Inhibitory Effects of Resveratrol and Piceid against Pathogens of Rice Plant, and Disease Resistance Assay of Transgenic Rice Plant Transformed with Stilbene Synthase Gene

Sang-Mi Yu¹, Ha Kyung Lee¹, Ui-Seon Jeong¹, So Hyeon Baek², Tae-Hwan Noh²,
Soon Jong Kwon³ and Yong Hoon Lee^{1,4*}

¹Division of Biotechnology, Chonbuk National University, Iksan 570-752, Korea

²Department of Rice and Winter Cereal Crop, National Institute of Crop Science, Rural Development Administration, Iksan 570-080, Korea

³Department of Agricultural Biotechnology, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Korea

⁴Advanced Institute of Environment and Bioscience, and Plant Medical Research Center, Chonbuk National University, Korea

(Received on June 23, 2013; Revised on July 11, 2013; Accepted on July 13, 2013)

Resveratrol has been known to inhibit bacterial and fungal growth *in vitro*, and can be accumulated in plant to concentrations necessary to inhibit microbial pathogens. Hence, stilbene synthase gene has been used to transform to synthesize resveratrol in heterologous plant species to enhance resistance against pathogens. In the present study, we investigated the antimicrobial activities of resveratrol and piceid to bacterial and fungal pathogens, which causing severe damages to rice plants. In addition, disease resistance was compared between transgenic rice varieties, Iksan 515 and Iksan 526 transformed with stilbene synthase gene and non-transgenic rice varieties, Dongjin and Nampyeong. Minimum inhibitory concentration of resveratrol for *Burkholderia glumae* was 437.5 μ M, and the mycelial growth of *Biplaris oryzae* was slightly inhibited at concentration of 10 μ M. However, other bacterial and fungal pathogens are not inhibited by resveratrol and piceid. The expression of the stilbene synthase gene in Iksan 515 and Iksan 526 did not significantly enhanced resistance against bacterial grain rot, bacterial leaf blight, sheath blight, and leaf blight. This study is the first report on the effect of resveratrol and piceid against pathogens of rice plant, and changes of disease resistance of transgenic rice plants transformed with stilbene synthase gene.

Keywords : Iksan 526, Piceid, Resveratrol, Stilbene synthase, Transgenic rice

Introduction

Phytoalexins have been known to be important natural components in the defense of plants against microbial pathogens. A lot of plants synthesize the stilbene-type phytoalexin resveratrol (trans-3,4,5-trihydroxy-stilbene) when attacked by pathogens (Adrian *et al.*, 1997; Jeandet *et al.*, 2002). Resveratrol is synthesized by the enzyme stilbene synthase, using as substrates one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA (Pezet *et al.*, 2004b). Resveratrol is also glycosylated as piceide (5,4'-dihydroxystilbene-3-O- β -glucopyranoside),

and pterostilbene (3,5-dimethoxy-4-hydroxystilbene) is a dimethylated resveratrol derivative (Jeandet *et al.*, 2002). Viniferins is oxidation product of resveratrol by 4-hydroxystilbene peroxidases. Recently, it was shown that an isomer of ϵ -viniferin, δ -viniferin is one of the major stilbenes produced from resveratrol oxidation in grapevine leaves infected by *Plasmopara viticola* (Pezet *et al.*, 2003).

A number of *in vitro* studies demonstrated the toxicity of stilbenes on fungal and bacterial pathogens. Resveratrol inhibited conidial germination of *Botrytis cinerea* (Adrian *et al.*, 1997). More recently, Pezet *et al.* (2004a) assayed the toxicity of the stilbenes against zoospores of *P. viticola* and reported that δ -viniferin and pterostilbene were the most toxic stilbenes. In addition, resveratrol exhibited inhibitory activities against bacterial pathogens of plants, such as *Xylella fastidiosa* (Maddox *et al.*,

*Corresponding author

Phone) +82-63-850-0841, Fax) +82-63-850-0834

Email) yonghoonlee@jbnu.ac.kr

2010), and human pathogens, such as *Bacillus cereus*, *Staphylococcus aureus*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa* (Paulo *et al.*, 2010; Tegos *et al.*, 2002).

