J. Pestic. Sci., **35**(4), 412–418 (2010)
DOI: 10.1584/jpestics.G09-63

Original Article

Effects of cytokinin on production of diterpenoid phytoalexins in rice

Kwang-Wook Ko,[1] Kazunori Okada,[1,*] Jinichiro Koga,[2] Yusuke Jikumaru,[3] Hideaki Nojiri[1] and Hisakazu Yamane[1,*]

[1] *Biotechnology Research Center, The University of Tokyo, 1–1–1 Yayoi, Bunkyo-ku, Tokyo 113–8657, Japan*
[2] *Food and Health R&D Laboratories, Meiji Seika Kaisha, Ltd., Sakado, Saitama 350–0289, Japan*
[3] *RIKEN Plant Science Center, Tsurumi-ku, Yokohama, Kanagawa 230–0045, Japan*

(Received November 18, 2009; Accepted May 13, 2010)

The production of phytoalexins is a well-documented defense response against pathogen infection in rice. Although jasmonic acid has been suggested to be involved in the production of phytoalexins in both suspension-cultured rice cells and rice leaves, there has been almost no information on other secondary signaling molecules that regulate the production of phytoalexins in rice. In this study, the production of the major diterpenoid phytoalexins, momilactones and phytocassanes, was found to be induced by cytokinin treatment in both suspension-cultured rice cells and rice leaves, and also the upregulation of phytoalexin biosynthetic genes was found to be induced by cytokinin treatment. The roles of cytokinins in the production of diterpenoid phytoalexins in rice infected with a pathogen are discussed. © Pesticide Science Society of Japan

Keywords: cytokinin, defense responses, diterpene cyclase genes, diterpenoid phytoalexins, rice.

Introduction

Higher plants that are attacked by pathogenic microorganisms respond with a variety of defense reactions, including the production of secondary metabolites called phytoalexins, which serve as plant antibiotics.

In rice, 15 compounds have been identified as phytoalexins in suspension-cultured cells treated with a biotic elicitor such as a chitin oligosaccharide or a cerebroside[1–2)] and/or in leaves either infected with the blast fungus *Magnaporthe oryzae* or irradiated with UV light.[3–10)] Except for the flavonoid sakuranetin, they are all diterpenoids. The rice diterpenoid phytoalexins are classified into four groups based on their basic carbon frameworks: phytocassanes A–E,[8,9,11)] oryzalexins A–F,[4–6,12)] momilactones A and B,[3,13)] and oryzalexin S,[7)] the major ones being phytocassanes and momilactones.

As shown in Fig. 1, the common precursor geranylgeranyl diphosphate (GGDP) is cyclized to *ent*-copalyl diphosphate (*ent*-CDP) and then to *ent*-cassa-12,15-diene and *ent*-sandaracopimaradiene, leading to phytocassanes A to E and oryzalexins A to F, respectively. GGDP is also cyclized to *syn*-CDP and then to 9βH-pimara-7,15-diene and stemar-13-ene, leading to momilactones A and B and oryzalexin S, respectively. By utilizing the information from the rice genome database that recently opened to the public, six diterpene cyclases have been demonstrated to be involved in the conversion of GGDP to the four diterpene hydrocarbon precursors *via ent*- or *syn*-CDP. *ent*-CDP synthase (OsCPS2) and *syn*-CDP synthase (OsCPS4) catalyze the conversion of GGDP to *ent*-CDP and *syn*-CDP (14), and *ent*-cassa-12,15-diene synthase (OsKSL7), *ent*-sandaracopimaradien synthase (OsKSL10), 9βH-pimara-7,15-diene synthase (OsKSL4), and stemar-13-ene synthase (OsKSL8) catalyze the conversion of *ent*-CDP or *syn*-CDP to the four diterpene hydrocarbons *ent*-cassa-12,15-diene, *ent*-sandaracopimaradiene, 9βH-pimara-7,15-diene, and stemar-13-ene, respectively.[15–17)] The methylerythritol phosphate (MEP) pathway genes have been shown to be responsible for chitin oligosaccharide elicitor-inducible production of diterpenoid phytoalexins in suspension-cultured rice cells,[18)] and microsomal P450s and a dehydrogenase have also been shown to be involved in the downstream oxidation of diterpene hydrocarbons.[19)]

On the other hand, secondary signaling molecules, including plant hormones, have been indicated to be involved in the defense responses caused by pathogen attacks. For example, salicylic acid, jasmonic acid (JA), and ethylene have been investigated extensively and shown to play crucial roles as sec-

* To whom correspondence should be addressed.
E-mail: ayamane@mail.ecc.u-tokyo.ac.jp (H.Y.);
ukokada@mail.ecc.u-tokyo.ac.jp (K.O.)
Published online June 18, 2010

