

Minireview

Rhizobial Strategies to Enhance Symbiotic Interactions: Rhizobitoxine and 1-Aminocyclopropane-1-Carboxylate Deaminase

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Nitrogen-fixing nodules are formed as a result of a series of interactions between rhizobia and leguminous plants. *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 and nodulation competitiveness by inhibiting ethylene biosynthesis in host roots. In addition, the gene for 1-aminocyclopropane-1-carboxylate (ACC) deaminase was recently found in some rhizobia, such as *Mesorhizobium loti*, *Bradyrhizobium japonicum* and *Rhizobium* sp.. ACC deaminase also facilitates symbiosis by decreasing ethylene levels in host roots. 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. In this review, we compare these strategies and discuss how they function and have evolved in terms of genetics, biochemistry, and ecology. These rhizobial strategies might be utilized as tools in agriculture and biotechnology.

Key words: ACC deaminase, ethylene, legume, rhizobia, rhizobitoxine

In the symbiotic nitrogen-fixation process, bacteria of the family *Rhizobiaceae* convert atmospheric dinitrogen (N₂) to ammonia (NH₃), which can be effectively utilized by host legume plants. The taxonomy within the family *Rhizobiaceae* is still in a state of flux, although the major taxa are members of the alpha-subdivision of the *Proteobacteria*^{73,89,90}. 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 plant⁴². Symbiotic

nitrogen fixation is of great importance not only in the production of leguminous crops but also in the global nitrogen cycle. Nitrogen is a major limiting factor for most plant species in the biosphere. Application of N fertilizers to fields increases crop production. However, excessive use of N fertilizers often causes environmental problems, including water pollution by NO₃⁻ and the evolution of a greenhouse gas, nitrous oxide (N₂O), into the atmosphere^{34,53}. Effective use of biological nitrogen fixation results in an ideal cropping system with decreased impact on the environment through decreased application of N fertilizers³.

Outline of nodulation process by rhizobia

Nitrogen-fixing nodules are formed as a consequence of a series of interactions between rhizobia and leguminous host plants¹⁵. The symbiotic partners show a high degree of mutual specificity, mediated by the exchange of chemical

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AVG, aminoethoxyvinylglycine.

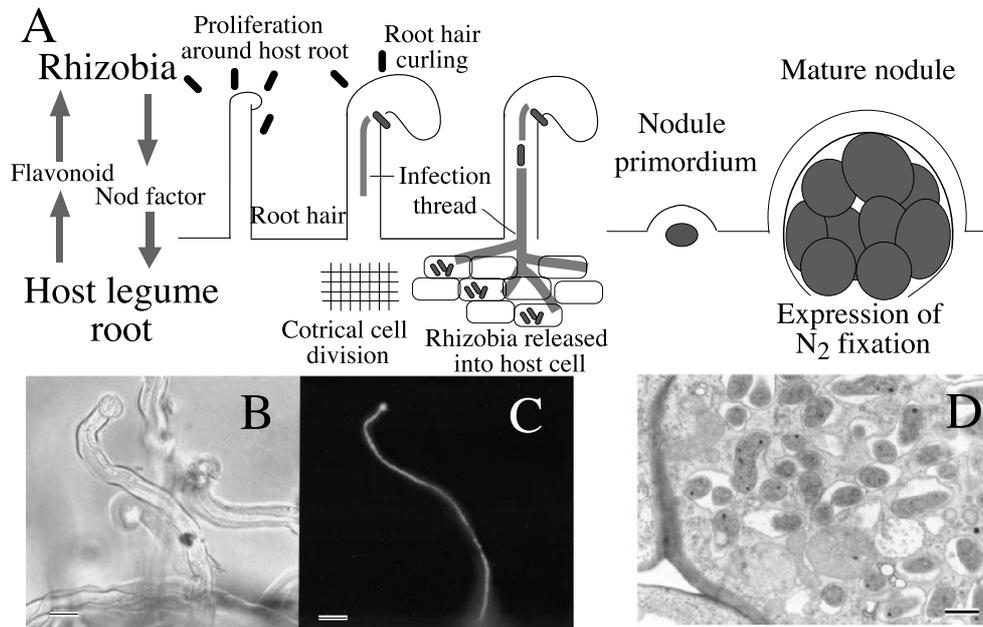


Fig. 1. Nodulation by rhizobia. (A) Scheme of chemical signal exchanges and infection processes involving rhizobia. (B) Root-hair curling in *Lotus japonicus*. (C) *Mesorhizobium loti* cells tagged with constitutive *gfp* gene in an infection thread in the root hair shown in panel (B). (D) *M. loti* bacteroids in infected cells of a mature *L. japonicus* nodule. Bar indicates 10 μm (B, C) and 1 μm (D).

