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Rhizobitoxine-induced Chlorosis Occurs in Coincidence with Methionine Deficiency in Soybeans

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• *Background and Aims* Rhizobitoxine, produced by the legume symbiont *Bradyrhizobium elkanii*, inhibits cystathionine-β-lyase (EC 4·4·1·8) in methionine biosynthesis and 1-aminocyclopropane-1-carboxylate synthase (ACC) in ethylene biosynthesis. Rhizobitoxine production by *B. elkanii* enhances nodulation of host legumes via the inhibition of ethylene synthesis, but causes foliar chlorosis in susceptible soybeans, though how it does so remains to be investigated. The aim of this study was to examine the physiological basis of rhizobitoxine-induced chlorosis in soybeans.
• *Methods* Wild-type *B. elkanii* and a rhizobitoxine-deficient mutant were inoculated in *Glycine max* 'Lee'. Thirty days after inoculation, the upper parts of soybean shoots were analysed for amino acid contents. Chlorotic soybeans inoculated with wild-type *B. elkanii* were treated with methionine and ACC to assess the effects of the chemicals on the chlorosis.
• *Key Results* Chlorotic upper shoots of soybeans inoculated with wild-type *B. elkanii* had a lower methionine content and higher accumulation of the methionine precursors than those with the rhizobitoxine-deficient mutant. In addition, the foliar chlorosis was alleviated by the application of methionine.
• *Conclusions* Rhizobitoxine-induced chlorosis occurs in coincidence with methionine deficiency as a result of cystathione-β-lyase inhibition during methionine biosynthesis.

Key words: *Bradyrhizobium elkanii*, chlorosis, methionine, nodulation, rhizobitoxine, *Glycine max*, soybean.

INTRODUCTION

Rhizobitoxine [2-amino-4-(2-amino-3-hydropropoxy)-*trans*-but-3-enoic acid] is produced by the legume symbiont *Bradyrhizobium elkanii* (Owens *et al.*, 1972) and the plant pathogen *Burkholderia andropogonis* (Mitchell *et al.*, 1986). Rhizobitoxine inhibits cystathionine-β-lyase (EC 4·4·1·8) in methionine biosynthesis (Owens *et al.*, 1968; Giovanelli *et al.*, 1972) and 1-aminocyclopropane-1-carboxylate (ACC) synthase in ethylene biosynthesis (Yasuta *et al.*, 1999). Several genes responsible for rhizobitoxine production are known in *B. elkanii* (Ruan and Peters, 1992; Yasuta *et al.*, 2001).

Rhizobitoxine production by *B. elkanii* enhances nodulation of the host legume species *Vigna radiata* (mung bean) (Duodu *et al.*, 1999), *Macroptilium atropurpureum* (siratro) (Yuhashi *et al.*, 2000; Okazaki *et al.*, 2003) and *Amphicarpaea edgeworthii* (Parker and Peters, 2001). Inoculation with rhizobitoxine-producing *B. elkanii* caused lower ethylene evolution in host legumes, whereas the loss of rhizobitoxine production resulted in the restoration of ethylene evolution in the host (Yuhashi *et al.*, 2000) and in fewer nodules and lower nodulation competitiveness of the bacteria (Duodu *et al.*, 1999; Yuhashi *et al.*, 2000; Parker and Peters, 2001; Okazaki *et al.*, 2003; Sugawara *et al.*, 2006). Ethylene suppresses nodulation in most legume species, including *M. atropurpureum* (Okazaki *et al.*, 2004*a*) and *Lotus japonicus* (Nukui *et al.*, 2000, 2004). Rhizobitoxine production by *B. elkanii* enhances ethylene-sensitive nodulation in the host plants as a result of inhibition of ACC synthase and blocking of ethylene biosynthesis of the hosts.