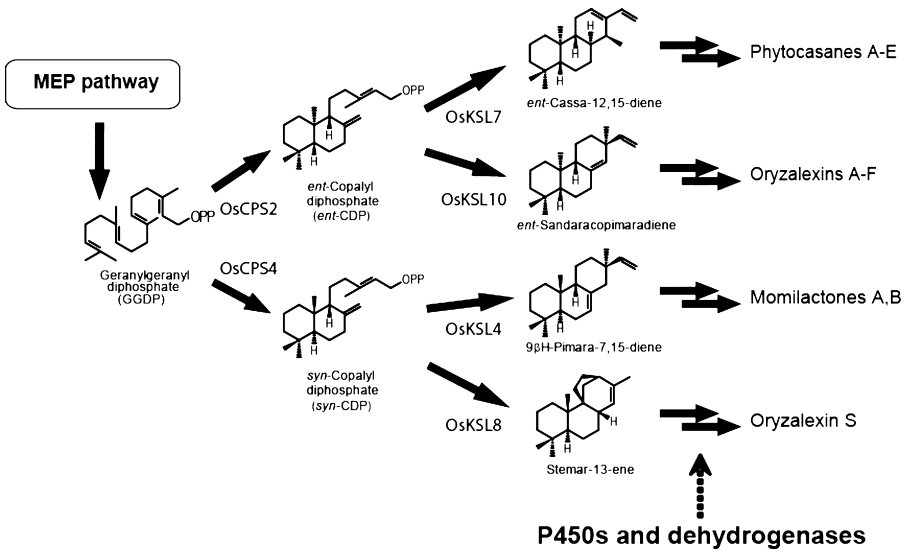

Fig. 1. Biosynthetic pathways of diterpenoid phytoalexins in rice.

ondary signaling molecules in plant defense mechanisms.[20,21] With regard to phytoalexin production in rice, the involvement of JA has been suggested in both suspension-cultured cells and leaves[22,23]; however, there has been almost no information on other secondary signaling molecules that regulate phytoalexin production in rice.

The plant hormone cytokinin is also believed to play important roles in plant disease resistance as described below. The formation of green islands on rust-infected cereal is a defense response in which cytokinin is involved.[24,25] Cytokinin is suggested to act as a molecular switch regulating downstream events of a small GTP-binding protein in defense signal transduction pathways in tobacco.[26] In this study, we found that exogenously applied cytokinins induced the production of the major diterpenoid phytoalexins momilactones and phytocassanes in both suspension-cultured rice cells and rice leaves. We herein report the details of this finding and discuss the roles of cytokinin in the production of diterpenoid phytoalexins in rice infected with a pathogen.

Materials and Methods

1. Plant material

Calli of *Oryza sativa* L. cv. Nipponbare were maintained on gerangum (0.3%) plates using modified N6 medium supplemented with 1 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), as described previously.[15] For suspension cultures, approximately 1-ml aliquots of rice cells were transferred to 100 ml Erlenmeyer flasks containing 30 ml each of fresh N6 medium with 1 mg/l 2,4-D. The cultures were maintained on a rotary shaker (120 rpm) at 25°C in the dark. Suspension-cultured cells were collected every week and filtered through a 20-mesh screen to collect fine aggregates. At the time of each collection, 1-ml aliquots of the resultant cells were transferred to new 100 ml Erlenmeyer flasks containing 30 ml each of the above medium and cultured further for the following experiments.

To grow rice plants (*O. sativa* L. cv. Nipponbare), rice seeds were sterilized with 2.5% sodium hypochlorite solution (Kanto Chem., Tokyo, Japan) for 30 min, and then washed with sterilized water 10 times. Surface-sterilized seeds were soaked in water and incubated for 5 d at 28°C in the dark. Germinated seeds were then transplanted into a mixture of vermiculite and artificial compost, Bonsol (Sumitomo Chemical, Japan) to grow until the seven- or eight-leaf stage at 28°C under a 16 hr light/8 hr dark cycle condition.

2. Dose response experiments

Approximately 1-ml aliquots of fresh cells were transferred to 100-ml Erlenmeyer flasks containing 30 ml each of the culture medium with 2,4-D (1 mg/l), and cultured on a rotary shaker (120 rpm) for 6 d at 25°C in the dark.[15] 6-Bezylaminopurine (BA) and *trans*-zeatin (*t*Z) stock solutions that had been sterilized through a 0.20-μm filter were added to the rice cell suspension cultures at the given final concentrations, respectively. Each treated cell culture was incubated on a rotary shaker (120 rpm) at 25°C in the dark. The medium was collected 72 hr after treatment and used for quantification of rice diterpenoid phytoalexins by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry (HPLC-ESI-MS/MS).

