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Probenazole Promotes Root Growth and Suppresses Expression of Pathogenesis-related Proteins in Roots of Rice Seedlings

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Ⅰ. Introduction

Although rice is the staple food for more than half of the human population, its cultivation is hampered by problems such as low temperatures, fungal infections, insect damage and drought, which all serve to reduce crop yield. In cool and humid climatic conditions, rice blast fungus (*Magnaporthe grisea*) often causes severe damage to rice production. Under these conditions, rice crops are often treated with an agrochemical such as Oryzemate (Meiji Seika, Japan) to counter rice blast fungal infection. The active ingredient of Oryzemate is probenazole (IWAI *et al*., 2007), and is applied as a granular treatment to the paddy field or used as a seedling box treatment (WALTERS and FOUNTAIN, 2009). Probenazole is absorbed by the roots and is then transferred systemically throughout the plant (IWATA, 2001). In the cells, probenazole is converted into 1,2-benzisothiazole-1,1-dioxide where it induces salicylic acid (SA) accumulation and induces expression of pathogenesis-related genes (YOSHIOKA *et al*., 2001; MIDOH and IWATA, 1996; IWAI *et al*., 2007; UMEMURA *et al*., 2009). SA acts as a key endogenous signaling molecule that mediates systemic acquired resistance (UMEMURA *et al*., 2009).

SA also affects plant growth and development (VICENTE and PLASENCIA 2011). Growth promotion occurs following SA treatment in soybean (GUTIERREZ-CORONADO *et al*., 1998), wheat (SHAKIROVA *et al*., 2003), maize (GUNES *et al*., 2007), chamomile (KOVACIK *et al*., 2009), rice (MOHAMMED 2009) and fennel (HASHMI *et al*., 2012). In soybean, foliar spray with SA in the range 10 nM to 10 mM not only promotes shoot growth but also accelerates root growth. In wheat, a presowing treatment of seeds with 50 μM SA promotes germination and subsequent seedling growth, and results in an increased yield. In maize, application of 0.1 – 1 mM SA to the soil increases the dry yield of plants under both saline and non-saline conditions. Chamomile plants grown in a solution containing 50 μM SA exhibit increased biomass of leaf rosettes and roots, whereas, in a solution containing 250 μM SA, they show inhibition of growth. The treatment of rice leaves with 1 mM SA increases the number of filled grains per panicle and produces an increased grain yield. Foliar spray of fennel plants with 0.1 mM SA significantly increases fresh and dry weights of shoots and roots, seed yield and oil yield. Increased plant growth has also been reported in rice and sunflowers that have higher levels of endogenous SA accumulation following a bacterial infection (SAIKIA *et al*., 2006; FORCHETTI *et al*., 2010). In rice, growth was promoted by infection with *Pseudomonas aeruginosa* that induces the accumulation of endogenous SA in roots. In sunflowers under water stress, seedling growth is promoted by SA produced by endophytic bacteria.

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However, exogenous SA has also been reported to have a negative effect on growth. For example, PANCHEVA *et al*., (1996) reported that leaf and root growth are reduced in barley plants and TRAW and BERGELSON (2003) found that SA (100 μM and 1 mM) reduces trichome density and number in Arabidopsis. High endogenous levels of SA are associated with a dwarf phenotype in Arabidopsis mutants (BOWLING *et al*., 1997; RATE *et al*., 1999; RATE and GREENBERG 2001). KUREPIN *et al*., (2012) reported that in the shade ecotype of *Stellaria* longipes, endogenous SA levels are increased and dry shoot biomass is decreased in plants grown under normal compared to reduced light quality.

Although probenazole is known to induce SA accumulation, which might affect plant growth and development as described above, and has been used to protect rice plants from rice blast fungus for over three decades, to date no study has examined its effect on the growth of rice plants. Here, we treated rice seedlings with probenazole and monitored root and shoot growth. We found that root growth was significantly accelerated by probenazole treatment. In addition, we found that probenazole treatment altered endogenous SA levels, and expression of genes related to SA metabolism enzymes and of pathogenesis related proteins.

