$ by one-way analysis of variance, with Tukey's posttest). Letters indicate statistical significance as follows: a, between F8-polysaccharide-treated samples at indicated time to F8-polysaccharide-treated samples collected at 0 hpt; b, between H₂O and F8-polysaccharide-treated samples at each time course; c, between H₂O-treated mock-inoculated and F8-treated mock-inoculated; d, between H₂O-treated TMV-inoculated and F8-treated TMV-inoculated; e, between H₂O-treated mock-inoculated and H₂O-treated TMV-inoculated; f, between F8-treated mock-inoculated and F8-treated TMV-inoculated; g, indicates statistical significance to H₂O-treated mock-inoculated at 0 h postinoculation.

Table 3. ¹H and ¹³C nuclear magnetic resonance chemical shifts (δ , D₂O, 323 K) of F8-polysaccharide

(1 $\rightarrow$ 4)- α -D-Glcp-(1 $\rightarrow$ 4)- ^a	1	2	3	4	5	6
¹ H	5.63	3.88	4.21	3.90	4.10	4.08; 4.11
¹³ C	99.9	71.8	73.6	77.4	71.8	60.8

^a The configuration was determined based on the $J_{H1-C1} = 173.3$ Hz.

filtrate treatment did not confer any observable growth defect (Fig. 6C and D).

The origin of F8-polysaccharide is intriguing. The composition and structure have not been previously revealed. The F8-polysaccharide is obviously not directly derived from original VB culture medium because VB did not induce as effective a resistance in plants, as compared with F8 culture filtrate treatment (Figs. 1 and 6B). Also, F8 pellets (contain mostly F8 fungi cell debris) do not induce as effective a resistance as F8 culture filtrate on *N. benthamiana*. F8-polysaccharide may come from the fermentation process between VB and F8 fungi, but its origin remains elusive.

Our results provide several new insights into the application of induced plant resistance against viruses, which will be important to further develop management strategies to protect plants against virus infection.

MATERIALS AND METHODS

Isolation of soil microorganisms and preparation of culture filtrate for antiviral activity assay.

Soil samples (10 to 20 g) were collected from a depth of 5 to 10 cm in Taipei, Taiwan. Soil microorganisms were isolated with selective medium as reported by Ko et al. (2010), except we adjusted the pH of VB to 4.7 for the culture of F8 fungi and the VB was filtrated through a 25- μ m filter before storing at -80°C .

Plant materials and inoculation of viruses.

Plants were grown in a growth chamber with 10,000 lux fluorescent light and a 16-h light and 8-h dark cycle. *N. glutinosa* (6 to 7 weeks old, eight- to ten-leaf stage), *N. benthamiana* (3 to 4 weeks old, five- to six-leaf stage) and *Brassica juncea* (14 days old, two to three true-leaf stage) were used for one or both treatment and inoculation. For virus inoculation, plants were dusted with 600-grit silicon carbide powder (Sigma-Aldrich, St. Louis) and were mechanically inoculated. Virus inoculum was prepared with 100 ml of 0.1 M potassium phosphate (KP) buffer (61.5% 0.1 M K_2HPO_4 and 38.5% 0.1 M KH_2PO_4 , pH 7.0) mixed with 0.1 g of lyophilized leaves of plants infected with TMV (Figs. 1, 4A to E, 8, and 9; Supplementary Fig. S6) or TMV tagged with green fluorescence protein (TMV-GFP) (Fig. 5; Table 1) (Rabindran and Dawson 2001). Different concentrations of TMV-purified particle inoculum was diluted with 0.1 M KP buffer (61.5% 0.1 M K_2HPO_4 and 38.5% 0.1 M KH_2PO_4 , pH 7.0) (Figs. 2, 3, and 4F; Table 2). TuMV inoculum was prepared with 0.1 g of fresh leaves from plants infected with TuMV ground with 10 ml of 0.1 M sodium phosphate buffer (0.1 M NaH_2PO_4 , 0.1 M Na_2HPO_4 , and 0.5% Na_2SO_3 , pH 7.4). The severity of TMV or TuMV symptoms of infected plants were scored based on external phenotype (Supplementary Fig. S6). Transmission electron microscope (Philips Electronic Instruments, Mahwah, NJ, U.S.A.) was used to examine TMV particles of different inoculums.

Purification of TMV particles.