Nodulation by *B. elkanii* causes foliar chlorosis in soybean (*Glycine max*) (Erdman *et al.*, 1956; Owens and Wright, 1965; Teaney and Fuhrmann, 1992). Plants show yellow leaves mainly in newly developing leaves, lower chlorophyll content, growth inhibition (Eaglesham and Hassouna, 1982; Fuhrmann, 1990; Bruce and Fuhrmann, 1993) and higher accumulation of rhizobitoxine in leaves, stems and nodules (Minamisawa and Kume, 1987). Rhizobitoxine production by *B. elkanii* is responsible for the induction of foliar chlorosis, because the loss of rhizobitoxine production by *B. elkanii* abolishes the chlorosis induction (Ruan and Peters, 1992; Okazaki *et al.*, 2004*b*). Okazaki *et al.* (2004*b*) reported that the *rtxC* gene mutant of *B. elkanii* USDA94, designated Δ*rtxC*, produced dihydrorhizobitoxine but no rhizobitoxine. Dihydrorhizobitoxine, the oxidative form of rhizobitoxine, was accumulated in the upper shoot of *G. max* inoculated with Δ*rtxC* but revealed no chlorosis on the plant. The wild-type strain of *B. elkanii* USDA94 resulted in the accumulation of rhizobitoxine in the upper shoot and caused severe chlorosis on *G. max*. However, the mechanism of chlorosis induction remains to be solved.

The induction of foliar chlorosis should involve methionine or ACC in the host plants, because rhizobitoxine inhibits the biosynthesis of both (Yasuta *et al.*, 1999). Here, the accumulation of rhizobitoxine-related amino acids in host shoots and the effect of methionine and ACC application on foliar chlorosis induction are assessed.

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MATERIALS AND METHODS

Bacterial strains, plasmids and growth conditions

Wild-type *B. elkanii* USDA94 and the *rtxC* mutant Δ*rtxC* (Okazaki *et al.*, 2004*b*) were grown aerobically at 30 °C for 7 d in HM medium (Cole and Elkan, 1973) supplemented with 0·1 % arabinose and 0·025 % yeast extract (and kanamycin at 150 mg L^{-1} for culturing the mutant). Bacterial cells were collected by centrifugation at 5000 *g* for 10 min at room temperature and washed twice with sterile water. The bacterial cell suspension was adjusted to 1×10^7 cells mL^{-1} in sterile water just before inoculation (Miyamoto *et al.*, 2004).

Soybean cultivation

Seeds of *Glycine max* 'Lee' were surface-sterilized with 0·5 % hydrogen peroxide for 1 min and washed ten times with sterile distilled water. The seeds were sown in sterile vermiculite with a nitrogen-free plant nutrient solution (Akao and Kouchi, 1989) in sterile Leonard jar assemblies composed of two 300-mL plant boxes (Ye *et al.*, 2005; Saito and Minamisawa, 2006). The seeds were inoculated with bacterial cell suspension at 1×10^7 cells per seed. The plants were cultivated at 25 °C under a light–dark cycle of 16-h light and 8-h dark in a plant growth cabinet (LH300; NK Systems Co. Ltd, Osaka, Japan) that provided 65 μmol photons m^{-2} s^{-1} of photosynthetically active radiation (You *et al.*, 2006).

Chemical application

Methionine, ACC, aspartate, glutamate and potassium nitrate (KNO_3) were dissolved in plant nutrient solution at 500 μM each. These solutions were applied to the lower reservoir of the Leonard jar assemblies, and changed every 2 d from 16 d after inoculation.

Amino acid content in soybean

Thirty days after sowing, the upper part of the shoots (from the shoot tip to the third trifoliate leaf position) were harvested and weighed. The materials were homogenized in hot 80 % methanol and extracted at 80 °C for 1 h. The extract was centrifuged at 5000 *g* for 15 min, and the supernatant was collected. The pellet was re-extracted twice with hot methanol. The combined supernatant was dried *in vacuo* and re-dissolved in distilled water (3 mL of distilled water per gram fresh weight of initial plant materials). The samples were derivatized with phenylisothiocyanate (PITC) and analysed by liquid chromatography and mass spectrometry (LC/MS) to quantify major amino acids according to Yasuta *et al.* (2001) as follows. A 500-μL aliquot of the samples was mixed with 10 nmol of aminoethoxyvinylglycine (a structural analogue of rhizobitoxine), used as an internal standard, before PITC derivatization. PITC derivatization was carried out according to the method of Yamaya and Matsumoto (1988). A 50-μL aliquot of the sample solution was evaporated *in vacuo* in a 1·5 mL tube, and the pellet was dissolved in 20 μL of ethanol–triethylamine–water (2:1:2, v/v/v). After evaporation, the pellet was dissolved in 10 μL of ethanol-triethylamine-water-PITC (7:1:1:1, v/v), incubated for 20 min at room temperature, and then evaporated to dryness. Each pellet of PITC derivative was dissolved in 100 μL of deionized water and passed through a 0·2-μm cellulose nitrate filter prior to LC/MS analysis. A JMS-LCmate (JEOL, Tokyo, Japan) equipped with an electrospray ionization system and high-performance liquid chromatograph (HP-1100; Hewlett Packard, Waldbronn, Germany) was used for analysis of PITC-labelled amino acids under the following conditions: column, Inertsil ODS-2 (1·5 × 150 mm; GL Sciences Inc., Tokyo, Japan); column temperature, 40 °C; flow rate, 0·1 ml min^{-1}; mobile phase, a linear gradient from 30 % solvent B (100 % MeCN) in solvent A (0·1 % HCOOH) to 100 % solvent B for 15 min. The concentrations of each amino acid were calculated according to the ratio between the peak area of PITC-aminoethoxyvinylglycine (m/z 431) and the peak area of the PITC derivative of each amino acid. PITC-derivatives of measured amino acids (m/z in parentheses) were methionine (285), aspartate (269), homoserine (255), serine (241), leucine (267), isoleucine (267), valine (253), alanine (225), glycine (211) and glutamate (283).

