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Research review paper

## Rhizobitoxine modulates plant–microbe interactions by ethylene inhibition

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### Abstract

*Bradyrhizobium elkanii* produces rhizobitoxine, an enol-ether amino acid, which has been regarded as a phytotoxin because it causes chlorosis in soybeans. However, recent studies have revealed that rhizobitoxine plays a positive role in establishing symbiosis between *B. elkanii* and host legumes: rhizobitoxine enhances the nodulation process by inhibiting ACC (1-aminocyclopropane-1-carboxylate) synthase in the ethylene biosynthesis of host roots. *B. elkanii* *rtxA* and *rtxC* genes are required for rhizobitoxine production. In particular, *rtxC* gene is involved in the desaturation of dihydrorhizobitoxine into rhizobitoxine. A legume with a mutated ethylene receptor gene produced markedly higher numbers of rhizobial infection threads and nodule primordia. Thus, endogenous ethylene in legume roots negatively regulates the formation of nodule primordia, which is overcome by rhizobitoxine. Although a plant pathogen *Burkholderia andropogonis* has been known to produce rhizobitoxine, the genome sequence of *Xanthomonas oryzae* showed the existence of a putative rhizobitoxine transposon in the genome. The cumulative evidence suggests that rhizobitoxine-producing bacteria modulate plant–microbe interactions via ethylene in the rhizosphere and phyllosphere environments. In addition, rhizobitoxine-producing capability might be utilized as tools in agriculture and biotechnology.

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**Keywords:** *Bradyrhizobium elkanii*; Ethylene; Legume; Rhizobia; Rhizobitoxine

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## 1. Introduction

In the symbiotic nitrogen-fixation process, bacteria of the family *Rhizobiaceae* convert atmospheric dinitrogen ( $N_2$ ) to ammonia ( $NH_3$ ), which can be effectively utilized by host legume plants. The family *Rhizobiaceae* currently includes six genera: *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Allorhizobium*, *Azorhizobium*, and *Bradyrhizobium*, which are collectively referred to as rhizobia. They have the ability to infect the roots of legumes and to produce nodules. The differentiated forms of rhizobia in the nodule, called bacteroids, fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plant (Long, 1989). Symbiotic nitrogen fixation is of great importance not only in the production of leguminous crops but also in the global nitrogen cycle.

Nitrogen-fixing nodules are formed as a consequence of a series of interactions between rhizobia and leguminous host plants (Fisher and Long, 1992). The symbiotic partners show a high degree of mutual specificity, mediated by the exchange of chemical signals (Spaink, 1996, 2000). The plant signals, flavonoids excreted by the roots, activate the expression of nodulation genes in rhizobia, resulting in the production of rhizobial lipochitooligosaccharide signals called Nod factors. Although legumes form root nodules mainly in response to Nod factors, it has been thought that the plant perception of endogenous signals, particularly plant hormones, is also important for the establishment of proper symbiotic interactions between rhizobia and legumes (Caetano-Anolles and Gresshoff, 1991).

## 2. Effect of ethylene on nodulation

Ethylene is produced and sensed in response to a wide variety of environmental and developmental cues, including germination, flowering, drought, pathogen attack, and nodulation (Abeles et al., 1992; Spaink, 1997). In particular, ethylene inhibits infection of rhizobia and nodulation of most legumes, as shown by many studies (Okazaki et al., 2004a). Exogenously applied ethylene or ethylene precursor inhibits nodulation in many legumes. In *Pisum sativum*, exogenous ethylene application inhibits elongation of infection threads into the inner cortex (Lee and LaRue, 1992b). In *P. sativum*, endogenous ethylene exerts positions on nodule meristem morphogenesis (Heidstra et al., 1997).

Inhibition of ethylene synthesis and responses has been shown to enhance nodule formation in *Medicago truncatula* (Oldroyd et al., 2001), *Medicago sativa* (Nukui et al., 2000; Peters and Crist-Estes, 1989), *Lotus japonicus* (Bras et al., 2000; Nukui et al., 2000), *P. sativum* (Guinel and Sloetjes, 2000; Lorteau et al., 2001), and *Macroptilium atropurpureum* (Nukui et al., 2000). In addition, a hypernodulating mutant of *M. truncatula* has ethylene-insensitive phenotypes (Penmetsa and Cook, 1997). Unlike in most legume species, however, ethylene may not play a significant role in the nodulation of modern soybean cultivars (Hunter, 1993; Schmidt et al., 1999). Ethylene evolution in legume roots increases after application of rhizobial cells (Ligero et al., 1986; Sukanuma et al., 1995), Nod factor (van Spronsen et al., 1995), nitrate (Caba et al., 1998; Ligero et al., 1987), and illumination (Lee and LaRue, 1992a), suggesting that these environmental factors control nodulation through their effects on the levels of ethylene.