Resveratrol is synthesized from coumaroyl CoA and malonyl CoA by the enzyme stilbene synthase. Since these two substrates are commonly present in plants, introduction of a single gene encoding stilbene synthase may be sufficient to synthesize resveratrol in heterologous plant species, and consequently to enhance natural resistance against fungal pathogens. 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). In transformed tomato plants, stilbene synthase also reduced 65% in disease incidence by *Phytophthora infestans* (Thomzik *et al.* 1997). Transgenic alfalfa had significantly increased resistance to *Phoma medicaginis* (Hipskind and Paiva, 2000). However, the stilbene synthase gene did not augment resistance against *B. cinerea* in transgenic kiwi (Kobayashi *et al.*, 2000).

The antimicrobial activity of plant stilbenes strongly suggests that these compounds are important components for disease resistance. However, the results are variable and cannot be predicted, which indicating the activities in transgenic plants must be evaluated empirically. In the present study, we investigated the antimicrobial activities of resveratrol and piceid to bacterial and fungal pathogens, which causing severe damages to rice plants. Moreover, disease resistance was evaluated in the transgenic rice varieties (Baek *et al.*, 2013), which transformed with stilbene synthase gene.

Materials and Methods

In vitro growth inhibition assay on bacterial pathogens. Growth inhibition activity on bacterial pathogens, *Burkholderia glumae* and *Xanthomonas oryzae* pv. *oryzae* was assayed following Maddox *et al.* (2010) with small modifications. Resveratrol (Sigma, USA) and piceid (polydatin; Sigma) were dissolved in 5% ethanol to a final concentration of 320 mM and filtered through 0.22 µm syringe filters (PALL Life Sciences, USA). The stock solutions were diluted in sterilized distilled water (DW), added to the LB media at 50°C, mixed rapidly, and poured into each well of 24-well plates (Corning, USA). The final concentrations of each compounds in the growth media were 10, 20, 40, 80, 100, 200, 400, 800 µM, and 1, 5, and 10 mM. The wells without resveratrol or piceid were used as control. Ten microliter of bacterial cell suspensions (10^4 colony forming unit (CFU)/ml) was inoculated in the

center of each media. The lowest concentrations of each compound that completely inhibited bacterial growth were considered to be minimum inhibitory concentrations (MICs). Each treatment was replicated three times and the experiment was repeated twice.

Assay on mycelial growth inhibition of fungal pathogens. Inhibition activity on mycelial growth of fungal pathogens, *Bipolaris oryzae*, *Pyricularia oryzae*, *Rhizoctonia solani*, and *Fusarium moniliforme* was evaluated using potato dextrose agar (PDA) medium containing various concentrations of resveratrol and piceid as described above. Resveratrol and piceid solutions were prepared as described above and added to PDA at 50°C to give final concentrations ranging from 0, 10, 100 and 1000 µM. A small plug of agar (6 mm diameter) from a 7–10 day old mycelial culture was placed in the center of each well of 24-well microtiter dish. The diameter of mycelium was measured 5 days after inoculation (DAI). The inhibition of fungal growth was compared to the mycelial colony of 0 mM resveratrol or piceid control. Each treatment was replicated three times and the experiment was repeated twice.

Rice varieties and cultivation. The transgenic rice varieties, Iksan 515 and Iksan 526 (Baek *et al.*, 2013), which was transformed with stilbene synthase gene in variety Dongjin, and non-transgenic rice varieties, Dongjin and Nampyeong, were compared for the difference of disease resistance. The seeds of each variety were sown in pots filled with sterilized nursery bed soil (Pungnong, Korea), and each pot received 20 ml of 1/3 (v/v) strength Hoagland's solution once a week, watered daily and did not receive additional fertilization. Seedlings were raised in plant growth room (light/dark, 16/8 hr, 27°C).