Ⅱ. Materials and Methods

1. Plant materials and probenazole treatment

Rice (*Oryza sativa* Japonica group cultivar 'Oborozuki') seeds were washed with sterilized water and then soaked in water for two days at 28℃ in the dark. After being soaked, germinating seeds were placed in hydroponic conditions in the dark. Nine seeds were placed on a plastic grid (ca. 35 x 35 mm), which was then floated in a plastic cup holding 100 ml of distilled water with various concentrations of Oryzemate (48% probenazole (w/w), Meiji Seika, Japan) or SA. Acetone has been used as a solvent to dissolve probenazole (IWAI *et al*., 2007); however, we found that even a low concentration of acetone had an adverse effect on rice root growth. We therefore used water-soluble Oryzemate instead of probenazole. Since Oryzemate contains substances such as minerals other than probenazole, the control solution should have contained those additive substances. However, we used distilled water as a control because details of those additive substances are not disclosed by the manufacturer. SA was initially dissolved in 100 μL dimethyl sulfoxide (DMSO) and a 100 mM stock solution was made up with distilled water and adjusted to pH 5.8 with KOH. Hydroponic culture was performed in a growth chamber (continuous dark, 25℃) for 1, 4 or 7 days. After incubation, the lengths of the primary roots and shoots were measured.

2. Quantitative reverse transcriptional (QRT)-PCR

Root and shoot samples were collected from 4-day-old plants grown with or without 100 μM probenazole or 10 μM SA in the dark. Tissue samples were frozen with liquid N_2 and stored in a -80℃ freezer until use. Total RNA was extracted from 100~200 mg of tissue samples using FastRNA PRO GREEN KIT (MP Biomedicals, USA). RNA samples were treated with DNase I (TaKaRa Bio, Japan) to remove genomic DNA and then subjected to chloroform purification to remove the DNase I. Complementary DNA (cDNA) was synthesized from 0.5 μg samples of the RNA using PrimeScript RT master mix (TaKaRa Bio, Japan). The levels of expression of the following groups of genes were examined after probenazole or SA treatment by QRT-PCR with a LightCycler Carousel-based system (Roche, Germany) using LightCycler TaqMan Master (Roche, Germany): the pathogenesis related genes, *PR2* (Os01g0940700), *PR3* (Os10g0416500), *PR5* (Os12g0628600), *PBZ1* (Os12g0555500), and *RSOsPR10* (Os12g0555000); genes associated with SA metabolism, isochorismate synthase (Os09g0361500), phenylalanine ammonia-lyase (Os02g0627100, Os02g0626100, Os05g0427400), chorismate mutase (Os01g0764400, Os12g0578200) and salicylate

glycosyl transferase (Os09g0518200). The gene-specific primer sets and universal TaqMan probes were designed using information from the website https://www.roche-applied-science.com/sis/rtpcr/upl/index.jsp?id=UP030000. The primer sequences and corresponding universal probes are listed in Table 1. Relative mRNA abundance was normalized against the level of ubiquitin (CHEN *et al*., 2009). Three biological replicates were performed.

3. Salicylic acid quantification

Extraction and quantification of endogenous SA levels were performed as described by MALAMY *et al*., (1992) and YASUDA *et al*., (2008) with minor changes. Plant tissues (approximately 100 mg) were homogenized in 2 ml of 90% methanol (v/v) and centrifuged at 13000 x *g* for 5 minutes. The pellet was extracted again by adding 100% methanol and centrifuged. Methanol extracts were combined and dried under vacuum. Dried samples were suspended with 1 ml of distilled water at 80℃ for 5 minutes. Samples were divided into two portions for analyzing free SA and total SA (sum of free and conjugated SA). For free SA extraction, a 250 μl aliquot was acidified by adding 10 μl of HCl and then vigorously mixed with 500 μl

Table 1. Primer and probe sets used for QRT-PCR

Locus ID	Description	Universal Probe	Primer sequence
Os09g0361500	Isochorismate synthase (ICS)	#83	F: GGGCCAAAATGCTTATCAGT
			R: AGTATTTCCGATGAAATAATTGCTACT
Os02g0627100	Phenylalanine ammonia-lyase (PAL)	#25	F: GCACCGACGGTCATGTTT
			R: ACCGGATGCCGGAGTATC
Os05g0427400	Phenylalanine ammonia-lyase (PAL)	#53	F: CTCATGTCCTCCACCTTCCT
			R: GACGTTCTCCTCGATGTGG
Os02g0626100	Phenylalanine ammonia-lyase (PAL)	#152	F: CATCGTCAATGGCACGTC
			R: CATCACCTCGCAGAACACC
Os01g0764400	Chorismate mutase (CM)	#136	F: TCAACAAGGAGATTTGGAAAATG
			R: CTTCCTTCTTTCACTAATCTTGGAA
Os12g0578200	Chorismate mutase (CM)	#67	F: AGGTACAAGAGCCCGGATG
			R: CATACTCAACAGATGGCTCCAC
Os09g0518200	Salicylate glycosyl transferase (SA-GTase)	#5	F: CCTCGTCAACTCCTTCTACGA
			R:GAGGCGGTTGTCGAGGTA
Os01g0940700	PR2	#88	F: TACATCACGGTCGGCAAC
			R: GCTGTTCATGTTCTGCATGG
Os10g0416500	PR3	#141	F: CCATGGTCAGCAGCTACAAG
			R: TCGATGGACGATCAGTGG
Os12g0628600	PR5	#149	F: CTCTTCCGCTGTCCTCGT
			R: GGTTGGTGATGGTGAAGGTC
Os12g0555500	PBZ1	#34	F: CTGCCGAATACGCCTAAGAT
			R: CATTTCTGCGGCTCTCATTA
Os12g0555000	RSOsPR10	#38	F: TGCTTAAAAATTGTCATAAACCAAA
			R: GGATTTGTCGTGGCTCACA
Os03g0234200	Ubiquitin	#42	F: TGGATGTTGTTGAACCGTGT
			R: GACCAATATAGTTTGCTGCCAAT