## 3. Biochemical functions of rhizobitoxine

In 1956, Erdman et al. (1956) first reported that certain strains of rhizobia induce chlorosis in new leaves of soybeans. Owens and colleagues (Owens and Wright, 1965a,b) purified the toxic compound from nodule extracts and culture media of *Rhizobium japonicum* (now *Bradyrhizobium elkanii*) and demonstrated that the compound is phytotoxic to soybeans (Johnson et al., 1959; Owens, 1973). The toxic compound was later called rhizobitoxine. Among soybean bradyrhizobia, all strains of *B. elkanii* (formerly DNA homology group II) produce rhizobitoxine, whereas those of *B. japonicum* (formerly DNA homology group I) do not produce rhizobitoxine at all (Hollis et al., 1981; Minamisawa, 1989, 1990; Minamisawa and Mitsui, 2000). Rhizobitoxine has therefore been regarded as a phytotoxin that causes chlorosis symptoms in soybeans.

The structure of rhizobitoxine was solved in 1972 (Owens et al., 1972b). Rhizobitoxine is an enol-ether amino acid (2-amino-4-[2-amino-3-hydroxypropoxy]-*trans*-3-butenic acid) with a molecular weight of 190 (Fig. 1). The first biochemical function identified for rhizobitoxine was that of an inhibitor of  $\beta$ -cystathionase in the methionine biosynthesis pathway (cystathionine  $\beta$ -lyase, E.C.4.4.1.8) (Fig. 1).  $\beta$ -Cystathionase catalyzes the conversion of cystathionine to homocysteine, pyruvate,

and ammonium. As an analog of cystathionine, rhizobitoxine irreversibly inhibits  $\beta$ -cystathionase in bacteria (Owens et al., 1968) and plants (Giovanelli et al., 1972).

Yasuta et al. (1999) found that rhizobitoxine also strongly inhibits 1-aminocyclopropane-1-carboxylate (ACC) synthase (E.C.4.4.1.14) in the ethylene biosynthesis pathway (Fig. 1), which can explain the early observation of rhizobitoxine inhibition of ethylene evolution in apple tissues (Owens et al., 1971). ACC synthase is the rate-limiting enzyme in ethylene biosynthesis in plants, catalyzing the conversion of *S*-adenosylmethionine to ACC. A closely related ethoxy analog of rhizobitoxine, aminoethoxyvinylglycine (AVG), also inhibits ACC synthase (Devine and Breithaut, 1980; Yasuta et al., 1999). The oxidized form of rhizobitoxine (dihydrorhizobitoxine) is approximately 99% less potent than rhizobitoxine as an inhibitor of ACC synthase, so the double bond between C3 and C4 may be critical for the inhibition of ACC synthase (Yasuta et al., 1999).

#### 4. Positive role of rhizobitoxine production in symbiosis

Rhizobitoxine has been regarded as a phytotoxin ever since the discovery that it induces chlorosis to soybeans. However, recent studies have shown a positive role of rhizobitoxine in the symbiosis between *B. elkanii* strains and their host legumes. Yuhashi et al. reported that rhizobitoxine production in *B. elkanii* USDA94 reduces ethylene evolution from the associated roots of *M.*

*atropurpureum* (siratro) and enhances nodule formation by using *B. elkanii* USDA94 and its mutant lacking rhizobitoxine production (Yuhashi et al., 2000). In particular, the nodulation competitiveness of *B. elkanii* USDA94 was significantly enhanced by rhizobitoxine production. Duodu et al. also reported a positive role of rhizobitoxine in the symbiosis between *B. elkanii* USDA61 and *Vigna radiata* (mungbean) (Duodu et al., 1999). Rhizobitoxine-minus mutants of USDA61 formed dramatically fewer mature nodules than the wild-type strain. Interestingly, the rhizobitoxine-minus mutants induced many immature nodules, indicating that the initial stages of root hair infection were not impaired but that the development to mature nodules was obstructed. Therefore, ethylene might affect abortion of the developing nodules in the association between USDA61 and mungbean, and rhizobitoxine production by the bacterium would overcome this effect by inhibiting ethylene biosynthesis.