Disease resistance assay. The transgenic and non-transgenic rice seeds were surface sterilized with 2% sodium hypochlorite for 2 min and washed with sterilized DW. The bacterial suspension of *B. glumae* cultured in LB medium for 24 hr at 28°C and 180 rpm was adjusted to 1×10^8 CFU/ml. The surface-sterilized rice seeds were soaked in the *B. glumae* suspension (5 g/100 ml) amended with 0.2% carboxymethyl cellulose (CMC) at 28°C and 150 rpm for 12 hr (Fang *et al.*, 2009). The challenged seeds were air-dried for 12 hr and then sown in the pots as described above. At 7 days after seeding, disease incidence was scored as follows; 0 = seedlings with no symptom, 1 = seedlings with pale yellow leaves, 2 = seedlings with severe chlorosis and stunting, 3 = seedlings killed soon after germination, 4 = seeds rooted without germination. Disease severity was calculated by the following formula: disease index = $(0n_0 + 1n_1 + 3n_2 + 5n_3 + 7n_4)/7N \times 100$; where n_{0-5} is the

number of leaves in each degree (0 to 4) and N is the number of total seedlings investigated. Each treatment consisted of 3 replicates of 50 seeds each (10 seeds/pot) and the experiment was repeated three times.

X. o. pv. oryzae was inoculated following the procedures described by Kauffman *et al.* (1973). The K1 race (KACC10332) of *X. o. pv. oryzae* were cultured on peptone sucrose agar (PSA) for 2 days at 28°C, adjusted to 10⁸ CFU/ml in 10 mM MgCl₂. The three- to four-week old-seedlings were clip inoculated 3 cm below the tip with sterile scissors dipped in the bacterial suspension. The inoculated plants were covered with a polythene hood for 24 hr, and the seedlings were grown as described above. The length of lesion was recorded 14 DAI and the mean lesion length was calculated. Two separate experiments were conducted with ten plants for each treatment.

R. solani was inoculated following Park *et al.* (2008) with small modifications. The fungal isolate was grown on PDA for 7 days at 28°C, and agar blocks (0.5 cm squares) were cut from the outer edge of the culture. The agar block was placed on the beneath of the leaf sheath of 4 weeks old rice plants. The inoculated sheath was covered immediately with aluminum foil. When typical lesions appeared around 3 DAI, the aluminum foil was removed. The infected rice plants were left in a humidity chamber made of clear plastic for 2 weeks to allow for disease development. The lesion length was recorded from each culm and the mean lesion length was calculated. Each treatment consisted of 2 replicates of five plants and the experiment was repeated three times.

P. oryzae (KI197, KI1113) was cultured on rice flour agar and incubated at 25°C under fluorescent lights with a 12 hr photoperiod for 2 weeks (Naureen *et al.*, 2009). Spores were harvested and adjusted to 5 × 10⁵ spores/ml. Four week old rice plants were inoculated by spraying the spore suspensions. Immediately after inoculation, the plants were covered with a polythene hood for 24 hr in the dark. After 7 days of inoculation, the disease incidence was determined by estimating the diseased leaf area from zero percent which represents healthy plants (no chlorosis and necrosis) to 100% which completely diseased plants (intense yellowing and chlorosis). Each treatment consisted of 2 replicates of 5 plants and the experiment was repeated three times.

Statistical analysis. The analysis of variance of each disease incidence was performed with SAS software (Statistical Analysis System 9.2, NC, USA) using Tukey's test. Means were compared by least significant difference (LSD) at *P* < 0.05.

Results and Discussion

***In vitro* bacterial growth-inhibitory activity of resveratrol and piceid.** Plant stilbenes, which are considered as phytoalexins can accumulate in plant tissues to concentrations necessary to inhibit growth of microbial pathogens. Hence, stilbenes have been known to inhibit bacterial and fungal growth *in vitro*, and attracted much attention as a candidate to increase resistance in transgenic plants. However, there is no report on their toxic activities on the pathogens of rice plant.