RESULTS AND DISCUSSION

The *rtxC* gene is involved in the final step of rhizobitoxine biosynthesis, desaturation of dihydrorhizobitoxine, to produce biologically active rhizobitoxine in *B. elkanii* (Okazaki *et al.*, 2004*b*). Therefore, by comparing the wild type and the *rtxC* mutant of *B. elkanii* USDA94 it is possible to determine the biological effects of rhizobitoxine in host plants. The soybeans inoculated with the wild-type strain showed foliar chlorosis in the upper shoots and growth inhibition (Fig. 1A), typical host responses in the *B. elkanii*–soybean nodulation interaction (Teaney and Fuhrmann, 1992). On the other hand, plants inoculated with the *rtxC* mutant (Δ*rtxC*) did not show these negative phenotypes (Fig. 1D). The shoot fresh weight of soybeans inoculated with the wild-type strain was 3·4 ± 0·4 g per plant, whereas that of soybeans inoculated with Δ*rtxC* was at 5·0 ± 0·4 g $plant^{-1}$.

Amino acid analysis showed differences in amino acid accumulation in soybean shoots between inoculations with the wild-type and Δ*rtxC* (Table 1). The methionine content of plants inoculated with the wild-type strain was significantly lower, and the aspartate content was significantly higher, than that of plants inoculated with Δ*rtxC*.

Because aspartate and homoserine are generally metabolized in methionine biosynthesis as precursors of cystathionine, the substrate of cystathione-β-lyase (Yasuta *et al.*, 2001) (Fig. 2), the ratios of aspartate and homoserine contents to methionine content were calculated. The ratio of homoserine to methionine was increased in the plants inoculated with the wild-type strain more than in the plants inoculated with Δ*rtxC*. This difference indicates a higher accumulation of homoserine, suggesting the inhibition of cystathionine-β-lyase in methionine biosynthesis