Another study on rhizobitoxine and nodulation was carried out by Parker et al. using *Amphicarpaea* (Parker and Peters, 2001). Nodulation experiments using *B. elkanii* USDA61 and its rhizobitoxine-minus mutants revealed that efficient nodulation in *A. edgeworthii* but not in *A. bracteata* is highly dependent on rhizobitoxine production.

#### 5. Rhizobitoxine biosynthesis genes and pathway

The genes involved in rhizobitoxine biosynthesis have been identified in two *B. elkanii* strains, USDA61 (Ruan and Peters, 1992) and USDA94 (Yasuta et al.,

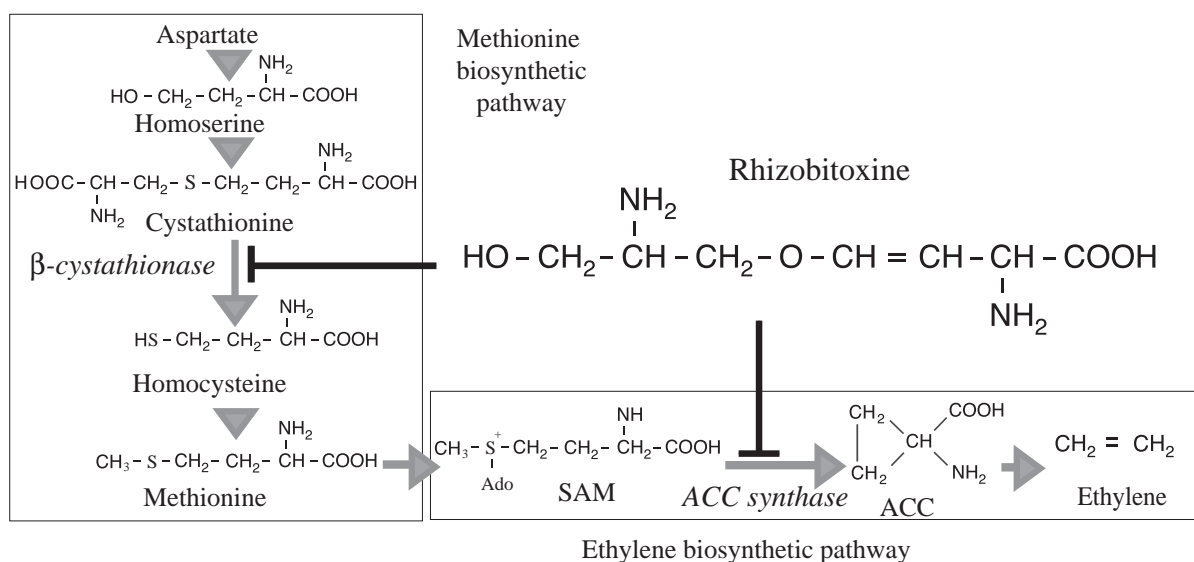


Fig. 1. Rhizobitoxine inhibition in methionine and ethylene biosynthetic pathway. SAM, *S*-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate.

2001). Ruan et al. isolated Tn5-induced rhizobitoxine-null mutants of *B. elkanii* USDA61 and identified the *rtxA* gene, which is responsible for rhizobitoxine biosynthesis in culture and *in planta* (Ruan and Peters, 1992). Yasuta et al. (2001) cloned and sequenced the genetic locus involved in rhizobitoxine biosynthesis from *B. elkanii* USDA94, which produces more rhizobitoxine than USDA61 in culture. *B. elkanii* USDA94 possesses several open reading frames (ORFs) downstream of the *rtxA* gene. A large deletion mutant of *B. elkanii*, USDA94 $\Delta$ *rtx:: $\Omega$ 1, which lacks *rtxA*, *rtxC*, and the downstream ORFs, does not produce rhizobitoxine, dihydrorhizobitoxine, or serinol. The latter two compounds have been found to be co-produced with rhizobitoxine and are regarded as intermediates (Minamisawa and Watanabe, 1986; Owens et al., 1972a; Yasuta et al., 2001). Complementation analyses with cosmids containing an insertional mutagenesis within the putative *rtx* operon suggested that (i) at least *rtxA* and *rtxC* are necessary for rhizobitoxine production in *B. elkanii*, (ii) the newly discovered *rtxC* gene, which is located immediately downstream of the *rtxA* gene, is involved in desaturation of dihydrorhizobitoxine into rhizobitoxine, and (iii) other ORFs downstream of *rtxC* affect the levels of production of rhizobitoxine and its intermediates by unknown functions.*

Large amounts of dihydrorhizobitoxine are generally co-produced with rhizobitoxine (Minamisawa and Kume, 1987; Owens et al., 1972a). However, no conclusive data have been reported on the biological effects of dihydrorhizobitoxine *in planta*. Okazaki et al. (2004b) demonstrated that dihydrorhizobitoxine production does not affect the plant phenotypes of *B. elkanii* USDA94 in terms of nodulation competitiveness and chlorosis induction. They thus concluded that desaturation of

dihydrorhizobitoxine by an *rtxC*-encoded protein is essential for the bacterium to elicit these phenotypes *in planta*.