A leaf disk (6 mm in diameter) from the uppermost leaves at the seven- or eight-leaf stage of rice plants was floated on 200 μl of an assay solution in each well of 96-well plastic plates. The assay solutions were prepared by diluting the BA and *t*Z stock solutions with dH_2O to the given final concentrations, respectively. The respective leaf disks were harvested 72 hr after treatment and used for the quantification of rice diterpenoid phytoalexins by HPLC-ESI-MS/MS.

3. Time–course experiments

Similarly to "dose response experiments", rice cell suspension cultures were prepared. BA stock solution was added to rice cell suspension cultures at a final concentration of 100 μM. An *N*-acetylchitooctaose stock solution was added to rice cell suspension cultures at a final concentration of 0.6 μM as a positive control. As a negative control, the same volume of dH_2O was added instead of the *N*-acetylchitooctaose solution and BA stock solution. Each cell suspension was incubated on a rotary shaker (120 rpm) at 25°C in the dark. The cells and medium were collected at given hours after treatment. The cells were used for expression analysis of diterpene cyclase genes involved in the biosynthesis of diterpenoid phytoalexins, and the medium was used for quantification of rice diterpenoid phytoalexins by HPLC-ESI-MS/MS.

Leaf disks are prepared as described above. BA stock solution was diluted with dH_2O to give assay solutions at a final concentration of 100 μM. Leaf disks treated with 500 μM JA solution were used as positive controls, and those treated with dH_2O were used as negative controls. The respective leaf disks were harvested 48 hr and 72 hr after treatment and used for the quantification of rice diterpenoid phytoalexins by HPLC-ESI-MS/MS and expression analysis of diterpene cyclase genes involved in their biosynthesis.

4. Quantification of diterpenoid phytoalexins by HPLC-ESI-MS/MS

To quantify diterpenoid phytoalexins in the culture medium of the rice cell suspension treated with the chitin oligosaccharide elicitor, BA, or *t*Z, a 0.3-ml aliquot of each culture medium was collected at given hours after treatment. The culture medium was extracted three times with the same volume of ethyl acetate at an approximate pH of 5.8. The combined ethyl acetate extracts were evaporated to dryness. The residues were dissolved in 3 ml of 79% aqueous ethanol containing 7% acetonitrile, and 0.01% acetic acid, and subjected to HPLC-ESI-MS/MS, as described by Shimizu *et al*.[27]

To quantify diterpenoid phytoalexins in a leaf disk treated with JA, BA, or *t*Z, the leaf disk was collected 48 hr and 72 hr after treatment. The leaf disk was extracted in 150 μl of 79% aqueous ethanol containing 7% acetonitrile, and 0.01% acetic acid at 4°C overnight. The extract was subjected to HPLC-ESI-MS/MS.

HPLC: An Agilent 1100 separation module (Agilent Technologies, Palo Alto, CA, USA) equipped with a Pegasil C18 column (150×2.1 mm in diameter; Senshu Scientific, Tokyo, Japan) was used with 70% aqueous acetonitrile containing 0.1% acetic acid as a solvent at a flow rate of 0.2 ml/min.

ESI-MS/MS: An API-3000 quadrupole tandem mass spectrometer (Applied Biosystems Instruments, Foster City, CA, USA) outfitted with an electrospray ion source in positive-ion mode was used. Nitrogen was used as the collision gas. The ES capillary was set at 3.0 kV and the source temperature was 400°C. Other parameters were optimized by spectrometer software (Applied Biosystems Instruments). The diterpenoid phytoalexins was determined with combinations of precursor/product ions: *m/z* 315/271 for momilactone A, *m/z* 331/269 for momilactone B, *m/z* 317/299 for phytocassane A, D, and E, *m/z* 335/317 for phytocassane B and *m/z* 319/301 for phytocassane C in the multiple reaction monitoring mode.

5. Expression analysis of diterpene cyclase genes involved in the biosynthesis of rice diterpenoid phytoalexins

Total RNA was extracted from the plant materials using Sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan). Rice cells and rice leaf disks were homogenized with 1 ml Sepasol-RNA I Super and mixed with 200 μl chloroform ($CHCl_3$). After centrifugation at 12,000 *g* for 15 min at 4°C, the supernatant was mixed with 500 μl of 2-propanol and centrifuged at 12,000 *g* for 10 min at 4°C. After washing the pellet with 1 ml of 70% ethanol, the pellet was dissolved in 50 μl RNA secure solution by heating for 10 min at 60°C. The RNA concentration was determined by measuring A_{260} of a 1 : 50 dilution of the solution with a SmartSpec 3000 spectrophotometer (BIO-RAD, Hercules, CA, USA).