Leaves of TMV-infected *N. benthamiana* were harvested approximately 5 days after inoculation. The initial purification of TMV virus particles followed the protocol described by Gooding and Hebert (1967), with modifications. Briefly, polyethylene glycol with molecular weight 8,000 (PEG 8000) (Sigma-Aldrich) was used instead of PEG 6000. In addition, the semi-purified preparation was submitted to linear density sucrose gradient (0 to 18%) and was ultracentrifuged at $228,000 \times g$ for 1.5 h. The light scattering region was collected and diluted with 100 ml of suspension buffer (35 mM Na_2HPO_4 , 15 mM NaH_2PO_4 , and 0.05% 2-mercaptoethanol, pH 7.2). NaCl (0.4 g)

and PEG 8000 (4 g) were added and were stirred for at least 1 h on ice, then, centrifuged at $9,000 \times g$ for 15 min at 4°C . The pellets were resuspended in 1 ml of suspension buffer. The concentration of the purified TMV suspension was determined ($A_{260} = 0.3$ for 1 mg of TMV per milliliter) with a Nanodrop (ND-1000; Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Antiviral activity assays.

Whole plants were sprayed with 0.4 ml of H_2O , VB, culture filtrate derived from microorganisms, or 5 mM SA (Sigma-Aldrich) three times, with a 24-h interval between each spray. Twenty-four hours after the final treatment, plants were rubbed with the virus inoculum.

Measurement of area of TMV-GFP infection foci.

Photographs of TMV-GFP infection foci in *N. benthamiana* leaves were taken under UV light at 5 and 7 dpi, and the level of fluorescence as well as the areas of fluorescent infection sites in each leaf were measured, using ImageJ v1.47. All imaging was conducted under identical illumination and exposure conditions to allow for comparisons. Mean values of areas of all infection foci per treatment were calculated.

RNA extraction and gene expression analysis.

Total RNA was isolated by use of Trizol (Invitrogen, Bethesda, MD, U.S.A.), according to the manufacturer's instruction. Residual DNA was removed using the Turbo DNA-free kit (Ambion, Austin TX, U.S.A.). cDNA was synthesized using the PrimeScript RT-PCR kit (Takara Bio, Shiga, Japan). qRT-PCR involved the SYBR protocol (Life Technologies, Gaithersburg, MD, U.S.A.) and the ABI 7100 real-time PCR system (Applied Biosystems, Carlsbad, CA, U.S.A.). For quantitative analysis, actin was used as an input control. Relative fold change in mRNA expression was determined by calculating cycle threshold ($2^{-\Delta\Delta\text{CT}}$).

Identification of fungi and phylogenetic analysis.

Genomic DNA was extracted by the CTAB method (Chang et al. 1993). Internal transcribed spacer or 26S rDNA region sequences were amplified with specific primers designed for the ITS1-5.8S-ITS2 region (ITS5e-F and ITS4e-R) and the D1/D2 domain (LR1-F and LR3-R), respectively. The sequence alignment involved use of CLUSTAL X (1.81) (Thompson et al. 1997). All the phylogenetic trees in this study were generated using MEGA 5 (Tamura et al. 2011). Phylogenetic analyses involved NJ and ML methods. The branch supports of NJ and ML methods were analyzed by using PAUP* 4.0 v10 (Swofford 2003) with bootstrapping. NJ involved a Kimura 2-parameter model and node support was analyzed by bootstrapping with 1,000 replicates. ML involved a Jukes-Cantor model and bootstrapping with 1,000 replicates. Bootstrap minimal concordance was set to 80%. These sequence data have been submitted to the GenBank databases under accession number MG757354 and MG752969.

Measurement of leaf area and fresh weight.

N. benthamiana (16 days after seeding) and *Brassica juncea* (10 days after seeding) were treated with the F8 culture filtrate, as described in the antiviral activity assay. All shoot tissues of individual plants were measured for fresh weight. Leaves were randomly collected from six individual *N. benthamiana* or *Brassica juncea* plants with or without F8 culture filtrate treatment at 20 or 25 days posttreatment, respectively. Photos were taken from every collected leaf using identical illumination and exposure conditions. Each leaf area was measured using Image J.

Identifying the active functional compound of F8 culture filtrate for inducing plant resistance.

To purify and characterize the active functional compound, freeze-dried powder (from 90 ml of F8 culture filtrate) was dissolved in 90 ml of distilled H₂O. After centrifugation at 6,000 × g for 10 min, the supernatant was separated on a prepacked gel filtration Sephadex LH-20 column (GE Healthcare Life Sciences, Uppsala, Sweden) eluted with 500 ml of H₂O per fraction. Eight fractions derived from the F8 culture filtrate (F8-fr1 to F8-fr8) were collected and freeze-dried, then, were dissolved in 90 ml of distilled H₂O and were tested for antiviral activity.