## 6. Rhizobitoxine-producing pathogens

A plant pathogen, *Burkholderia andropogonis*, is also known to be a rhizobitoxine producer. The pathogen causes chlorotic symptoms in corn and sorghum, presumably as a result of rhizobitoxine production *in planta* (Mitchell and Frey, 1988). Recently, *rtx* genes found in the genome of *Xanthomonas oryzae* pathovar *oryzae* KACC10331 (Lee et al., 2005), a bacterium that causes bacterial blight in rice (*Oryza sativa* L.), although the *rtx* genes were misannotated (Lee et al., 2005). Amino acid sequences of *rtxA* and *rtxC* genes are highly homologous with those of *B. elkanii* (Fig. 2). The *rtx* gene clusters in *X. oryzae* and *B. elkanii*, contained additional two genes that are relevant to glutamine (ORF2; Glutamine amidotransferase, ORF4; Glutamine synthetase). These genes probably supply glutamine-related substrates for rhizobitoxine biosynthesis (Sugawara et al., unpublished results). From the comparison, we expect rhizobitoxine production in *X. oryzae* KACC10331. Interestingly, the *rtx* gene cluster in *X. oryzae* pv. *oryzae* KACC10331 resides between two insertion sequences (ISs), and formed a composite transposon structure for rhizobitoxine biosynthesis (Fig. 2). Therefore, such rhizobitoxine transposon may transfer to other plant-associated bacteria in the environments.

## 7. Nodulation enhancement via ethylene perception

Rhizobitoxine is secreted outside rhizobial cells and delivered to the plant. In fact, rhizobitoxine has been

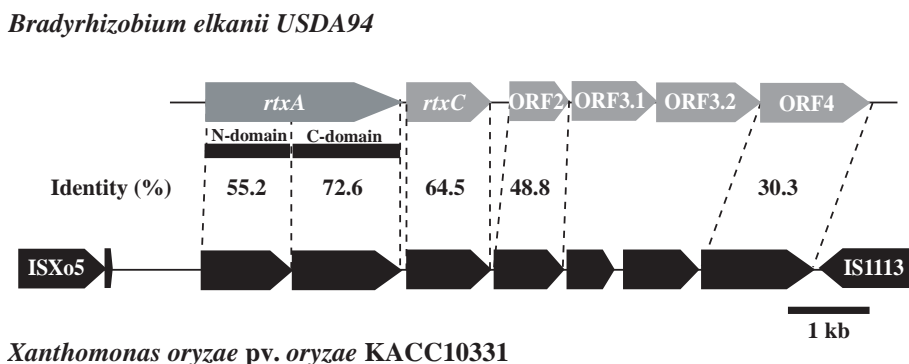


Fig. 2. Comparison of *rtx* genes in *Bradyrhizobium elkanii* USDA94 and *Xanthomonas oryzae* pv. *oryzae* KACC10331. Sequence comparison were carried out using sequence of *rtx* genes in *B. elkanii* USDA94 (accession no. AB062279) and *X. oryzae* pv. *oryzae* KACC10331 (accession no. AE013598). Arrows show *rtx* genes and predicted open reading frame (ORFs). Numbers in the middle space indicate percent identity based on the amino acids sequences. ISXo5 and IS113 are insertion sequences with transposase gene (Lee et al., 2005).

detected in leaves as well as in nodules of soybeans inoculated with *B. elkanii* (Minamisawa and Kume, 1987). Moreover, ethylene evolution is blocked in legume root systems inoculated with *B. elkanii* (Yuhashi et al., 2000). Therefore, the explanation of how rhizobitoxine decreases endogenous levels of ethylene in legume roots appears straightforward.