For each sample, 1 μg total RNA was used for reverse transcription (RT) to synthesize first-strand cDNA using the QuantiTect reverse transcription kit (Qiagen, Tokyo, Japan). One microgram of total RNA was treated with 2 μl genomic DNA wipeout buffer to remove contaminating genomic DNA, and then used for RT. The cDNA sample was diluted to the same concentration, and then subjected to PCR using TAKARA PCR Thermal cycler® Dice (TAKARA BIO, Otsu, Shiga, Japan) for *OsCPS2, OsCPS4, OsKSL4, OsKSL7*, and *UBQ* with the following cycling program: 95°C for 2 min, 95°C for 30 sec; 55°C for 30 sec; 72°C for 30 sec; 28 cycles (*UBQ*) or 30 cycles (*OsCPS2, OsCPS4, OsKSL4,* and *OsKSL7*), 72°C for 4 min. The primers used in this study are shown in Table 1.

Table 1. Primers used in the present study

Gene name	Primer name	Primer sequence
OsCPS2	OsCPS2 F	5′-TTAGGAAAATGGTTGACTA-3′
	OsCPS2 R	5′-ATCGACTAAATTCATCTCAC-3′
OsCPS4	OsCPS4 F	5′-TGACGAGGCTGGGCATATC-3′
	OsCPS4 R	5′-TCTGGAGTCCAGTTCCTGAAA-3′
OsKSL4	OsKSL4 F	5′-CGCTTTGTAACTCTAAGGTA-3′
	OsKSL4 R	5′-ACGTAAAAGGCTTGTATATC-3′
OsKSL7	OsKSL7 F	5′-TTCATCTCTGTCACTTTTTCTTTTT-3′
	OsKSL7 R	5′-ATCCCAACGAAGTCATCCAC-3′
UBQ	OsUBQ F	5′-GGACTGGTTAAATCAATCGTCA-3′
	OsUBQ R	5′-CCATATACCACGACCGTCAAAA-3′

Results and Discussion

1. *Effects of cytokinin on accumulation of diterpenoid phytoalexins in rice*

The possibility that cytokinin is involved in plant defense responses against pathogens has been indicated; however, there has been little evidence regarding the relationships between cytokinin and disease resistance in plants. In this study, we investigated whether cytokinin is involved in phytoalexin production, a well-documented defense response, in rice. We first examined the effects of the synthetic cytokinin BA and the biologically active natural cytokinin *t*Z on the accumulation of the major diterpenoid phytoalexins momilactones and phytocassanes in rice.

In suspension-cultured rice cells, BA treatment induced a dose-dependent accumulation of phytocassanes until 100 μM, although the phytocassane level greatly decreased at 1000 μM. BA treatment also induced a dose-dependent accumulation of momilactones until 100 μM, similarly to the BA-induced accumulation of phytocassanes, the maximum level of momilactones being about 20% that of the maximum level of phytocassanes (Fig. 2a).

In rice leaf disks, BA treatment induced the dose-dependent accumulation of phytocassanes and momilactones, at least until 100 μM, although the levels of phytocassanes and momilactones greatly decreased at 1000 μM. These BA effects on phytoalexin levels in leaf disks were similar to those in suspension-cultured cells, but differed in that the momilactones levels were higher than the phytocassanes levels in leaf disks (Fig. 2b).

The biologically active natural cytokinin *t*Z had similar activity to BA in inducing the accumulation of diterpenoid phytoalexins at 100 μM in both suspension-cultured rice cells and rice leaf disks (Fig. 2c and 2d). These results indicate that BA and *t*Z have similar phytoalexin-inducing activity in both suspension-cultured rice cells and rice leaves.

Interestingly, cytokinin treatments at 1000 μM markedly reduced phytoalexin levels. A possible reason for the reduction of phytoalexin levels at higher concentration is that cytokinin may act as a strong free radical scavenger at this concentration. Previous studies suggested that active oxygen species play a role in the activation of plant defense responses, including the production of phytoalexins.[28] Tamogami *et al.*[29] reported that *t*Z counteracts JA-inducible