Primer design and universal probe selection were performed using data from the Roche Applied Science website.

of 50% cyclohexane/50% acetate (v/v). The upper organic phase was subsequently collected into a new tube. The partitioning step was repeated twice and the combined organic phase was dried by vacuum. The dried residue was dissolved with 20 mM sodium acetate (pH 5.0) containing 20% methanol (v/v).

To extract total SA, the second 250 μl aliquot was mixed with an equal volume of 10 unit/ml β-glucosidase (Oriental Yeast Co. Japan). The mixture was incubated at 37℃ for 10 hours. After the glucosidase digestion, samples were partitioned using organic solvents as described above. The resultant extracts were analyzed using the HPLC CLASS-LC10 system (SHIMAZU, Japan) equipped with a TSK-gel ODS-80 column (4.6 x 150 mm; Tosoh, Japan). The amount of SA O-beta-glucoside (SAG) was estimated by subtracting the measured free SA from the measured total SA.

Ⅲ. Results

1. Probenazole promotes root growth

Rice seedlings were grown under hydroponic conditions and treated with probenazole for 4 days. The probenazole-treated seedlings had longer seminal roots than the control seedlings, but there was no significant difference between the two groups for shoot lengths (Fig. 1a). As the largest effect on root growth was observed using 100 μM probenazole, this concentration was selected for all subsequent experiments.

Next, germinated rice seeds were treated with 100 μM probenazole for 1, 4 or 7 days and seminal root and shoot lengths were measured. The probenazole treated seedlings had longer roots after 4 and 7 days of culture compared to control (untreated) seedlings (Fig. 1b). As described above in the initial experiment, probenazole had no significant effect on shoot lengths (Fig. 1c).

The effects of exogenous SA on root growth were examined by treating rice seedlings with 10 or 100 μM SA. Root elongation in seedlings treated with 10 μM SA was found to be greater than controls and to be similar to that observed after treatment with 100 μM probenazole; however, 100 μM SA severely inhibited root growth (Fig. 1d).

2. Probenazole reduces endogenous SA levels in roots

Endogenous SA levels were measured in control seedlings and in seedlings grown in100 μM probenazole for 4 days. The roots and shoots were separated for analysis of endogenous SA contents. In the root tissue, free SA was decreased by the probenazole treatment (Fig. 2a), whereas the level of free SA was not altered in shoots after probenazole treatment (Fig. 2b). The amount of SAG in roots was not affected by probenazole treatment (Fig. 2a), however, the amount of SAG in shoots was increased (Fig. 2b).

We examined the effect of probenazole treatment on expression of genes associated with SA metabolism by QRT-PCR (see Materials and Methods and Fig. 3). The salicylate glycosyl transferase (SA-GTase) gene (Os09g0518200) was found to show significantly increased expression in roots after probenazole treatment; there was also a significant increase in expression in shoots, albeit from a markedly lower level than in roots (Fig. 3g). The phenylalanine ammonia-lyase (PAL) gene (Os02g06260100) and the chorismate mutase (CM) gene (Os12g0578200) showed increased expression in roots but not shoots after probenazole treatment (Fig. 3c and 3f). Probenazole treatment did not affect the level of expression of the isochorismate synthase (ICS) gene (Os09g0361500) in either roots or shoots (Fig. 3a).