Isolation of polysaccharides.

The F8-fr was mixed with a ratio of ethanol to H₂O/ethanol (80:20 [vol/vol]) and was stirred vigorously at 4°C. The precipitate was centrifuged at 6,000 × g for 10 min. The supernatant was precipitated again by the addition of a ratio of ethanol to H₂O/ethanol (50:50 [vol/vol]) and H₂O/ethanol (20:80 [vol/vol]). All the precipitate was dissolved in 90 ml of distilled H₂O, then, tested for antiviral activity. To remove minor impurities, the F8-fr-2-80% EtOH pellets were further purified by preparative HPLC with a TSKgel P2500GW column (21.5 mm × 60 cm; Tosoh Bioscience LLC, Tokyo) for structure elucidation. The HPLC-purified F8-frs were dissolved in distilled H₂O, then, tested for antiviral activity.

Polysaccharide structure elucidation.

For sugar composition analysis, the purified polysaccharide underwent methanolysis by 3N Methanolic-HCl (Supelco, Bellefonte, PA, U.S.A.) for 14 h at 85°C in a sealed glass tube. After drying with N₂, the residue was then acetylated with 50 µl acetic anhydride and 10 µl pyridine in 500 µl methanol at room temperature for 20 min. All solvent was removed by a steady stream of nitrogen, then, the Sylon HTP kit (Supelco) was used for trimethylation at room temperature for 30 min. The dried sample was dissolved with 800 µl of *n*-hexane (HPLC grade; Sigma) and, then, analyzed by GC-MS. All data were collected by GC-MS (Bruker) with a HP-5MS silica capillary column (30 m × 0.25 mm I.D., Agilent) and temperature gradient of 1 min at 60°C, 2 min at 60 to 90°C, 9 min at 90 to 290°C, 5 min at 290°C, 36 min at 290 to 300°C and kept for 2 min at 300°C.

For the linkage analysis, the polysaccharide was lyophilized, then dissolved in 1 ml of dimethyl sulfoxide (DMSO) overnight at 80°C. NaOH pellets were ground with a mortar and were added into the sample solution for a 2-h incubation in a sealed tube. The sample was methylated by using CH₃I and powder NaOH in DMSO. Furthermore, the dried sample was partitioned with H₂O and CHCl₃ to give the organic layer. The dried organic layer residue was further hydrolyzed with 2 M trifluoroacetic acid (TFA) at 120°C for 1 h, to give the partially methylated monosaccharides. Then, the sample was reduced by NaBD₄ at room temperature for 1 h and was neutralized with AcOH. The solution was coevaporated with MeOH to remove the boric acid. After that, the sample was acetylated with 1:2 pyridine-Ac₂O at 80°C for 1 h and the mixture was partitioned with H₂O-CHCl₃. The organic layer containing methyl alditol acetates was analyzed by GC-MS on a HP-5MS silica capillary column (30 m × 0.25 mm I.D., Agilent) temperature gradient of 1 min at 60°C, 2 min at 60 to 90°C, 9 min at 90 to 290°C, 5 min at 290°C, 36 min at 290 to 300°C and kept for 2 min at 300°C. The polysaccharide consisted of terminal-D-glucopyranosyl residue, 4-linked-D-glucopyranosyl residue, and 4,6-linked-D-glucopyranosyl residue.

The one- and two-dimensional (2D) NMR spectra were collected using a Bruker AVANCE 600 spectrometer in D₂O at 323 K. All 2D NMR experiments were carried out with standard pulse sequences provided by Bruker. For the NMR data, the major composition of the polysaccharide was determined to

be α-1,4-glucan. The assignments of protons and carbons of the sample are given in Table 3.

MALDI-TOF MS.

A 1-µl sample was spotted on a MTP 384 ground steel plate and 1 µl of saturated universal matrix solution (Sigma) (dissolved in 50% acetonitrile with 0.1% TFA) was spotted on top of the sample for MALDI-TOF MS analysis. A Bruker Autoflex Speed MALDI-TOF/TOF MS (Bruker, Bremen, Germany) equipped with a Smartbeam laser (1,000 Hz) was used for MALDI-TOF MS analysis. All data were collected in positive ion liner mode and were analyzed using Daltonics flexAnalysis 3.0 (Bruker).

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