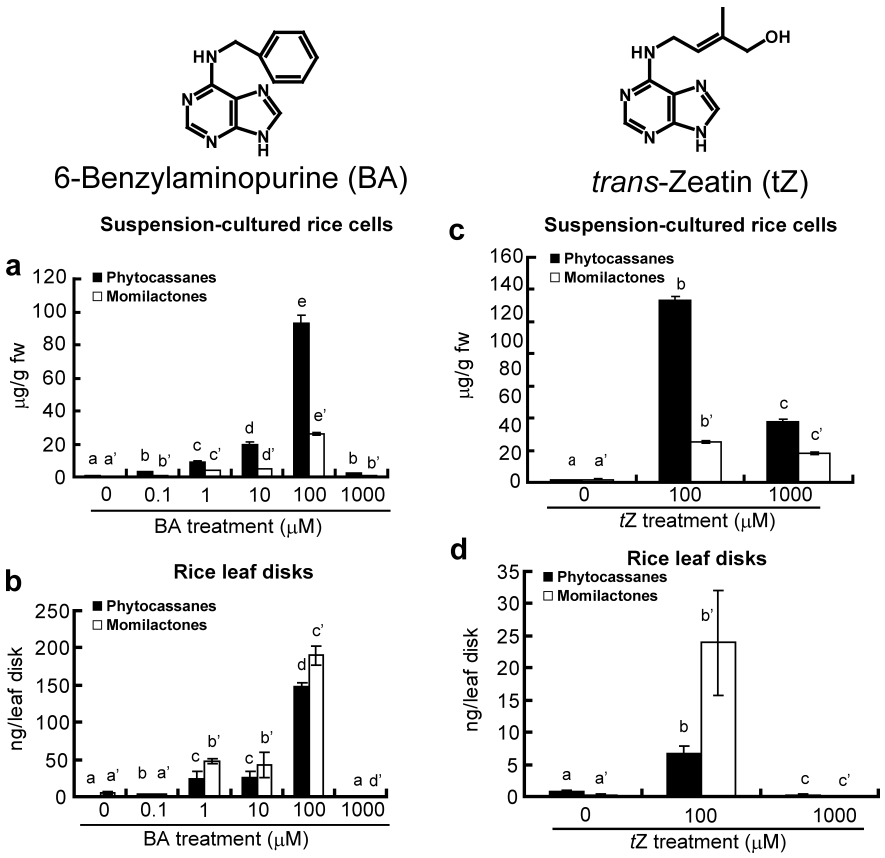

Fig. 2. Dose–response effects of BA and *t*Z on accumulation of phytocassanes and momilactones in the media of suspension-cultured rice cells (**a** (BA) and **c** (*t*Z)) and rice leaf disks (**b** (BA) and **d** (*t*Z)) 72 hr after treatment. Phytoalexin contents were determined by HPLC-ESI-MS/MS. The results are expressed as the mean±standard error of three replicates (suspension-cultured rice cells) or those of five replicates (rice leaf disks). Different letters indicate significant differences at $p<0.05$ (Student's *t*-test).

production of the flavonoid phytoalexin sakuranetin in rice leaves at 250 μM. This evidence suggests that up to 100 μM cytokinin may induce the production of diterpenoid phytoalexins but a higher concentration of cytokinin (1000 μM) may also act as a strong free radical scavenger, resulting in the decreased production of diterpenoid phytoalexins.

2. Time–course analysis of accumulation of diterpenoid phytoalexins and expression of their biosynthetic genes in suspension-cultured rice cells and rice leaves

Since treatments with BA and *t*Z showed similar effects on the accumulation of diterpenoid phytoalexins in rice, BA was used in the following time–course study. Suspension-cultured rice cells were treated with either 100 μM BA or 0.6 μM of the chitin oligosaccharide elicitor *N*-acetylchitooctaose, which was used as a positive control of the induction of diterpenoid phytoalexins.[22,27)]

As shown in Fig. 3a, chitin oligosaccharide treatment led to an increase, beginning at 8 hr, of the phytocassane level, which reached more than 500 μg/g fresh weight at 48 hr, being maintained until 96 hr. The momilactone level also increased beginning at 8 hr, reaching nearly 200 μg/g fresh weight at 48 hr, and this level was maintained until 96 hr. BA treatment led to an increase in the phytocassane level beginning at 24 hr, and the level continued to increase to the final measurement at 96 hr. The momilactone level also continued to increase from 24 hr through 96 hr in response to BA. The levels of phytocassanes and momilactones at 96 hr were 242 and 50 μg/g fresh weight, respectively.

These results revealed that accumulation of the diterpenoid phytoalexins was induced by treatment with BA, although the induction of phytoalexins by BA was lower than that by the chitin oligosaccharide, and the time–course curves of the induction levels by BA were quite different from those by the chitin oligosaccharide. To examine whether the BA-induced accumulation of phytoalexins was caused by the activation of *de novo* biosynthesis of the phytoalexins, time–course analysis of the expression of phytoalexin biosynthetic genes in suspension-cultured rice cells treated with 100 μM BA was also performed by semiquantitative RT-PCR.