In the present study, the antibacterial effect of resveratrol and piceid to *B. glumae* and *X. o. pv. oryzae*, which causing bacterial grain rot and bacterial leaf blight (BLB), respectively was assayed using an agar diffusion method. There was no growth inhibition in the control which containing traces of ethanol. Resveratrol suppressed the growth of *B. glumae*, and the MIC of resveratrol to the pathogen was 437.7 µM (Table 1). In the previous reports, resveratrol showed low levels of inhibitory activity against gram-positive species, such as *B. cereus* and *S. aureus*, and gram-negative species, such as *Escherichia coli*, *S. typhimurium*, and *P. aeruginosa* (Paulo *et al.*, 2010; Tegos *et al.*, 2002). More recently, resveratrol exhibited very strong inhibitory activities (MIC = 200 µM) towards all four *X. fastidiosa* strains (Maddox *et al.*, 2010). However, in the present study, the growth of *X. o. pv. oryzae* was not inhibited by resveratrol. Its glucoside piceid showed no inhibitory effect on *B. glumae* and *X. o. pv. oryzae* either. It has been known that resveratrol and piceid are synthesized and accumulated in the leaves of the transgenic rice varieties 0–8.9 µg/g and 1.2–174.4 µg/g (Baek *et al.*, 2013). In addition, the grains of the transgenic rice contained 0.1–4.8 µg/g and 0.1–10.4 µg/g of resveratrol and piceid, respectively. In view of the accumulation amount of the stilbene, the biosynthesis of stilbene in the transgenic rice plant cannot exert inhibitory activity on the bacterial pathogens.

Effect on mycelial growth of fungal pathogens. Many studies reported that resveratrol is toxic to fungal

Table 1. MIC of resveratrol and piceid against bacterial pathogens of rice plant

Pathogens	Minimum inhibitory concentration (µM)	
	Resveratrol	Piceid
<i>B. glumae</i>	437.5 ± 10.5	NI ^a
<i>X. o. pv. oryzae</i>	NI	NI

^aNo inhibition in the concentration from 10 µM to 10 mM.

Fig. 1. Mycelial growth of *B. oryzae* (□), *F. moniliforme* (■), *M. grisea* (▤) and *R. solani* (▥) on culture medium containing (A) resveratrol and (B) piceid. Each fungus was inoculated on the PDA containing each compound. The control was supplemented with an equal amount of ethanol. The same letters are not significantly different at $P < 0.05$ according to Tukey's test. Vertical bars indicate mean $\pm$ standard deviation of the replications. The experiment was repeated two times with three replications.

pathogens. In this study, we investigated the antifungal activity of resveratrol and piceid against *B. oryzae*, *P. oryzae*, *R. solani* and *F. moniliforme*, which causing brown spot, leaf blast, sheath blight, and bakanae disease, respectively to rice plants. When 10 μ M of resveratrol was treated, the growth of *B. oryzae* was reduced to 84.2% compared to untreated control. However, the mycelial growth of *P. oryzae*, *R. solani* and *F. moniliforme* was not inhibited by resveratrol (Fig. 1).

Resveratrol inhibits conidial germination of *B. cinerea* which causing gray mold on grapes (Adrian *et al.*, 1997). When resveratrol was exogenously applied to apples, the penetration of cuticular membranes by *Venturia inaequalis*, which causing apple scab was inhibited.

Moreover, piceid completely inhibited penetration of *V. inaequalis* at concentrations between 200 and 400 g/ml (Schulze *et al.*, 2005). However, resveratrol and piceid have little or no inhibitory activity against *Plasmopara viticola* which causing downy mildew (Pezet *et al.*, 2004). In the same study, pterostilbene, the dimethylated form of resveratrol, had a 5-fold higher activity than resveratrol in inhibiting mycelial growth of *P. viticola* *in vitro*, and δ -viniferin and pterostilbene was the most toxic stilbenes concerning zoospore mobility and disease development. However, piceid has never shown any toxic activity against *P. viticola* zoospores, even at concentrations >1000 mM. Overall, the results including this study indicate that the antifungal activity of resveratrol is very limited and variable to fungal pathogen.

Disease resistance of transgenic rice cultivars. 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* (1997). However, they did not presented the accumulation of the phytoalexin resveratrol in the transgenic rice plant. In addition, the effectiveness of stilbene synthase gene in monocots is very limited.