3. Probenazole reduces the levels of expression of genes for pathogenesis related proteins in roots

We carried out a similar QRT-PCR analysis as above for expression of genes associated with pathogenesis related (PR) proteins in control, and probenazole or SA treated seedlings. The treated seedlings were exposed to 100 μM probenazole or 10 μM SA for 4 days, and roots and shoots were

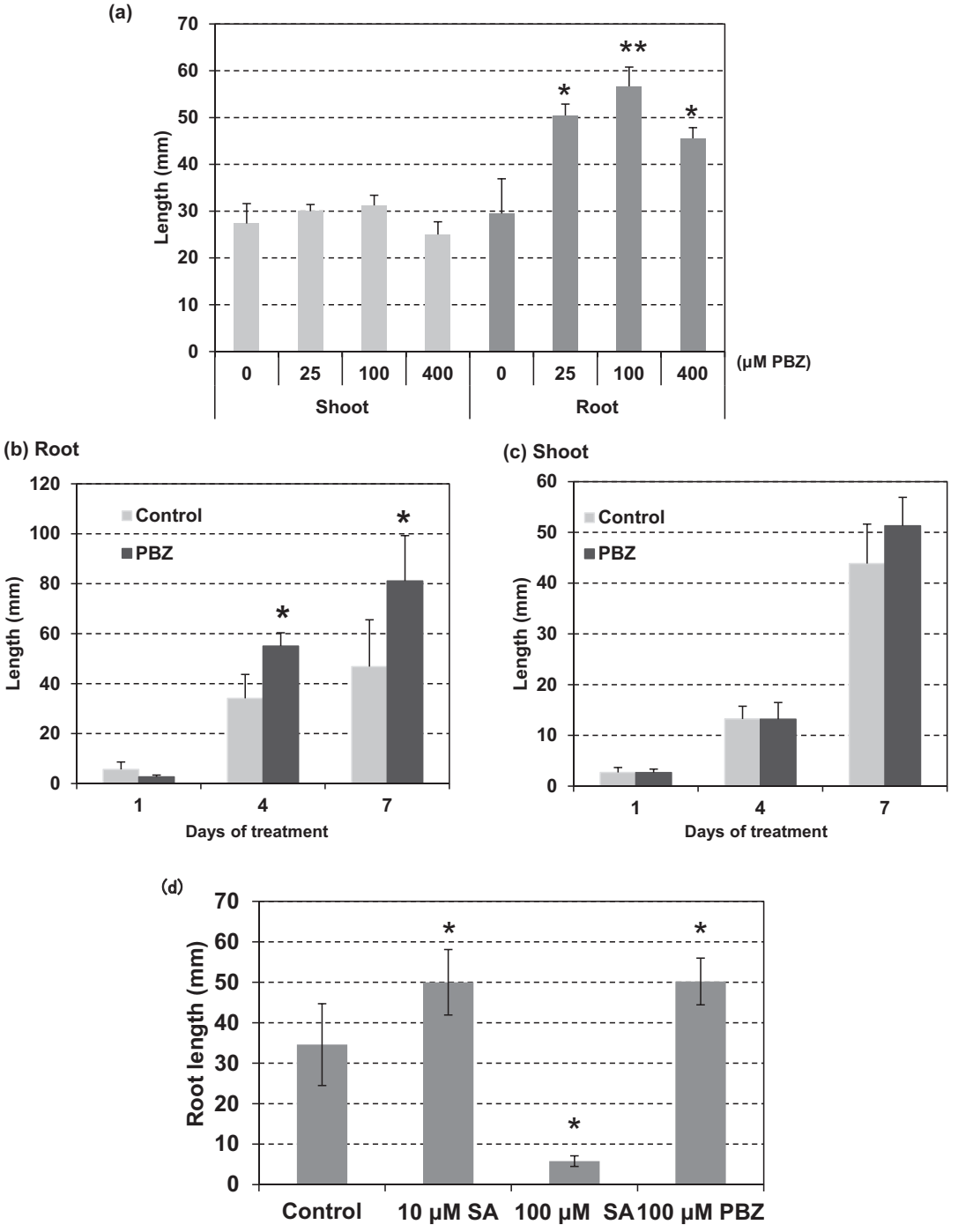

Fig. 1. The effect of probenazole on early growth of dark-grown rice seedlings.

(a) The effects of different doses of probenazole (PBZ) on root and shoot lengths. Shoot and root lengths were measured after 4 days. Error bars show standard deviation (SD) (n = 9). Asterisks indicate a significant difference from the control mean (*$P < 0.05$, **$P<0.01$, Student's t test).

(b, c) The effect of probenazole on growth rates in rice seedlings. Plants were grown in the dark for 1, 4 or 7 days with or without 100 μM PBZ. The lengths of roots (b) or shoots (c) were measured at each time point. Error bars show the SD (n = 27). Asterisks indicate significant differences in mean root lengths at 4 or 7 days compared to controls ($P < 0.05$, Student's t test).

(d) Effect of SA on seedlings grown for 4 days in the dark with or without 10 μM SA, 100 μM SA or 100 μM PBZ. Error bars show the SD (n = 9). Asterisks indicate a significant difference from the control mean ($P < 0.05$, Student's t test). The experiments were repeated three times with similar results.