signals^{77,79}). 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 (Fig. 1A). These Nod factors in return induce root-hair curling and cortical cell division in the roots of the host legume (Fig. 1A). Simultaneously, the Nod factors in the presence of rhizobia induce the root cells to form an infection thread (a tube-like structure growing from the root hair tip toward the root cell body) through which the rhizobia enter, travel down a root hair, and are thereafter released into the dividing cortical cells of legume roots (Fig. 1A–C). Rhizobial cells released in the plant cells differentiate into their symbiotic form, nitrogen-fixing bacteroids (Fig. 1AD). 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 legumes⁶.

Effect of ethylene on nodulation

Ethylene is a plant hormone that functions as a regulator in many aspects of plant life. Ethylene is produced and sensed in response to a wide variety of environmental and

developmental cues, including germination, flowering, drought, pathogen attack, and nodulation^{1,78}).

In particular, ethylene inhibits infection of rhizobia and nodulation of most legumes, as shown by many studies (Table 1). 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 cortex³⁷. In *P. sativum*, endogenous ethylene exerts positions on nodule meristem morphogenesis²⁷. Inhibition of ethylene synthesis and responses has been shown to enhance nodule formation in *Medicago truncatula*⁵⁹, *Medicago sativa*^{56,70}, *Lotus japonicus*^{4,56}, *P. sativum*^{25,43}, and *Macroptilium atropurpureum*⁵⁶. In addition, a hypernodulating mutant of *Medicago truncatula* has ethylene-insensitive phenotypes⁶⁸. Unlike in most legume species, however, ethylene may not play a significant role in the nodulation of modern soybean cultivars^{30,75}.

Ethylene evolution in legume roots increases after application of rhizobial cells^{38,82}, Nod factor⁹¹, nitrate^{5,39}, and illumination³⁶, suggesting that these environmental factors control nodulation through their effects on the levels of ethylene.

Table 1. Effects of ethylene, ethylene precursors, and inhibitors on nodulation of legumes by rhizobia

Legume (rhizobia)	Cultivar, ecotype, mutant	Reagent or treatment ^a	Effects on nodulation	Reference
<i>Glycine max</i> L. (<i>Bradyrhizobium japonicum</i>)	cv. Ransom	Ethylene gas	No effect on nodule number	37
	cv. Tracy	Ethephon	No effect on nodule number	30
	cv. Hakuchou	Rhizobial inoculation	Enhanced production of ethylene	82
	cv. Bai tie jia qing	Ethylene gas	No effect on nodule number	94
	cv. Gong jiao 6308-1		Decrease in nodule number	
	cv. Bragg	STS	Restoration of nitrate inhibition of nodulation	41
	cv. Hobbit 87	ACC/STS	No effect on nodule number	75
	cv. Enrei	ACC/AVG/STS	No effect on nodule number	56
<i>Lotus japonicus</i> (<i>Mesorhizobium loti</i>)	Ecotype B-129 Gifu	ACC	Decrease in nodule number	56
		AVG/STS	Increase in nodule number	
		AVG	Increase in nodule number	4
		AVG, STS	Enhancement of <i>NIN</i> gene expression	55
<i>Macroptilium atropurpureum</i> (<i>Bradyrhizobium japonicum</i>)	cv. Siratro	ACC	Decrease in nodule number	56
		AVG/STS	Increase in nodule number	
<i>Medicago sativa</i> L. (<i>Sinorhizobium meliloti</i>)	cv. Aragon	Rhizobial inoculation	Enhanced production of ethylene	38
	cv. AS-R3	AVG	Increase in nodule number	70
	cv. Aragon	AVG	Restoration of nitrate inhibition of nodulation	40
		STS	Restoration of nitrate inhibition of nodulation	5
	cv. Du Puits	ACC	Decrease in nodule number	56
<i>Medicago truncatula</i> (<i>Sinorhizobium meliloti</i>)	cv. Jemalong genotype A17	ACC	Decrease in nodule number	68
			Decrease in nodule number	59
			Fewer infection threads	
		AVG	Increase in nodule number More infection threads	
<i>Phaseolus vulgaris</i> L. (<i>Rhizobium</i> sp.)	var. Pencil Podded Black Wax	Ethylene gas	Decrease in nodule number Decrease in nitrogen fixation	22
	cv. Harvester	Ethephon	Decrease in nodule number	84
		AVG/AOA/cobalt	Increase in nodule number	
<i>Melilotus alba</i> (<i>Sinorhizobium meliloti</i>)	U389	Ethylene gas	Decrease in nodule number	37
<i>Pisum sativum</i> L. (<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>)	cv. Sutton's Show Perfection	Ethrel	Decrease in nodule number Local regulation	9
	cv. Feltham First	Ethylene gas	Inhibition of root extension	19
			Decrease in nodule number Decrease in nitrogen fixation	
	cv. Sparkle	STS	Restoration of nitrate/light inhibition of nodulation	36
		Nitrate	Enhanced production of ethylene	
		Ethylene gas	Decrease in nodule number, blockage of infection thread elongation in inner cortex	37
	AVG/Ag ₂ SO ₄	Control of the position of nodule primordium formation	27	