In the present study, resistance or susceptibility of transgenic rice cultivar, Iksan 515 and Iksan 526 on bacterial grain rot, BLB, and sheath blight was evaluated in growth room conditions. The transgenic rice cultivars didn't show any difference of resistance to bacterial grain rot compared with Dongjin and Nampyeong (data not shown).

The incidence of BLB was evaluated by clip inoculation of *X. o. pv. oryzae* to each rice cultivar. Though disease severity in Iksan 526 was slightly reduced compared to the incidence of Nampyeong, all of the tested varieties was susceptible to BLB (Fig. 2). The results indicated that the transgenic rice didn't acquire any resistance to BLB.

The susceptibility on sheath blight was compared by lesion length after artificial inoculation of *R. solani*. The transgenic rice varieties and non-transgenic control varieties were very susceptible to sheath blight (Fig. 2, Fig. 3). The results indicated that the biosynthesis of resveratrol in Iksan 515 and Iksan 526 did not increase the resistance to sheath blight. In addition the incidence of blast in transgenic rice varieties didn't show any significant difference in comparison to non-transgenic varieties.

Stilbene synthase genes isolated from grapevine have been transformed into tobacco (Hain *et al.*, 1993), tomato (Thomzik *et al.*, 1997), rice (Stark-Lorenzen *et al.*, 1997), barley and wheat (Leckband and Lorz, 1998), kiwi (Kobayashi *et al.*, 2000), alfalfa (Hipskind and

Fig. 2. Severity of bacterial leaf blight (**A**) and sheath blight (**B**) by artificial inoculation of *X. o. pv. oryzae* and *R. solani*, respectively. The picture was taken 14 DAI in transgenic rice (Iksan 515, Iksan 526) and non-transgenic rice varieties (Dongjin, Nampyeong).

Fig. 3. Comparison of sheath blight incidence between transgenic (Iksan 515, Iksan 526) and non-transgenic rice varieties (Dongjin, Nampyeong). Each plant was inoculated by mycelial plug of *R. solani*. The lesion length was recorded at 14 DAI.

Paiva, 2000), and apple (Szankowski *et al.*, 2003). Though the resistance was increased in most of cases, in some cases, expression of stilbene synthase did not result in an improved pathogen resistance. For instance, Kiwi fruits transformed with the stilbene synthase gene produced

piceid but did not increase resistance to *B. cinerea* (Kobayashi *et al.*, 2000). In addition, transgenic white poplars expressing *Vitis* stilbene synthase did not showed any effects in *in vitro* bioassays against *Melampsora pulcherrima*, a rust pathogen (Giorcelli *et al.*, 2004). Pezet *et al.* (2004b) reported that resistance of grape leaves to *P. viticola* is associated with the conversion of resveratrol to viniferins while susceptibility is associated with the formation of piceid from resveratrol.

The results of this study indicated that the expression of stilbene synthase gene did not induce any changes of mechanisms to increase disease resistance. No stimulation of disease resistance may be related to the production of the glucosylated form of resveratrol, viz. piceid or low concentration of resveratrol (0–8.9 µg/g leaf).

The main goal of this investigation was to assess toxic effects of resveratrol and piceid on pathogens which causing devastating damage to rice plants and changes in disease resistance of the transgenic rice varieties which producing stilbenes. Overall, though the resveratrol inhibited the growth of some pathogens, both compounds showed no significant inhibitory activity on the growth of bacterial and fungal pathogens of rice plants. In addition, the expression of the stilbene synthase gene did not enhance resistance to diseases in transgenic rice cultivars. To our knowledge, this is the first report that the effect of resveratrol and piceid was specifically tested against pathogens of rice plant. The *in planta* production and modifications of resveratrol after infection of pathogens need more investigation.

Acknowledgment

This study was supported by a grant from the Next-Generation BioGreen 21 Program (No. PJ0095282013), and S. M. Yu was partly supported by Cooperative Research Program for Agriculture Science & Technology Development (Project No. PJ0083112011), Rural Development Administration, Republic of Korea.

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