The next question is how a decreased ethylene level enhances nodulation. Recently, several models have been proposed depicting the relationships between signal transduction, ethylene sensing, and the development of nodulation (Gresshoff et al., 2003; Guinel and Geil, 2002; Stearns and Glick, 2003). One promising approach will be to construct transgenic legumes with altered ethylene sensitivities. The expression of ethylene receptors that cannot bind ethylene confers reduced ethylene sensitivity to heterologous plants in a genetically dominant manner (Bleecker, 1999). In petunia and tomato, transformation with a mutant *Arabidopsis* ethylene receptor gene (*etr1-1*) conferred reduced sensitivity to ethylene (Wilkinson et al., 1997).

To address the mechanism by which ethylene inhibits nodulation, Nukui et al. (2004) transformed *L. japonicus* B-129 'Giftu' (Handberg and Stougaard, 1992) with a mutated ethylene receptor gene *Cm-ERS1/H70A*: A point mutation was introduced into the melon ethylene receptor *Cm-ERS1* (Sato-Nara et al., 1999) by changing the 70th amino acid, histidine, to alanine, which abolished its ethylene-binding ability. The resultant transgenic *L. japonicus* plants showed reduced ethylene sensitivity.

When inoculated with *M. loti*, the transgenic *L. japonicus* produced markedly higher numbers of infection threads and nodule primordia on their roots than did either wild-type or azygous plants without the transgene. In addition, the amount of transcripts of *NIN*, a gene governing formation of infection threads (Schäuser et al., 1999), increased in the inoculated transgenic plants as compared with the wild-type plants. These results imply that endogenous ethylene in *L. japonicus* roots inhibits the formation of nodule primordia, as well as other infection processes (Nukui et al., 2004).

## 8. Alternative strategy for decreasing ethylene

The ability to synthesize rhizobitoxine is confined to the slow-growing *B. elkanii* among rhizobia so far. Thus, Yasuta et al. (1999) addressed the question as to whether fast-growing rhizobia possess another inhibitory factor for ethylene biosynthesis of host plants, because it could enhance nodulation (Nukui et al., 2000). They sought potential enzymes and compounds for reducing ethylene biosynthesis from the entire

genome of the fast-growing *M. loti* MAFF303099 presented in a database (Kaneko et al., 2000), and identified ACC deaminase gene as a candidate (Uchiumi et al., 2004). Indeed, ACC deaminase was verified to enhance nodulation and competitiveness to the host legume by using a disruption mutant of ACC deaminase gene (*mlr5932*) (Uchiumi et al., 2004). Recently, genes encoding ACC deaminase have been also found in other rhizobia, such as *Rhizobium leguminosarum* and *B. japonicum* (Kaneko et al., 2002; Ma et al., 2003a,b). ACC deaminase from *R. leguminosarum* bv. *viciae* has been confirmed to enhance nodulation of *P. sativum* as well (Ma et al., 2003a). These recent findings on ACC deaminase suggest that rhizobia can adopt more than one strategy to reduce the amount of ethylene synthesized by their host legumes and thus enhance nodule formation. Indeed, since many processes in nodule formation are blocked by ethylene, it seems reasonable that, in order to enable efficient nodulation, rhizobia would decrease ethylene levels by producing both ACC deaminase and rhizobitoxine.

## 9. Concluding remarks

The cumulative evidence reveals general strategies by which rhizobia produce an inhibitor and an enzyme to decrease ethylene levels in host roots and thereby enhance nodulation. Plant pathogens, such as *B. andropogonis* and *X. oryzae*, probably produce rhizobitoxine to inhibit ethylene biosynthesis and reduce defense reactions by the host plants. The existence of putative rhizobitoxine transposon in *X. oryzae* genome prompts us to speculate that more plant-associated bacteria produce rhizobitoxine and modulate plant–microbe interactions in the rhizosphere and phyllosphere environments.

If some rhizobial strains lack the ability to decrease ethylene levels in host legumes, the introduction of genes for rhizobitoxine biosynthesis and ACC deaminase into these rhizobia ought to enhance their symbiotic interactions with host legumes. This idea may also be extended to other plant-associated bacteria, such as *Rhizobium radiobacter* (formerly *Agrobacterium tumefaciens*), which has been used for plant transformation. Indeed, application of AVG, an analog of rhizobitoxine, increased the efficiency of *Agrobacterium*-mediated gene transfer to explants of melons (Ezura et al., 2000). The ethylene-decreasing strategies of rhizobia are interesting and suggestive for the further understanding of plant–microbe interactions and should be a promising tool for overcoming the problem of rhizobial competition and for making further progress toward



developing more environmentally friendly biotechnology and agriculture.

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