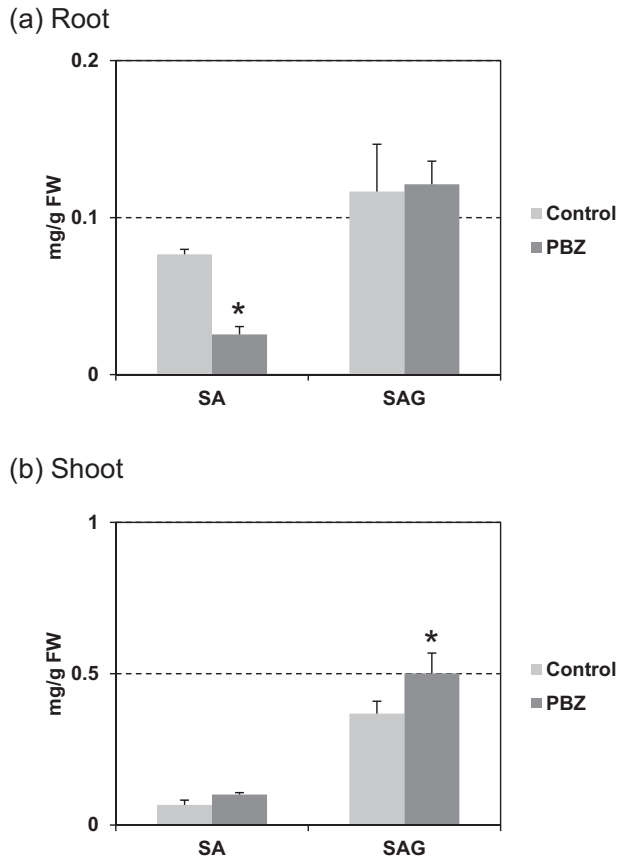

Fig. 2 Determination of endogenous SA levels in probenazole treated roots.

SA levels in roots (a) and shoots (b) were measured in 4-day-old seedlings grown in the presence of 0.1% DMSO (control), 100 μM probenazole (PBZ), 10 μM SA, or 100 μM SA in the dark. SA and SAG indicate free SA and SA O-beta-glucoside, respectively. Bars show the SD (n = 3). Asterisks indicate a significant difference from the control mean ($P < 0.05$, Student's *t* test).

separated for analysis. The levels of expression of the *PR2*, *PR3*, *PR5* and *RSOsPR10* genes were higher in roots than shoots of the control seedlings (Fig. 4). Probenazole or SA treatment caused a reduction in the levels of expression of *PR2*, *PR3*, *PR5*, *PBZ1* and *RSOsPR10* in roots but had no effect on expression levels in shoots (Fig. 4). Probenazole induced a greater reduction in expression of *PR2*, *PBZ1* and *RSOsPR10* than exogenous SA (Fig. 4).

Ⅳ. Discussion

In this study, we have demonstrated that increased root growth is induced in rice seedlings treated with probenazole (Fig. 1). Although SA has been reported to promote plant growth (Gutierrez-Coronado *et al*., 1998; Shakirova *et al*., 2003; Gunes *et al*., 2007; Kovacik *et al*., 2009; Mohammed 2009; Hashmi *et al*., 2012), this is the first study to show that probenazole promotes root growth. Since exogenous SA also promotes root growth (Fig. 1), probenazole probably affects root growth through SA biosynthesis or SA signals. In roots, probenazole induces increased expression of the PAL and CM genes (Fig. 3) that are related to the PAL mediated pathway for synthesis of SA (Dempsey *et al*., 2011); however, probenazole did not affect expression of the gene for ICS (Fig. 3), which is required for the isochorismate pathway for synthesis of SA (Dempsey *et al*., 2011). Although the gene for SA-GTase, which converts free SA to SAG, was up-regulated by probenazole both in shoots and roots, the increase was much larger in roots than in shoots (Fig 3). The level of free SA was reduced in roots after probenazole treatment but was not altered in shoots (Fig. 2). There are many reports showing that SA accumulates in probenazole-treated plants; however, none of these have suggested that free SA is reduced in the roots after treatment. The reduction in free SA levels in roots might result from the increased level of SA-GTase gene expression. However, SAG did not increase in the roots (Fig. 3). Further investigation will be required to elucidate the metabolic mechanisms of free SA reduction in roots by probenazole.