Table 1. Continued

Legume (rhizobium)	Cultivar, ecotype, mutant	Reagent or treatment ^a	Effects on nodulation	Reference
		AVG	Increase in mature nodule number	25
		BAP (cytokinin)	Decrease in nodule number	43
			Enhanced production of ethylene	
		AVG	Increase in nodule number	
		Ag ₂ SO ₄	Changed nodule distribution on root	
	cv. Rondo	Ethylene gas	Decrease in nodule number	37
<i>Sesbania rostrata</i> (<i>Azorhizobium caulinodans</i>)	cv. Brem	Ag ₂ SO ₄	Induction of indeterminate nodules	14
		ACC	Induction of determinate nodules	
<i>Trifolium repens</i> L. (<i>Rhizobium trifolii</i>)	cv. Huia	Ethylene gas	Decrease in nodule number Decrease in nitrogen fixation	19
<i>Vicia sativa</i> (<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>)	spp. <i>nigra</i>	AVG/Ag ₂ SO ₄	No effect on nod factor induced root hair tip growth	27
<i>Vigna radiata</i> L. (<i>Bradyrhizobium elkanii</i>)	cv. Berkan	Ethephon	Decrease in nodule number	10
		STS/cobalt	Increase in nodule number	
<i>Glycine max</i> L. (<i>Bradyrhizobium japonicum</i>)	cv. Hobbit 87 T119N54 (<i>etr1-1</i>) mutant		Normal nodulation	75
	cv. Williams 82 NOD1-3 (<i>rj7</i>) mutant (hypernodulation)	ACC	No effect on nodule number	
<i>Lotus japonicus</i> (<i>Mesorhizobium loti</i>)	Ecotype B-129 Gifu transformed with an ethylene receptor gene (reduced ethylene sensitivity)		Increase of infection threads and nodule primordia Enhancement of <i>NIN</i> gene expression	55
<i>Medicago truncatula</i> (<i>Sinorhizobium meliloti</i>)	<i>Sickle</i> mutant (ethylene insensitivity)		Hyperinfection	68
			Blockage of calcium spiking in root cells	59
			Control of the position of nodule primordium formation	69
	<i>sickle/sunn</i> double mutant		Control of the position of nodule primordium formation	
	<i>sunn</i> mutant (hypernodulation)	ACC	Decrease in nodule number	
<i>Pisum sativum</i> L. (<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>)	cv. Sparkle <i>sym5</i> mutant (low or non-nodulation)	AVG/Ag ₂ SO ₄ /Co(NO ₃) ₂	Restoration of nodulation in <i>sym5</i> mutants	13
	Sparkle E107 (<i>brz</i>) mutant (low nodulation mutant)	AVG/Ag ₂ SO ₄	Restoration of nodulation in <i>brz</i> mutants	24
	cv. Sparkle E135F (<i>sym13</i>) mutant (small ineffective nodule)	Ethylene gas	Decrease in nodule number	37
	cv. Rondo <i>nod-3</i> mutant (hypernodulation)	Ethylene gas	Decrease in nodule number	
	cv. Sparkle R50 (<i>sym16</i>) mutant (low nodulation)	AVG	Acceleration of nodulation development	25
<i>Vicia sativa</i> (<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>)	ssp. <i>nigra</i> <i>tsr</i> mutant (thick, short roots)	AVG	Restoration of normal nodulation in <i>tsr</i> mutant	99
		Ethephon	Tsr (thick, short root) phenotype	91
		AVG	Restoration of normal root phenotype	