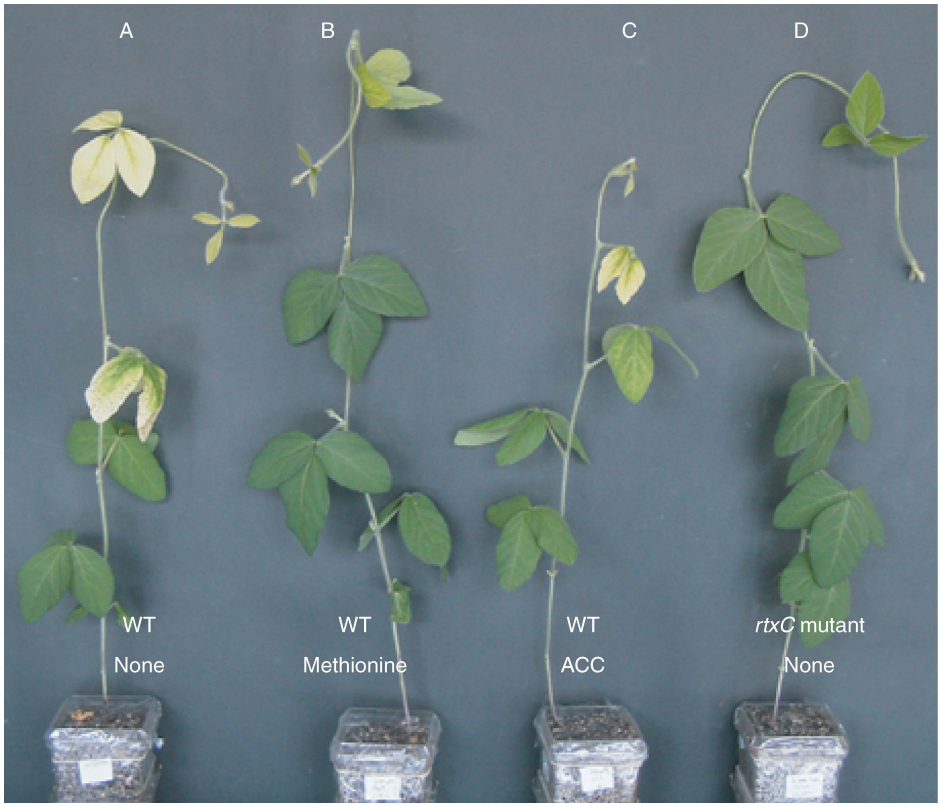

FIG. 1. Effects of methionine and ACC on rhizobitoxine-induced foliar chlorosis in soybean at 30 d after sowing and inoculation with *B. elkanii*. Methionine (500 μM) and ACC (500 μM) were supplied to plants every second day from 16 d after sowing. (A) Inoculation with wild-type (WT) *B. elkanii*; (B) 500 μM methionine application to (A); (C) 500 μM ACC application to (A); (D) inoculation with *rtxC* mutant.

by rhizobitoxine (Fig. 2). A similar increase was observed in the ratio of aspartate to methionine, although the aspartate level depends on the synthesis of methionine and other amino acids.

When 500 μM methionine was applied to soybeans from 16 d after inoculation with the wild-type strain (before the occurrence of rhizobitoxine-induced chlorosis), it alleviated the foliar chlorosis and plant growth inhibition (Fig. 1B). The application of 10 μM or 2 mM methionine showed either no discernible effect or inhibition of shoot growth (data not shown). The application of ACC led to a slight inhibition of shoot growth but did not alleviate the chlorosis (Fig. 1C). These results indicate that the chlorosis was due not to ACC deficiency but to methionine deficiency. The application of aspartate, glutamate and KNO_3 did not alter the chlorosis (data not shown).

These results show that the rhizobitoxine-induced foliar chlorosis was due to insufficient methionine biosynthesis in soybean shoots, which was supported by application of methionine (Fig. 1). The shoots showed lower content of methionine and higher accumulation of aspartate and homoserine (Table 1). These observations illustrated that rhizobitoxine-induced foliar chlorosis is the result of methionine deficiency due to inhibition of cystathione-β-lyase. In higher plants, most cystathionine-β-lyase is localized in chloroplasts (Wallsgrove *et al.*, 1983; Droux *et al.*, 1995, Ravanel *et al.*, 1998). Methionine is required as a precursor of *S*-adenosylmethionine for chlorophyll biosynthesis (Bollivar *et al.*, 1994). Soybean leaves with rhizobitoxine-induced foliar chlorosis had a lower chlorophyll content (Teaney and Fuhrmann, 1992). The inhibition by rhizobitoxine of cystathionine-β-lyase would affect chloroplast function owing to insufficient chlorophyll biosynthesis in soybean shoots deficient in methionine. Methionine depletion and subsequent amino acid imbalance may have caused impaired protein synthesis in the plant

TABLE 1. *Comparison of amino acid contents of soybean shoots between inoculations with the wild-type strain and an* rtxC *mutant (*Δrtx*C) of* Bradyrhizobium elkanii *USDA94*

	Content in soybean shoot (nmol g^{-1} f. wt)		Ratio of content relative to methionine†	
Amino acid	Wild-type	Δ*rtxC*	Wild-type	Δ*rtxC*
Methionine	0·9 ± 0·1	2·2 ± 1·1*	1	1
Aspartate	66·8 ± 10·4	37·6 ± 17·1*	71	17
Homoserine	68·1 ± 16·4	52 ± 28	76	24
Total	467 ± 86	382 ± 149	–	–

*Statistically significant difference between inoculations with wild-type USDA94 and Δ*rtxC* at 5 % level in *t*-test ($n = 4$).