One possible mechanism for the faster growth in roots after probenazole treatment is that the reduction in free SA diminishes the known negative effect of the hormone on plant growth (Bowling *et al*., 1997; Rate *et al*., 1999; Rate and Greenberg 2001; Kurepin *et al*., 2012). However, this mechanism seems unlikely because we observed accelerated root growth after SA

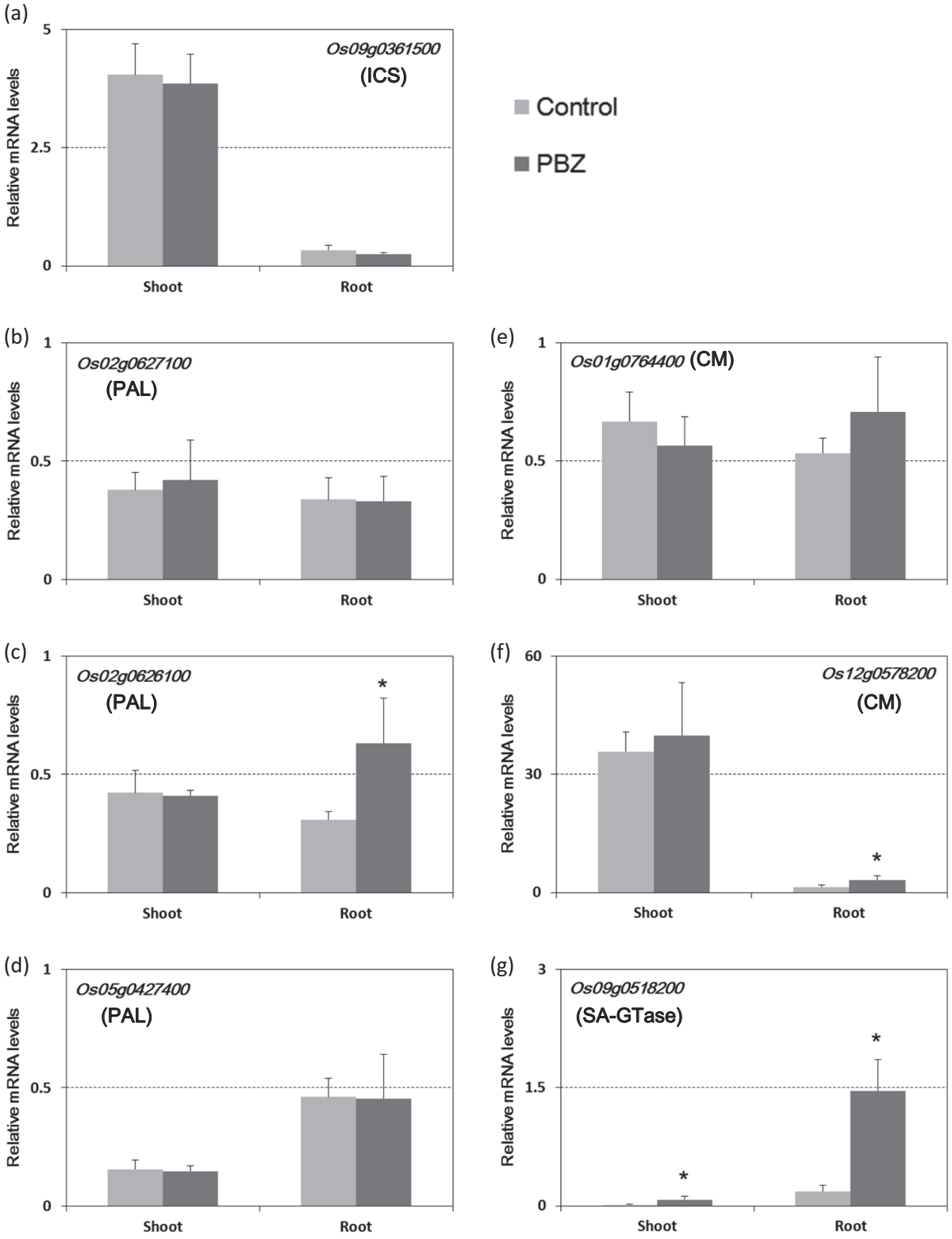

Fig. 3 The effect of probenazole on the expression of genes related to SA metabolism.

Rice seedlings were treated with or without 100 μM probenazole (PBZ) in the dark for 4 days and total RNAs extracted from their shoots or roots. Gene transcript levels were analyzed by QRT-PCR: (a) isochorismate synthase; (b, c and d) phenylalanine ammonia-lyase; (e and f) chorismate mutase; and (g) salicylate glycosyl transferase. Gene IDs are shown in each figure. Transcript levels were normalized against the ubiquitin gene. Bars show the SD (n = 3). Asterisks indicate a significant difference from the control mean ($P < 0.05$, Student's t test).

