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# Disease Occurrence in Transgenic Rice Plant Transformed with Silbene Synthase Gene and Evaluation of Possible Horizontal Gene Transfer to Plant Pathogens

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Genetic engineering is being used to enhance disease resistance and nutritional value of crops including rice plant. Considering the fast-growing agricultural biotechnology and rapidly increasing global area of transgenic crops, the risk evaluation on environment is necessary. In this study, we surveyed the difference of disease occurrence between transgenic rice variety, Iksan526 transformed with peanut stilbene synthase gene and non-transgenic rice varieties, Dongjin and Nampyeong in the field. Moreover, the possibility of gene transfer from transgenic rice to bacterial and fungal pathogens was investigated. The results of this study indicated that there was no significant difference in the occurrence and severity of the diseases between Iksan526 and Dongjin or Nampyeong. In addition, the results suggested that rice pathogen, such as *Xanthomonas oryzae* pv. *oryzae, Rhizoctonia solani* and *Magnaporthe grisea* did not take up stilbene synthase and *bar* genes under natural conditions. Moreover the transformed DNA was not transferred to the pathogens even in repetitive contacts.

**Keywords :** Gene transfer, Piceid, Resveratrol, Stilbene synthase, Transgenic rice

## Introduction

Rice (*Oryza sativa* L.) is one of the most important staple foods for the increasing world population, especially in Asia. Plant breeders are still trying to develop rice cultivars having high quality and quantity value. Nowadays, genetic engineering is among the various methods being used to enhance nutritional or functional value of rice plants, and confer resistance to pathogens (Stark-Lorenzen *et al*., 1997; Ye *et al*., 2003).

A number of previous studies demonstrated the toxic activity of resveratrol on fungal and bacterial pathogens (Adrian *et al*., 1997; Maddox *et al*., 2010; Yu *et al*., 2005). Resveratrol (trans-3,4,5-trihydroxy-stilbene) has also been shown to extend lifespan of yeast (*Saccharomyces cerevisiae*), worm (*Caenorhabditis elegans*), and fly (*Drosophila melanogaster*) under standard full diets, and also extend lifespan and improve health of a short-lived fish (*Nothobranchius furzeri*) and mice on a high-fat diet (Agarwal and Baur, 2011). Hence, a stilbene synthase gene from peanut was transformed to rice variety, Dongjin, and transgenic rice variety, Iksan526 which synthesizing resveratrol and piceid (5,4′-dihydroxystilbene-3-O-β-glucopyranoside) was developed to produce resveratrol-containing grain at National Institute of Crop Science, Rural Development Administration, Korea.

However, the increasing exploitation of transgenic plants has generated concerns over the ecological impact of engineered heterologous genes as these can be replicated and disseminated in the environment (Nielsen *et al*., 1997a, 1997b). The extensive use of antibiotic resistance genes as selectable markers in plants has further raised questions about the possible transfer of the

resistance genes to microbes in the environment. Prerequisites for natural transformation under soil conditions are the availability of free DNA, the development of competence, and the stable integration of the captured DNA into the genome of microorganisms. Hence, long-term persistence of even a small percentage of the released plant DNA is assumed to enhance the likelihood of transformation processes (Gebhard and Smalla, 1999). However, experimental evidence that horizontal gene transfer of genetic material from plants to microorganism can occur is still limited (Broer *et al*., 1999; Nielsen *et al*., 1997a, 1997b). It can be hypothesized that bacteria growing on rotting plant materials which allow a close contact between decaying plant cells releasing transgenic DNA and actively growing bacterial cells could be another scenario for horizontal gene transfer (Bertolla *et al*., 1997, 2000; Gebhard and Smalla, 1998; Lorenz and Wackernagel, 1994).

Diseases causing annual yield losses conservatively estimated at 5% (Song and Goodman, 2001) are among the most significant limiting factors that affect rice production. Thus, there is a need to develop strategies providing durable resistance against the diseases (Adrian *et al*., 1997; Maddox *et al*., 2010). In view of the importance of disease resistance, the transgenic rice plants developed to produce a certain ingredient need to be evaluated on the changes of resistance empirically.

This study was aimed to assess the difference of disease occurrence between transgenic rice variety, Iksan526 transformed with stilbene synthase gene (Baek *et al*., 2013) and non-transgenic rice varieties, Dongjin and Nampyeong in the field. Moreover, in view of the concerns about the transfer of engineered DNA from genetically engineered crop to plant pathogens, we investigated the possibility of horizontal gene transfer in natural or artificial infection conditions.