†Ratio of content of each amino acid to that of methionine.

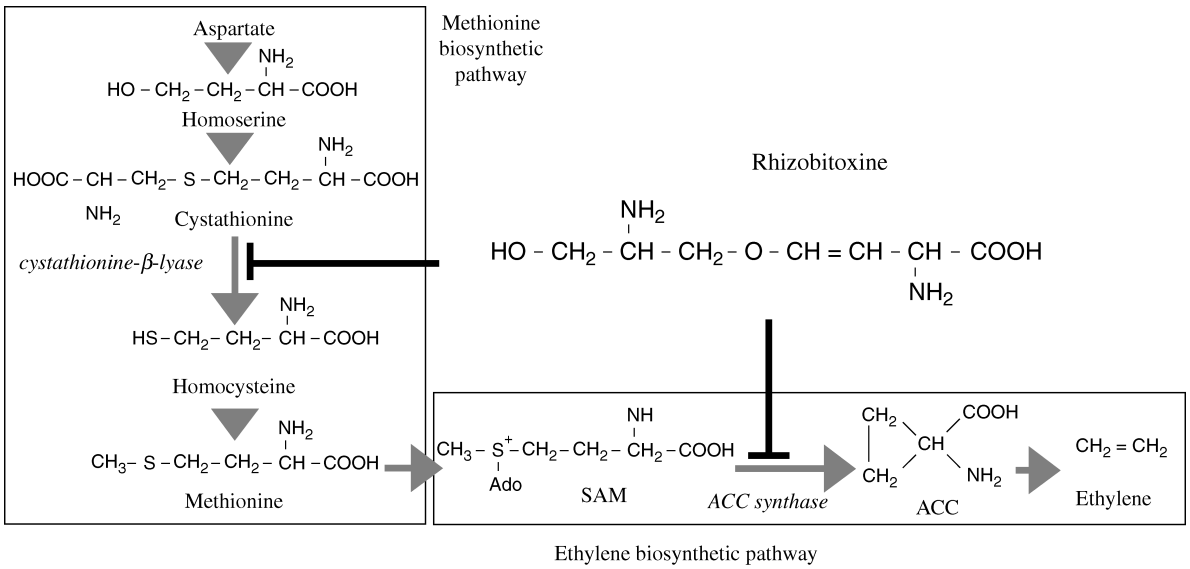

FIG. 2. Rhizobitoxine inhibition of methionine and ethylene biosynthesis pathways. SAM, *S*-Adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate.

cells. Indeed, inoculation of rhizobitoxine-producing *B. elkanii* resulted in significant reduction in total leaf protein content of *G. max* (Teaney and Fuhrmann, 1992). The limited protein synthesis may well have obstructed the synthesis and maintenance of photosynthesis-related proteins, resulting in the development of chlorosis.

Soybean cultivars differ in their susceptibility to rhizobitoxine-induced chlorosis (Erdman *et al.*, 1957; Owens and Wright, 1965). Since the foliar chlorosis is caused by a methionine deficiency due to inhibition of cystathionine-β-lyase, the basis of the rhizobitoxine resistance or susceptibility of soybeans should involve the overall sensitivity of cystathionine-β-lyase to rhizobitoxine. However, the rhizobitoxine inhibition constant of cystathionine-β-lyase partially purified from four soybean cultivars was not clearly correlated with the resistance or susceptibility of the cultivars to rhizobitoxine (Xiong and Fuhrmann, 1996). The different methionine demands and tolerance to methionine deficiency among soybean cultivars might also account for the susceptibility to chlorosis development.

Because rhizobitoxine-induced foliar chlorosis in *B. elkanii*–soybean symbiosis reduces soybean growth and yield (Vasilas and Fuhrmann, 1993), the present results will contribute to the diagnosis of chlorosis symptoms induced by rhizobitoxine and to the breeding of rhizobitoxine-resistant cultivars.

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LITERATURE CITED

Akao S, Kouchi H. 1989. Light microscopic observation of root hair curling of soybean induced by *Rhizobium* infection. *Japanese Journal of Soil Science and Plant Nutrition* **60**: 53–55 [in Japanese].