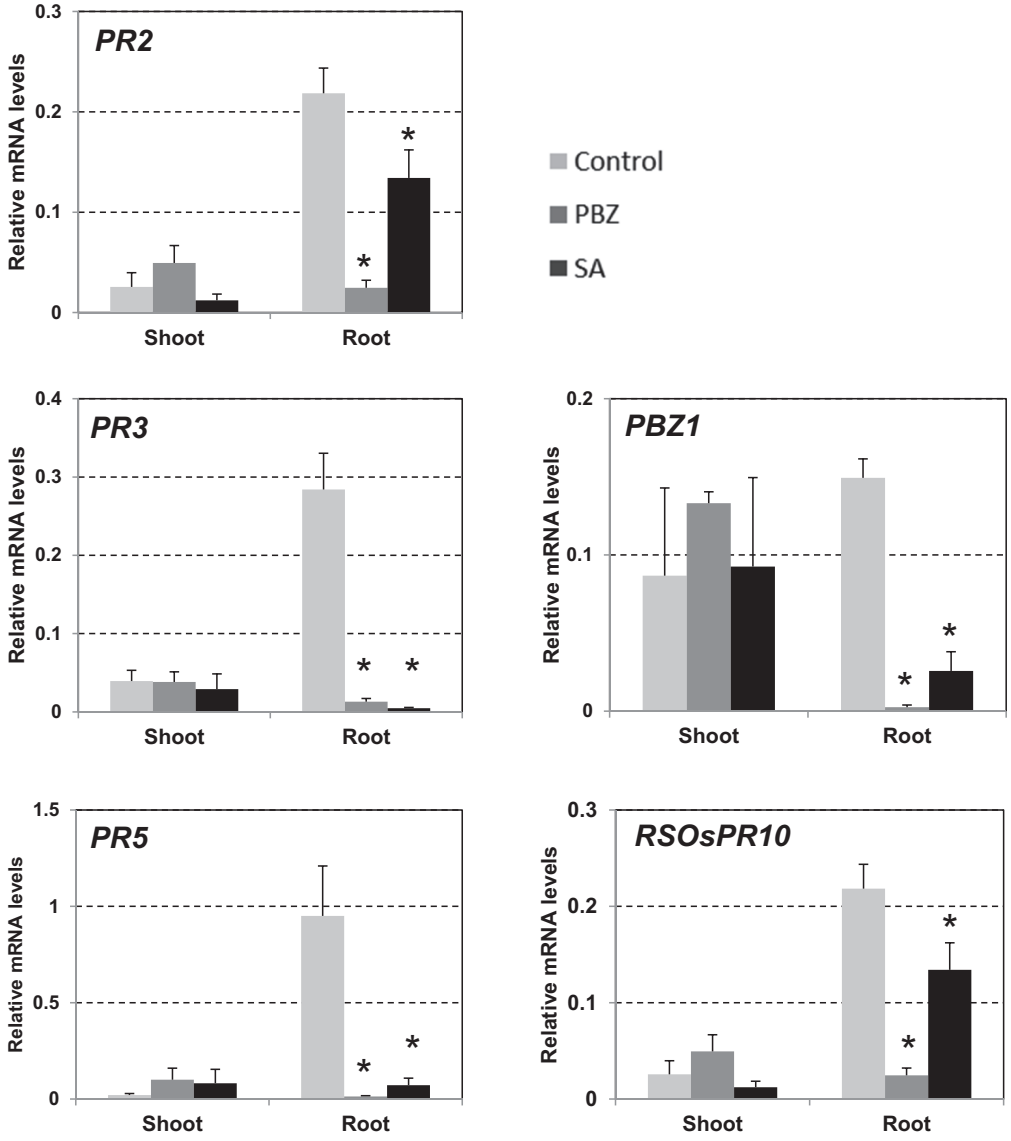

Fig. 4 Analysis of expression of genes for pathogenesis related proteins in probenazole or SA treated seedlings.

Rice seeds were treated for 4 days with 0.1% DMSO (control), 100 μM probenazole (PBZ) or 10 μM SA. Total RNAs from shoots or roots were used for QRT-PCR. Expression levels were normalized against the ubiquitin gene. Bars show the SD (n = 3). Asterisks indicate a significant difference from the control mean ($P < 0.05$, Student's *t* test).

treatment (Fig. 1). Treatment of roots with SA caused an increase in endogenous free SA content compared to control and probenazole treated roots (data not shown). Thus, there does not appear to be a clear correlation between endogenous SA levels and root growth. Possibly, further insight into the mechanism might be obtained through use of SA-deficient transgenic rice expressing nahG (Yang *et al.*, 2004).