## Materials and Methods

**Plant materials and cultivation in the confined field.** The transgenic rice variety Iksan526, which was transformed with stilbene synthase gene in variety Dongjin and non-transgenic rice varieties, Dongjin and Nampyeong, were cultivated from May to October 2012 and 2013 in confined rice fields of National Institute of Crop Science, Iksan, Korea. Rice seeds were sown on a nursery bed at April, and the seedlings were transplanted to the plots in mid-May. The other managements, such as irrigation and drainage, fertilization, and pest control were performed following Korean conventional practices for paddy rice plant cultivation.

**Survey of disease occurrence.** For the comparison of the difference of disease resistance between transgenic and non-transgenic rice cultivars, the disease incidence was evaluated from June to September. The average percentage of bacterial grain rot was surveyed by counting infected panicles during ripening stage. The incidence of sheath blight was scored by counting the infected culm and measuring the lesion length of the culum. The number of spots and percentage of infected leaves was also calculated to survey leaf blast and brown spot.

**Isolation of pathogens from transgenic rice variety in the field.** The possibility of horizontal gene transfer was primarily investigated by isolating bacterial and fungal pathogens from infected symptoms of transgenic rice plants. The grains showing grain rot was collected from transgenic rice variety, Iksan526, grounded with a sterile mortar and pestle, and then diluted with distilled water (DW). The 100 μl from each dilution was evenly spread on the plate of *Burkholderia glumae* differential medium S-PG (Tsushima *et al*., 1986) with 3 to 4 replications. Single colony was picked 3 days after incubation (DAI). The pathogen was identified following Fang *et al*. (2009).

For the isolation of *Rhizoctonia solani* and *Bipolaris oryzae,* the infected tissues having distinctive symptoms were collected, surface sterilized with 2% sodium hypochlorite for 2 min, and washed with sterilized DW. The surface sterilized samples of plant tissue were placed on water agar. The single isolates of the fungal pathogens were preliminarily obtained and identified by observing mycelia and spores.

**Repetitive inoculation of pathogens to transgenic rice variety.** To assay horizontal gene transfer, we inoculated pathogens to the transgenic rice variety Iksan526, isolated the pathogens from the inoculated plants, and then re-inoculated the isolated pathogens, which was performed several times (hereafter repetitive inoculation). The seeds of Iksan526 were sown in pots filled with nursery bed soil (Pungnong, Korea), and seedlings were raised in plant growth room (light/dark, 16/8 h, 28 ± 1°C), watered daily, and 20 ml of 1/3 (v/v) strength Hoagland''s solution was poured once a week. *Xanthomonas oryzae* pv. *oryzae* was inoculated following the procedures described by Kauffman *et al*. (1973). Briefly, the races (K1, K2, and K3, KACC10332, 10312, and 10860, respectively) of *X. o.* pv. *oryzae* were cultured on peptone sucrose agar at 28°C, adjusted to $10^8$ colony forming unit (CFU)/ml. The four-week old-seedlings were clip inoculated with sterile scissors dipped in the bacterial suspensions. The inoculated plants were covered with a polythene hood for 24 h, and the seedlings were grown as described above. *Xanthomonas oryzae* pv. *oryzae (X. o.* pv. *oryzae)* was re-isolated 10 to 14 DAI, and stored in −70°C until used for the repetitive inoculation and genomic DNA (gDNA) isolation.

*R. solani* was inoculated following Park *et al*. (2008) with small modifications. Briefly, agar blocks (8 mm diameter) were cut from the outer edge of the culture grown on potato dextrose agar (PDA) for 7 days at 28°C. The agar block was placed on the beneath of the leaf sheath of 4–5 weeks old rice plant. The inoculated sheath was covered with aluminum foil, which was removed when typical lesions appeared after 3 days. The infected plants were left in a humidity chamber to allow for disease development, and the fungal pathogen was re-isolated around

14 DAI, and stored in PDA slants at 4°C until used for the further experiment.

The *Pyricularia grisea* (Teleomorph; *Magnaporthe grisea)* was cultured on rice flour agar and incubated at 25°C under fluorescent lights with a 12 h photoperiod for 2 weeks (Naureen *et al*. 2009). Spores were harvested and adjusted to $5 \times 10^5$ spores/ml. Three weeks old rice plants were inoculated by spraying spore suspensions, and the inoculated plants were covered with a polythene hood for 24 h in the dark. The blast fungus was re-isolated around 14 DAI, and stored in PDA slants at 4°C until used for the next experiment. Each experiment included two replicates per treatment.