Bollivar DW, Jiang ZY, Bauer CE, Beale SI. 1994. Heterologous overexpression of the *bchM* gene product from *Rhodobacter capsulatus* and demonstration that it encodes for *S*-adenosyl-L-methionine: Mg-protoporphyrin methyltransferase. *Journal of Bacteriology* **176**: 5290–5296.

Bruce LV, Fuhrmann JJ. 1993. Field response of soybean to nodulation by a rhizobitoxine-producing strain of *Bradyrhizobium*. *Agronomy Journal* **85**: 302–305.

Cole MA, Elkan GH. 1973. Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrobial Agents and Chemotherapy* **4**: 248–53.

Droux M, Ravanel S, Douce R. 1995. Methionine biosynthesis in higher plants. II. Purification and characterization of cystathionine β-lyase from spinach chloroplasts. *Archives of Biochemistry and Biophysics* **316**: 585–595.

Duodu S, Bhuvaneswari TV, Stokkermans TJW, Peters NK. 1999. A positive role for rhizobitoxine in *Rhizobium*–legume symbiosis. *Molecular Plant–Microbe Interactions* **12**: 1082–1089.

Eaglesham ARJ, Hassouna S. 1982. Foliar chlorosis in legumes induced by cowpea rhizobia. *Plant and Soil* **65**: 425–428.

Erdman IW, Johnson HW, Clark FE. 1956. A bacterial-induced chlorosis in the Lee soybean. *Plant Disease Reports* **40**: 646.

Erdman LW, Johnson HW, Clark FE. 1957. Varietal responses of soybeans to a bacterial-induced chlorosis. *Agronomy Journal* **49**: 267–271.

Fuhrmann J. 1990. Symbiotic effectiveness of indigenous soybean bradyrhizobia as related to serological, morphological, rhizobitoxine, and hydrogenase phenotypes. *Applied and Environmental Microbiology* **56**: 224–229.

Giovanelli J, Owens LD, Mudd SH. 1972. β-Cystathionase – *in vivo* inactivation by rhizobitoxine and role of the enzyme in methionine biosynthesis in corn seedlings. *Plant Physiology* **51**: 492–503.

Minamisawa K, Kume N. 1987. Determination of rhizobitoxine and dihydrorhizobitoxine in soybean plants by amino acid analyzer. *Soil Science and Plant Nutrition* **33**: 645–649.

Mitchell RE, Frey EJ, Benn MK. 1986. Rhizobitoxine and 1-threohydroxythreonine production by the plant pathogen *Pseudomonas andropogonis*. *Phytochemistry* **25**: 2711–2715.

Miyamoto T, Kawahara M, Minamisawa K. 2004. Novel endophytic nitrogen-fixing clostridia from the grass *Miscanthus sinensis* as

revealed by terminal restriction fragment length polymorphism analysis. *Applied and Environmental Microbiology* **70**: 6580–6586.

Nukui N, Ezura H, Yuhashi K, Yasuta T, Minamisawa K. 2000. Effect of ethylene precursor and inhibitors for ethylene biosynthesis and perception on nodulation in *Lotus japonicus* and *Macroptilium atropurpureum*. *Plant and Cell Physiology* **41**: 893–897.

Nukui N, Ezura H, Minamisawa K. 2004. Transgenic *Lotus japonicus* with ethylene receptor gene Cm-ERS1/H70A enhances formation of infection threads and nodule primordia. *Plant and Cell Physiology* **45**: 427–435.

Okazaki S, Yuhashi K, Minamisawa K. 2003. Quantitative and time-course evaluation of nodulation competitiveness of rhizobitoxine-producing *Bradyrhizobium elkanii*. *FEMS Microbiology Ecology* **45**: 155–160.

Okazaki S, Nukui N, Sugawara M, Minamisawa K. 2004*a*. Rhizobial strategies to enhance symbiotic interactions: rhizobitoxine and 1-aminocyclopropane-1-carboxylate deaminase. *Microbes and Environments* **19**: 99–111.

Okazaki S, Sugawara M, Minamisawa K. 2004*b*. *Bradyrhizobium elkanii rtxC* gene is required for expression of symbiotic phenotypes in the final step of rhizobitoxine biosynthesis. *Applied and Environmental Microbiology* **70**: 535–541.