We examined the effects of probenazole and exogenous SA treatments on the levels of expression of genes for pathogenesis related proteins. Probenazole has previously been shown to induce increased expression of the *PR2, PR3*, *PR5* and *PBZ1* genes in leaves and of *RSOsPR10* in roots (MEI *et al.*, 2006; KANO *et al.*, 2010; MAHMOOD *et al.*, 2009; IWAI *et al.*, 2007; HASHIMOTO *et al.*, 2004). Here, we found a significant reduction in

expression of all these genes in roots but not shoots after either probenazole or exogenous SA treatment (Fig. 4). The non-induction of PR genes by probenazole in shoots might be due to the age of the plants when treated. IWAI *et al*., (2007) demonstrated that probenazole induces accumulation of PR proteins in adult rice plants but not in young seedlings. It is also possible that the dose of probenazole used may have affected the outcome. Here, we treated the seedlings with 100 μM probenazole, whereas previous studies used 450 – 500 μM probenazole (IWAI *et al*., 2007; MAHMOOD *et al*., 2009; HASHIMOTO *et al*., 2004). Although the unexpected failure to induce increased expression of PR genes in shoots might be ascribed to differences in plant ages and dose of probenazole, the suppression of their expression in roots cannot be explained by these factors.

MEI *et al*., (2006) reported that levels of expression of *PR2*, *PR2*, *PR3* and *PR5* genes increased in transgenic rice plants that accumulated a high concentration of endogenous jasmonic acid (JA) compared to wild type plants. HASHIMOTO *et al*., (2004) and TAKEUCHI *et al*., (2011) subsequently found that exogenous JA strongly induces *RSOsPR10* expression in roots. Analyses of mutant and transgenic plants have demonstrated that jasmonic acid has an important role in defense gene expression in tomato, tobacco, and potato (MEI *et al*., 2006). It is also well known that antagonistic interactions between the SA and JA signaling mechanisms modulate expression of defense genes (TAKAHASHI *et al*., 2004). Therefore, it is conceivable that probenazole and exogenous SA suppress the PR genes through antagonistic suppression of JA signals in roots of young rice seedlings.

The over-expression of *NH3* (an *NPR1* paraolog) driven by the *Ubi-1* promoter causes toxicity leading to lethality in transgenic rice (BAI *et al*., 2010). It has also been reported that transgenic rice plants over-expressing *WRKY45* show retarded growth (SHIMONO *et al*., 2007). Genes for PR proteins are probably expressed constitutively in these transgenic plants because *NH3* and *WRKY45* are positive regulators of defense response signals. Toxicity and retarded growth in these transgenic rice plants may be associated with this constitutive expression of genes for PR proteins. This suggests that the increased rate of root growth induced by probenazole or SA treatments might be caused by suppression of genes for PR proteins. Since we did not find any change in the levels of expression of these genes in shoots after probenazole and SA treatment, there may be another mechanism for regulation of gene expression by probenazole and SA in roots and shoots. A future study will be initiated to investigate the mechanisms of tissue-specific responses to probenazole and SA.

Ⅴ. Summary

Probenazole is the active ingredient of Oryzemate, an agrochemical that is widely used for protection of rice plants against the rice blast fungus *Magnaporthe grisea*. We found that treatment of rice seedlings with probenazole resulted in an acceleration of root growth. Although probenazole treatment has been shown previously to cause an accumulation of salicylic acid (SA), we found here that the SA levels in treated seedling roots were reduced. Expression of the gene for salicylate glycosyl transferase (SA-GTase), which converts SA to SA O-beta-glucoside (SAG), was induced in roots by probenazole treatment. Treatment of roots with either probenazole or SA significantly reduced expression of genes for the pathogenesis-related proteins PR2, PR3, PR5, PBZ1 and RSOsPR10.

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プロベナゾールはイネ実生の根の生育を促進し、感染特異的タンパク質遺伝子の発現を抑制する

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摘　要

プロベナゾールはイネいもち病防除のために広く用いられている農薬「オリゼメート」の主要成分である。我々は、プロベナゾール処理によりイネ幼苗の主根の伸長が促進されることを見出した。プロベナゾールは植物体内において、病害抵抗性を誘導することが知られているサリチル酸(SA)の蓄積を誘導することが報告されているが、本研究によってプロベナゾール処理された根ではSA濃度が減少することが明らかとなった。プロベナゾール処理された根では、SAをSA O-beta-glucoside（SAG）に変換するSalicylate glycosyl transferase（SA-GTase）遺伝子の発現が増加していることから、この変換によりSA濃度が低下した可能性がある。さらに、プロベナゾール処理された根では感染特異的(PR)タンパク質遺伝子であるPR2, PR3, PR5, PBZ1およびRSOsPR10タンパク質遺伝子の発現が顕著に減少していた。

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