**Isolation of gDNA from the pathogens.** For the isolation of gDNA from bacterial pathogens, each isolate of *X. o.* pv. *oryzae* was incubated in LB broth overnight, and 1 ml of cells was pelleted by centrifugation at 13,000 g for 5 min. The cell pellets were resuspended in 1 ml of sterile DW and centrifuged again. DNA was isolated from the pelleted bacterial cells using the QIAamp DNA Mini Kit (Qiagen, USA) following the manufacturer's instructions.

For the isolation of gDNA from fungal pathogens, *R. solani* and *P. oryzae* were cultured in Erlenmeyer flasks (250 ml) containing 50 ml of potato dextrose broth (PDB) at 25°C for 7 days. Mycelia were harvested by filtration through two layers of cheesecloth. Extraction buffer (0.2 ml, 3% SDS (w/v) containing 0.5 mM EDTA, 1.0 M NaCl, and 0.1 mM Tris-HCl, pH 8.0) was added to 10 mg of each fungal mycelium and the suspension shaken vigorously for 15 s. Chloroform-phenol mix (0.2 ml) was slowly added and incubated at 65°C for 5 min. The lysate was centrifuged at 10,000 g at 4°C for 15 min. The supernatant was transferred to fresh tubes. An equal volume of isopropanol was added to each sample, mixed well, and samples were incubated at –0°C for 10 min. Samples were then centrifuged for 20 min at 4°C, at 6000 rpm. The pellet was washed twice with 75% ethanol and centrifuged at 10,000 g, at 4°C for 5 min. The DNA pellets were air dried and resuspended in 100 μl of 1 × TE (10 mM Tris-HCl, 1 mM EDTA) buffer, pH 8.0. Concentration and quality of the isolated DNA were assayed using nanodrop (Shimadzu, Japan).

**PCR conditions.** The DNA amplification reactions were performed in volumes of 25 μl in a PTC100 thermocycler (Bio-Rad, USA). The primer sets, RV-F (5′-ATGGTGTCTGTGAGTG-GAATTC-3′) and RV-R (5′-CGTTATATGGCCACACTGC-3′), and BarF (5′-ATGAGCCCAGAACGACGCCCG-3′) and BarR (5′-TCA-GATCTCGGTGACGGGCAG-3′) were designed to specifically amplify 1,172 bp and 552 bp product by annealing to stilbene synthase and *bar* gene, respectively. The cycling conditions for PCR were as follows: preincubation at 95°C for 5 min; 30 cycles consisting of dsDNA denaturation at 95°C for 30 s, primer annealing at 60°C for 30 s, primer extension at 72°C for 30 s; final elongation at 72°C for 10 min. All amplification products were resolved in 1% agarose gel and stained with ethidium bromide. The 1-KB plus DNA ladder (Invitrogen, USA) was used as molecular size marker.

## Results and Discussion

**Overview of disease occurrence in the field.** In the present study, to compare the differences of disease resistance between transgenic rice and non-transgenic rice cultivars, we surveyed the incidence of rice diseases, such as bacterial grain rot (caused by *Burkholderia glumae*), bakanae disease (*Fusarium moliniforme*), brown spot (*B. oryzae*), sheath blight (*R. solani*), false smut (*Ustilaginoidea virens*), and leaf blast (*P. grisea*) from June to September in the confined rice filed for 2 consecutive years. The average temperature from June to August was 2–3°C higher compared to climatological normal at both years. In addition, because of frequent rainfall, the amount of precipitation at August in 2012 and July in 2013 was higher compared to climatological normal. Despite of the deviation from the normal, the climatic conditions during the survey period at Iksan have been considered optimal to survey the disease resistance of rice plants in the cultivated area.