Owens LD, Wright DA. 1965. Rhizobial-induced chlorosis in soybeans: isolation, production in nodules, and varietal specificity of the toxin. *Plant Physiology* **40**: 927–930.

Owens LD, Guggenheim S, Hilton JL. 1968. *Rhizobium*-synthesized phytotoxin: an inhibitor of β-cystathionase in *Salmonella typhimurium*. *Biochimica et Biophysica Acta* **158**, 219–225.

Owens LD, Thompson JF, Pitcher RG, Williams T. 1972. Structure of rhizobitoxine, an antimetabolic enol-ether amino-acid from *Rhizobium japonicum*. *Journal of the Chemical Society, Chemical Communications* **12**: 714.

Parker MA, Peters NK. 2001. Rhizobitoxine production and symbiotic compatibility of *Bradyrhizobium* from Asian and North American lineages of *Amphicarpaea*. *Canadian Journal of Microbiololgy* **47**: 1–6.

Ravanel S, Gakiere B, Job D, Douce R. 1998. The specific features of methonine biosynthesis and metabolism in plants. *Proceedings of the National Academy of Sciences of the USA* **95**: 7805–7812.

Ruan X, Peters NK. 1992. Isolation and characterization of rhizobitoxine mutants of *Bradyrhizobium japonicum*. *Journal of Bacteriology* **174**: 3467–3473.

Saito A, Minamisawa K. 2006. Evaluation of nitrogen fixation capability of endophytic clostridia by acetylene reduction and reverse transcription-PCR targeted to *nifH* transcript and ribosomal RNA. *Microbes and Environments* **21**: 23–35.

Sugawara M, Okazaki S, Nukui N, Ezura H, Mitsui H, Minamisawa K. 2006. Rhizobitoxine modulates plant–microbe interactions by ethylene inhibition. *Biotechnology Advances* **24**: 382–388.

Teaney GB III, Fuhrmann JJ. 1992. Soybean response to nodulation by bradyrhizobia differing in rhizobitoxine phenotype. *Plant and Soil* **145**: 275–285.

Vasilas BL, Fuhrmann JJ. 1993. Field response of soybean to nodulation by a rhizobitoxine-producing strain of *Bradyrhizobium*. *Agronomy Journal*. **85**: 302–305.

Wallsgrove RM, Lea PJ, Miflin BJ. 1983. Intracellular localization of aspartate kinase and the enzymes of threonine and methionine biosynthesis in green leaves. *Plant Physiology* **71**: 780–784.

Xiong K, Fuhrmann JJ. 1996. Comparison of rhizobitoxine-induced inhibition of β-cystathionase from different bradyrhizobia and soybean genotypes. *Plant and Soil* **186**: 53–61.

Yamaya T, Matsumoto H. 1988. Analysis of phenylthiocarbamyl-amino acids at pico-mole level by high performance liquid chromatography and application to plant materials. *Soil Science and Plant Nutrition* **34**: 297–302.

Yasuta T, Satoh S, Minamisawa K. 1999. New assay for rhizobitoxine based on inhibition of 1-aminocyclopropane-1-carboxylate synthase. *Applied and Environmental Microbiology* **65**: 849–852.

Yasuta T, Okazaki S, Mitsui H, Yuhashi K, Ezura H, Minamisawa K. 2001. DNA sequence and mutational analysis of rhizobitoxine biosynthesis genes in *Bradyrhizobium elkanii*. *Applied and Environmental Microbiology* **67**: 4999–5009.

Ye B, Saito A, Minamisawa K. 2005. Effect of inoculation with anaerobic nitrogen-fixing consortium on salt tolerance of *Miscanthus sinensis*. *Soil Science and Plant Nutrition* **51**: 243–249.

You M, Nishiguchi T, Saito A, Isawa T, Mitsui H, Minamisawa K. 2006. Expression of the *nifH* gene of a *Herbaspirillum* endophytes in wild rice species: daily rhythm during the light-dark cycle. *Applied and Environmental Microbiology* **71**: 8183–8190.

Yuhashi K, Ichikawa N, Ezura H, Akao S, Minakawa Y, Nukui N, *et al*. 2000. Rhizobitoxine production by *Bradyrhizobium elkanii* enhances nodulation and competitiveness on *Macroptilium atropurpureum*. *Applied and Environmental Microbiology* **66**: 2658–2663.