As shown in Fig. 3b, the chitin oligosaccharide led to an increased expression of the diterpene cyclase genes involved in the biosynthesis of the diterpenoid phytoalexins. As shown in Fig. 1, *OsCPS4* and *OsKSL4* are involved in momilactone

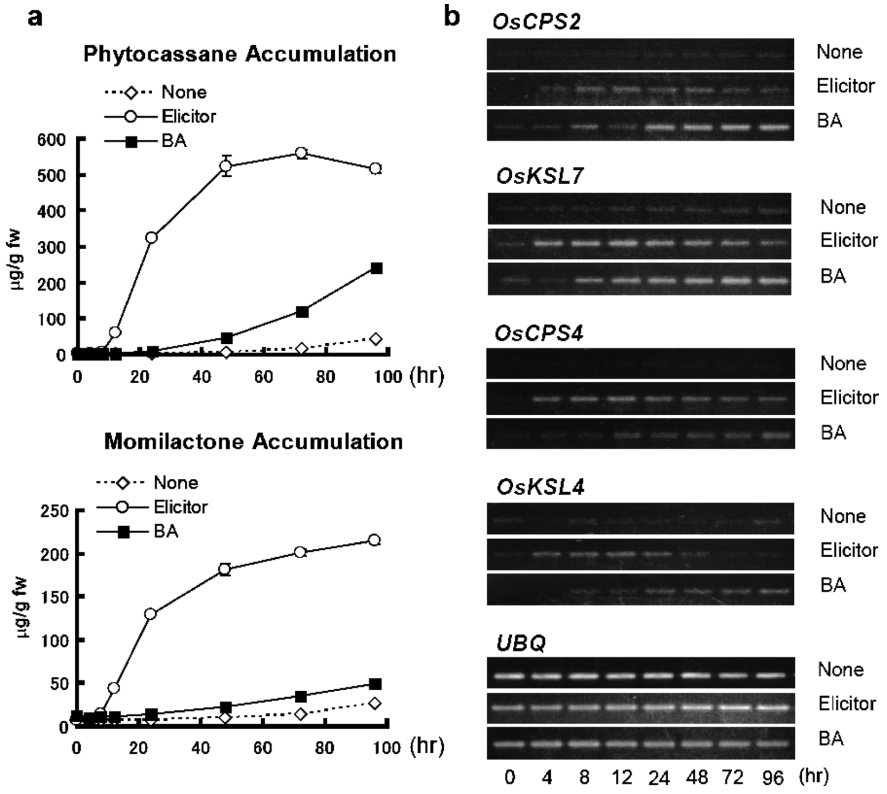

Fig. 3. Time–course analyses of the accumulation of phytocassanes and momilactones (**a**) and expression of diterpene cyclase genes (**b**) in suspension-cultured rice cells. The total amounts of phytocassanes and momilactones accumulated in the culture medium of rice cells from 0 to 96 hr after treatment with 0.6 μM chitin oligosaccharide or 100 μM BA were quantified using HPLC-ESI-MS/MS. The results are expressed as the mean±standard error of three experiments. The mRNA levels of the diterpene cyclase genes, *OsCPS2*, *OsCPS4*, *OsKSL4*, and *OsKSL7*, in suspension-cultured rice cells from 0 to 96 hr after treatment with 0.6 μM chitin oligosaccharide or 100 μM BA were determined by semiquantitative RT-PCR.