Diseases are considered as major constraints for rice production. Among the major diseases, bacterial grain rot, bacterial leaf blight, sheath blight, blast, and brown spot cause a significant loss in quality and quality of rice. Among them, sheath blight affects grain filling and panicle emergence, and about 28 to 30% yield reduction was estimated in susceptible cultivars. In this study, the percentage of infected culum was 50.7%, 47.6%, and 47.1% at August 30 in Iksan526, Dongjin, and Nampyeong, respectively (Fig. 1). In spite of application of fungicides which was conventional cultivation practice, the prevalent infection by *R. solani* at the vegetation stages has resulted from high

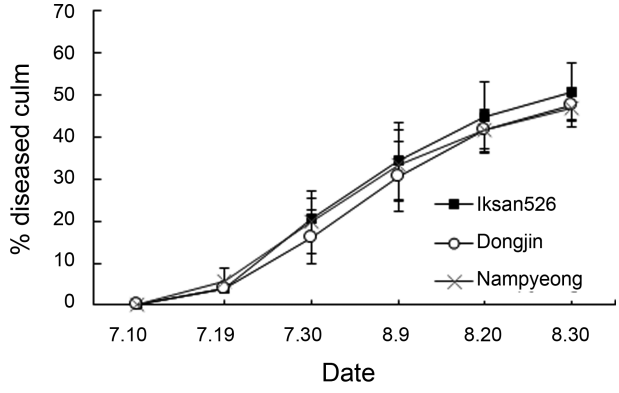


**Fig. 1.** Comparison of sheath blight occurrence between transgenic rice plant (Iksan526) and non-transgenic rice plants (Dongjin, Nampyeong). The severity of the disease was surveyed at the confined field for the experiment of genetically modified plants in Iksan, 2012 and 2013. The surveyed date was 7.10, 7.19 (7.20 in 2012), 7.30 (31), 8.9 (10), 8.20, and 8.30. The bars indicate standard deviations.

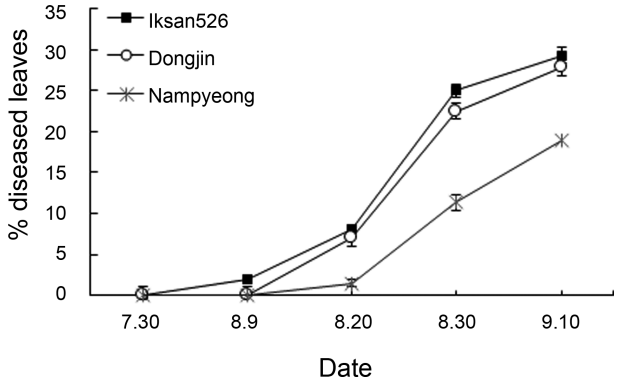


**Fig. 2.** Occurrence of brown spot in transgenic rice plant (Iksan526) and non-transgenic rice plants (Dongjin, Nampyeong). (A) The severity of the disease was surveyed at the confined field for the experiment of genetically modified plants in Iksan, 2012 and 2013. The surveyed date was 7.10, 7.19 (7.20 in 2012), 7.30 (31), 8.9 (10), 8.20, and 8.30. The bars indicate standard deviations.

temperature and constant excessive relative humidity from July to August. The results of this study indicated no significant difference in the periodical incidence and lesion length of sheath blight between transgenic and non-transgenic rice varieties.

The percentage of infected leaves by *B. oryzae* at September 10 was amount to 29.3%, 27.8%, and 19.0% in Iksan526, Dongjin, and Nampyeong, respectively (Fig. 2). There was no significant difference in the percentage of infected leaves between Iksan526 and Dongjin which is the mother plant for the Iksan526. However, Nampyeong showed significantly low infection ratio, which indicate that Nampyeong is more resistant to brown spot compared to Iksan526 and Dongjin. Overall, though the incidence of brown spot was too low (0.01–0.05%) to compare the difference of disease severity, the periodical incidence of brown spot showed similar pattern of increase between transgenic variety Iksan526 and non-transgenic rice variety, Dongjin.

The average percentage of infected panicles by bacterial grain rot at September 10 was lower than 4–5% in all surveyed cultivars (Table 1). Though the occurrence of bacterial grain rot was not severe, there was no difference in the disease severity among the cultivars. In addition, leaf blast, false smut, and bakanae disease was in low amount without any differences among the varieties. The incidence of other diseases have not detected in all rice varieties.

Resveratrol inhibited conidial germination of *Botrytis cinerea* (Adrian *et al*., 1997) and exhibited inhibitory activities against *Xylella fastidiosa* (Maddox *et al*., 2010). In addition, the expression of a stilbene synthase gene increased resistance to *B. cinerea* in transgenic tobacco, barley, and wheat plants (Hain *et al*., 1993; Leckband and Lorz, 1998; Serazetdinova *et al*., 2005). Furthermore, Stark-Lorenzen *et al*. (1997) reported that rice plants transformed with the grapevine stilbene synthase gene exhibited enhanced resistance against the rice blast pathogen *P. oryzae*. However, in transformed tomato, the stilbene synthase gene did not enhance resistance against *B. cinerea* (Kobayashi *et al*., 2000). Considering the data, the results for resistance by transforming with stilbene synthase gene are variable. Hence, the activities in transgenic plants must be evaluated empirically.

The results of this field study indicated that there was no significant difference in the incidence and prevalence of the major diseases between transgenic rice variety and non-transgenic varieties. The results of this study indicated that the production of stilbene may not induce any changes of mechanisms to increase disease resistance.

**Assay for possible horizontal gene transfer to pathogens.** Considering the possibility of horizontal gene transfer of genetic material from transgenic plants to microorganisms, much more favorable conditions could be encountered in plant tissues in which invading pathogens multiply actively (Bertolla *et al*., 1997, 2000; Gebhard and Smalla, 1998; Nielsen *et al*., 1997a, 1997b)). However, experimental evidence that horizontal gene transfer of genetic material from plants to pathogens can occur

**Table 1.** Disease incidence in the confined fields at Iksan during 2012 and 2013

| Disease | Survey date[a] | % occurrence | | | |
|---|---|---|---|---|---|
| | | Plants | Culms | Leaves (area) | Panicles |
| Bacterial grain rot | Sep. 10 | <4–5% | – | – | <0.5 |
| Sheath blight | Aug. 10(9) | 90–100% | 20–45% | – | – |
| | Aug. 30 | 100% | 45–55% | <0.1% | |
| Leaf blast | Aug. 20 | 0–3% | – | 0 | – |
| Brown spot | Aug. 20 | 10–40% | | 1–15% | |
| | Sep. 10 | 55–100% | 10–60% | 2–35% | – |
| False smut | Sep. 10 | <0.1 | – | – | – |
| Bakanae disease | Sep. 10 | <0.1 | – | – | – |

[a]The occurrence of the disease was surveyed at the confined field from June to September in Iksan, Korea, 2012 and 2013. Disease severities at the representative dates for each disease are shown at here. The dates for survey in 2013 are designated in parenthesis.
[b]Average occurrence is shown at here irrespective of varieties.

is still limited.

In this study, to assay the possibility of gene transfer from transgenic plants to bacterial and fungal pathogens, single isolates of *B. glumae, B. oryzae,* and *R. solani* were obtained from infected tissues of Iksan526 cultivated in the field. The stilbene synthase and *bar* gene was not detected from the gDNA of each isolate (Fig. 3), which indicating no transfer of both genes.

The increasing exploitation of transgenic plants in agriculture has generated concerns over the ecological impact of engineered heterologous genes in the environment (Nielsen *et al*., 1997a, 1997b). In addition, the extensive use of antibiotic resistance genes in plants has further raised questions about

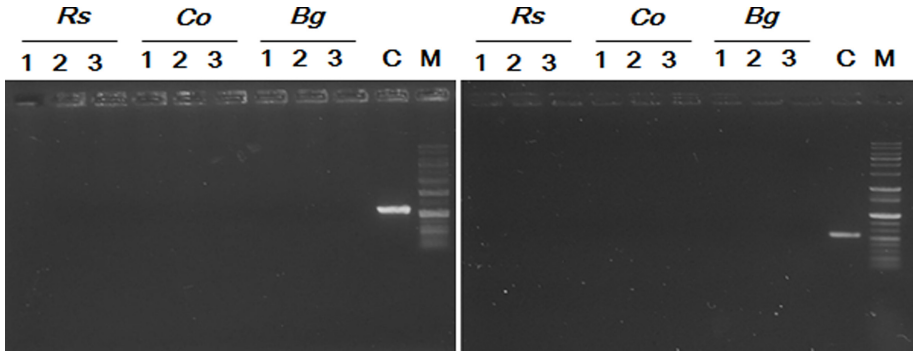


**Fig. 3.** Assay for the possible transfer of stilbene synthase (**left**) or *bar* (**right**) gene from transgenic rice plants to plant pathogens. The isolates of *Rhizoctonia solani* causing sheath blight (*Rs*), *Bipolaris oryzae* causing brown spot (*Co*), and *Burkholderia glumae* causing bacterial grain rot (*Bg*) were obtained from naturally infected rice plants in the field. The PCR was performed using the primers specific for stilbene synthase and *bar* gene. Representative pictures are shown at here.