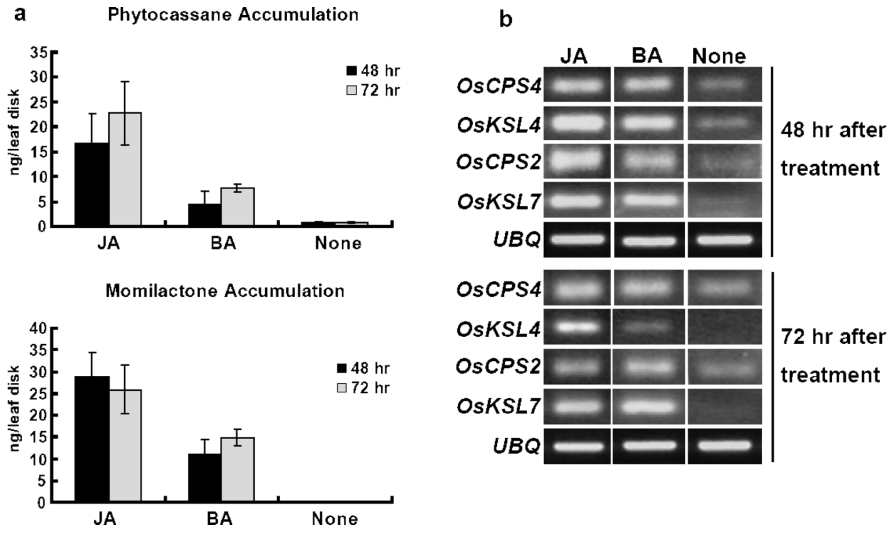

Fig. 4. Analyses of the accumulation of the diterpenoid phytoalexins phytocassanes and momilactones (**a**) and expression of diterpene cyclase genes (**b**) in rice leaf disks 48 and 72 hr after treatment with BA or JA. The total amounts of phytocassanes and momilactones accumulated in the rice leaf disks 48 and 72 hr after treatment with 500 μM JA, 100 μM BA, or dH_2O (none) were quantified using HPLC-ESI-MS/MS. The results are expressed as the mean±standard error of at least five experiments. The mRNA levels of the diterpene cyclase genes, *OsCPS2*, *OsCPS4*, *OsKSL4*, and *OsKSL7* in the leaf disks 48 and 72 hr after treatment with 0.6 μM JA, 100 μM BA, or dH_2O (none) were determined by semiquantitative RT-PCR.

biosynthesis, and *OsCPS2* and *OsKSL7* in phytocassane biosynthesis. The expression of these four diterpene cyclase genes peaked at 8–12 hr after treatment and thereafter decreased rapidly. On the other hand, BA led to an increased expression of the four diterpene cyclase genes from 8 hr or 12 hr through 96 hr, indicating differences in the time–course changes of the levels of diterpenoid phytoalexin biosynthetic gene expression between the responses to BA and the chitin oligosaccharide; that is, compared with the chitin oligosaccharide, BA induced a slower but prolonged expression of the four diterpene cyclase genes. These results suggest that the signal transduction pathway of cytokinin leading to phytoalexin production may be different from that of chitin oligosaccharide.

In rice leaf disks, the effect of BA was also examined. In this experiment, JA was used as a positive control,[23] because chitin oligosaccharide treatment induced little phytoalexin accumulation in rice leaf disks, probably due to low uptake by the leaf tissue (data not shown). Similarly to JA (500 μM), BA (100 μM) led to the increased accumulation of diterpenoid phytoalexins (Fig. 4a) and to the increased expression of *OsCPS2, OsCPS4, OsKSL4,* and *OsKSL7* at 48 hr and 72 hr after treatment (Fig. 4b), although the phytoalexin-inducing activity of cytokinin was lower than JA. These results indicate that BA-induced accumulation of the diterpenoid phytoalexins is caused by their *de novo* biosynthesis, not only in suspension-culture rice cells but in rice leaves.

In conclusion, this study indicates that the plant hormone cytokinin induces the production of the antimicrobial secondary metabolites phytoalexins in rice. As described in the introduction, evidence that cytokinin is involved in disease resistance in higher plants has been reported.[24–26] In rice plants, the production of diterpenoid phytoalexins was induced after infection with the pathogen *M. oryzae*.[3,8] In addition, endogenous cytokinin levels increased in response to infection with *M. oryzae* in rice plants (Takatsuji, personal communication). The results obtained in this study reveal that cytokinin may play a role in the production of diterpenoid phytoalexins in rice infected with a pathogen.

Acknowledgements

We thank Dr. N. Shibuya of Meiji University and Dr. M. Hasegawa of Ibaraki University for kindly providing the chitin oligosaccharide elicitor and samples of momilactones, respectively. We also thank Dr. H. Takatsuji of the National Institute of Agrobiological Sciences for helpful discussion about the contents of endogenous cytokinins after pathogen infection in rice. This work was supported in part by the Program for the Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN).

References

1) A. Yamada, N. Shibuya, O. Kodama and T. Akatsuka: *Biosci. Biotechnol. Biochem.* **57**, 405–409 (1993).
2) K. Umemura, N. Ogawa, J. Koga, M. Iwata and H. Usami: *Plant Cell Physiol.* **43**, 778–784 (2002).
3) D. W. Cartwright, P. W. Langcake, R. J. Pryce, D. P. Leworthy and J. P. Ride: *Phytochemistry* **20**, 535–537 (1981).
4) T. Akatsuka, N. Takahashi, O. Kodama, H. Sekido, Y. Kono and S. Takeuchi: *Agric. Biol. Chem.* **49**, 1689–1694 (1985).
5) H. Kato, O. Kodama and T. Akatsuka: *Phytochemistry* **33**, 79–