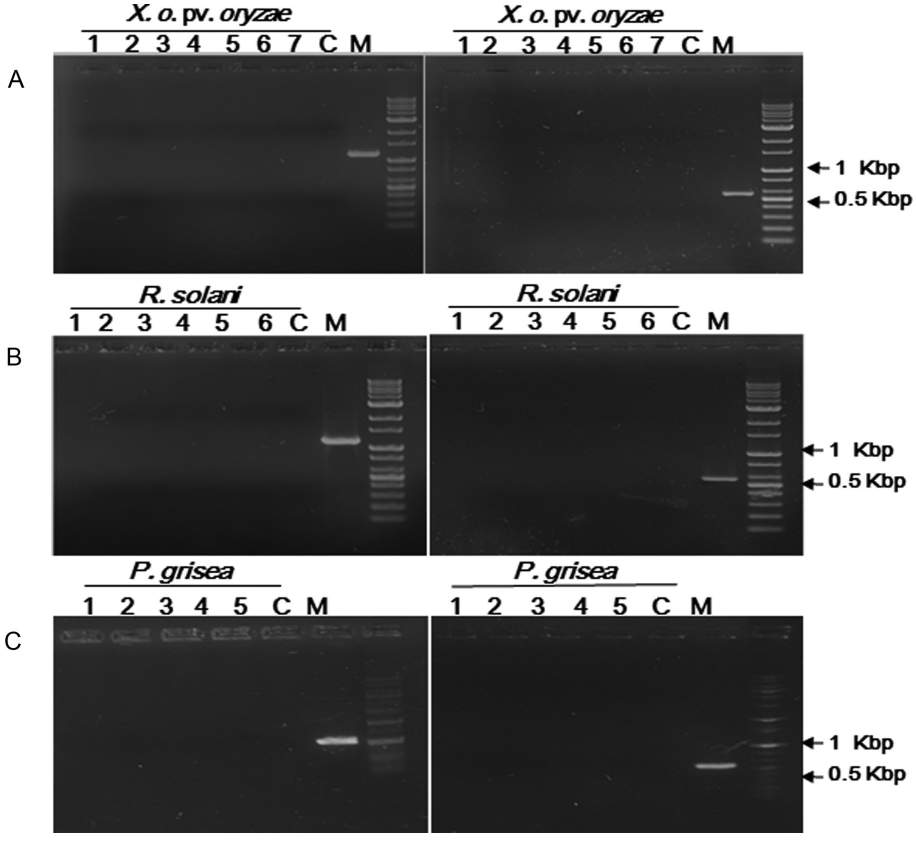


**Fig. 4.** Repetitive inoculation assay for the possible transfer of stilbene synthase (**left**) or *bar* (**right**) gene from resveratrol producing transgenic rice plants to plant pathogens. The *Rhizoctonia solani* (**A**), *Xanthomonas oryzae* pv. *oryzae* (**B**) and *Magnaporthe grisea* (**C**) was re-inoculated consecutively, and the genomic DNA isolated from the each isolate was subjected to PCR using the primers specific for stilbene synthase and *bar* gene.

the possible transfer of the resistance genes to microbes in the environment. (Gebhard and Smalla, 1999). Moreover, *Ralstonia solanacearum* is a naturally competent bacterium which was found to develop competence *in vitro* (Bertolla *et al*., 1997), and linear DNAs were effectively integrated by recombination requiring a minimum of 50 bp of homologous DNA. Therefore, we reasoned that the continual contact between transgenic plants and pathogens from generation to generation could increase the possibility of gene transfer. The possibility of transfer to stilbene synthase or *bar* gene from resveratrol producing transgenic rice Iksan526 to plant pathogens was investigated by repetitive inoculation of *X. o*. pv. *oryzae, R. solani,* and *P. grisea* (Fig. 4). Both genes were not detected from the bacterial and fungal pathogens, which repetitively inoculated at least 5 times. The results of this study indicated that the genes were not transferred to the pathogen.

In view of, about 40% of the world population consumes rice as staple food, development of rice varieties producing resveratrol which has functional value is very important (Baek *et al*., 2013). However, the assessment of possible changes in disease resistance should be evaluated before release of the transgenic variety to farmers. Taken together, the results of this study indicated that there was no significance changes of disease resistance in transgenic rice IKsan526. In addition, the results indicated that rice pathogen, such as *X. o*. pv. *oryzae, R. solani,* and *P. grisea* did not take up transformed DNA under natural and artificial conditions.

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