81 (1993).
6) H. Kato, O. Kodama and T. Akatsuka: *Phytochemistry* **36**, 299–301 (1994).
7) S. Tamogami, M. Mitani, O. Kodama and T. Akatsuka: *Tetrahedron* **49**, 2025–2032 (1993).
8) J. Koga, M. Shimura, K. Oshima, N. Ogawa, T. Yamauchi and N. Ogasawara: *Tetrahedron* **51**, 7907–7918 (1995).
9) J. Koga, N. Ogawa, T. Yamauchi, M. Kikuchi, N. Ogasawara and M. Shimura: *Phytochemistry* **44**, 249–253 (1997).
10) O. Kodama, J. Miyakawa, T. Akatsuka and S. Kiyosawa: *Phytochemistry* **31**, 3807–3809 (1992).
11) A. Yajima and K. Mori: *Eur. J. Org. Chem.* **2000**, 4079–4091 (2000).
12) Y. Kono, S. Takeuchi, O. Kodama and T. Akatsuka: *Agric. Biol. Chem.* **48**, 253–255 (1984).
13) T. Kato, C. Kabuto, N. Sasaki, M. Tsunagawa, H. Aizawa, K. Fujita, Y. Kato and Y. Kitahara: *Tetrahedron Lett*. **39**, 3861–3864 (1973).
14) K. Otomo, H. Kenmoku, H. Oikawa, W. A. König, H. Toshima, W. Mitsuhashi, H. Yamane, T. Sassa and T. Toyomasu: *Plant J.* **39**, 886–893 (2004).
15) E. M. Cho, A. Okada, H. Kenmoku, K. Otomo, T. Toyomasu, W. Mitsuhashi, T. Sassa, A. Yajima, G. Yabuta, K. Mori, H. Oikawa, H. Toshima, H. Shibuya, H. Nojiri, H. Omori, M. Nishiyama and H. Yamane: *Plant J.* **37**, 1–8 (2004).
16) K. Otomo, Y. Kanno, A. Motegi, H. Kenmoku, H. Yamane, W. Mitsuhashi, H. Oikawa, H. Toshima, H. Itoh, M. Matsuoka, T. Sassa and T. Toyomasu: *Biosci. Biotechnol. Biochem.* **68**, 2001–2006 (2004).
17) T. Nemoto, E. M. Cho, A. Okada, K. Okada, K. Otomo, Y. Kanno, T. Toyomasu, W. Mitsuhashi, T. Sassa, E. Minami, N. Shibuya, M. Nishiyama, H. Nojiri and H. Yamane: *FEBS Lett.* **571**, 182–186 (2004).
18) A. Okada, T. Shimizu, K. Okada, T. Kuzuyama, J. Koga, N. Shibuya, H. Nojiri and H. Yamane: *Plant Mol. Biol.* **65**, 177–187 (2007).
19) K. Shimura, A. Okada, K. Okada, Y. Jikumaru, K.-W. Ko, T. Toyomasu, T. Sassa, M. Hasegawa, O. Kodama, N. Shibuya, J. Koga, H. Nojiri and H. Yamane: *J. Biol. Chem.* **282**, 34013–34018 (2007).
20) A. Robert-Seilaniantz, L. Navarro, R. Bari and J. D. Jones: *Curr. Opin. Plant Biol.* **10**, 372–379 (2007).
21) R. Bari and J. D. G. Jones: *Plant Mol. Biol.* **69**, 473-488 (2009).
22) H. Nojiri, M. Sugimori, H. Yamane, Y. Nishimura, A. Yamada, N. Shibuya, N. Murofushi and T. Omori: *Plant Physiol.* **110**, 387–392 (1996).
23) R. Rakwal, S. Tamogami and O. Kodama: *Biosci. Biotechnol. Biochem.* **60**, 1046–1048 (1996).
24) R. Angra-Sharma and D. K. Sharma: *Mycopathologia* **148**, 87–95 (1999).
25) D. R. Walters and N. McRoberts: *Trends Plant Sci.* **11**, 581–586 (2006).
26) H. Sano and Y. Ohashi: *Proc. Natl. Acad. Sci. USA*. **92**, 4138–4144 (1995).
27) T. Shimizu, Y. Jikumaru, A. Okada, K. Okada, J. Koga, K. Umemura, E. Minami, N. Shibuya, M. Hasegawa, O. Kodama, H. Nojiri and H. Yamane: *Phytochemistry* **69**, 973–981 (2008).
28) T. Noritake, K. Kawakita and N. Doke: *Plant Cell Physiol.* **37**, 113–116 (1996).
29) S. Tamogami, R. Rakwal and O. Kodama: *FEBS Lett.* **412**, 61–64 (1997).