ACC, 1-aminocyclopropane-1-carboxylic acid; AOA, aminoxyacetic acid; AVG, L- α -(2-aminoethoxyvinyl) glycine; BAP, 6-benzyl-aminopurine; STS, silver thiosulfate

Chlorosis induction by rhizobitoxine produced by soybean bradyrhizobia

In 1956, Erdman *et al.*¹¹⁾ first reported that certain strains of rhizobia induce chlorosis in new leaves of soybeans. Owens and colleagues^{65,66)} 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 and other plant species^{31,60)}. The toxic compound was later called rhizobitoxine. Among soybean bradyrhizobia, all strains of *Bradyrhizobium elkanii* (formerly DNA homology group II) produce rhizobitoxine, whereas those of *B. japonicum* (formerly DNA homology group I) do not produce rhizobitoxine at all^{29,46,47,49)}. Thus, rhizobitoxine production is a general feature of *B. elkanii*, among which strains USDA94 and USDA76^T are high producers of rhizobitoxine in culture⁴⁶⁾. 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*⁵¹⁾.

Rhizobitoxine has therefore been regarded as a phytotoxin in that causes chlorosis symptoms in soybeans. However, recent studies have revealed that rhizobitoxine has a positive role in the establishment of symbiosis between *B. elkanii* strains and their host legumes (see below).

Biochemical functions of rhizobitoxine

The structure of rhizobitoxine was solved in 1972⁶⁴⁾. Rhizobitoxine is an enol-ether amino acid (2-amino-4-[2-amino-3-hydroxypropoxy]-*trans*-3-butenoic acid) with a molecular weight of 190. The first biochemical function identified for rhizobitoxine was that of an inhibitor of β -

cystathionase in the methionine biosynthesis pathway (β -cystathionase, E.C.4.4.1.8) (Fig. 2). β -Cystathionase catalyzes the conversion of cystathionine to homocysteine, pyruvate, and ammonium. As an analog of cystathionine, rhizobitoxine irreversibly inhibits β -cystathionase in bacteria⁶¹⁾ and plants¹⁶⁾.

Yasuta *et al.*⁹⁷⁾ found that rhizobitoxine also strongly inhibits 1-aminocyclopropane-1-carboxylate (ACC) synthase (E.C.4.4.1.14) in the ethylene biosynthesis pathway (Fig. 2), which can explain the early observation of rhizobitoxine inhibition of ethylene evolution in apple tissues⁶²⁾. 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^{7,97)}. The oxidized form of rhizobitoxine (dihydro-rhizobitoxine, Fig. 3) 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 synthase⁹⁷⁾.

Positive role of rhizobitoxine production in nodulation

Rhizobitoxine has been regarded as a phytotoxin ever since the discovery that it induces chlorosis. However, recent studies have shown a positive role of rhizobitoxine in the symbiosis between *B. elkanii* strains and their host legumes.

Yuhashi *et al.*⁹⁸⁾ reported that rhizobitoxine production in *B. elkanii* USDA94 reduces ethylene evolution from the associated roots of *Macroptilium atropurpureum* (Siratro) and enhances nodule formation by using *B. elkanii* USDA94 and its mutant lacking rhizobitoxine production⁹⁸⁾. In partic-

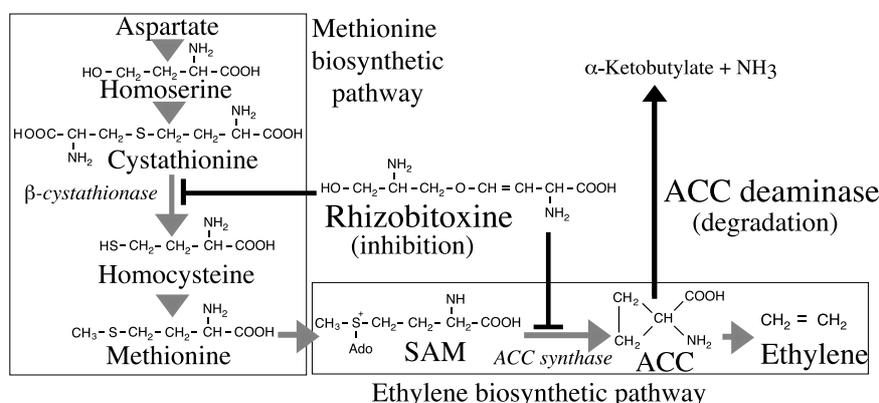


Fig. 2. Target reactions of rhizobitoxine and ACC deaminase. SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylate.

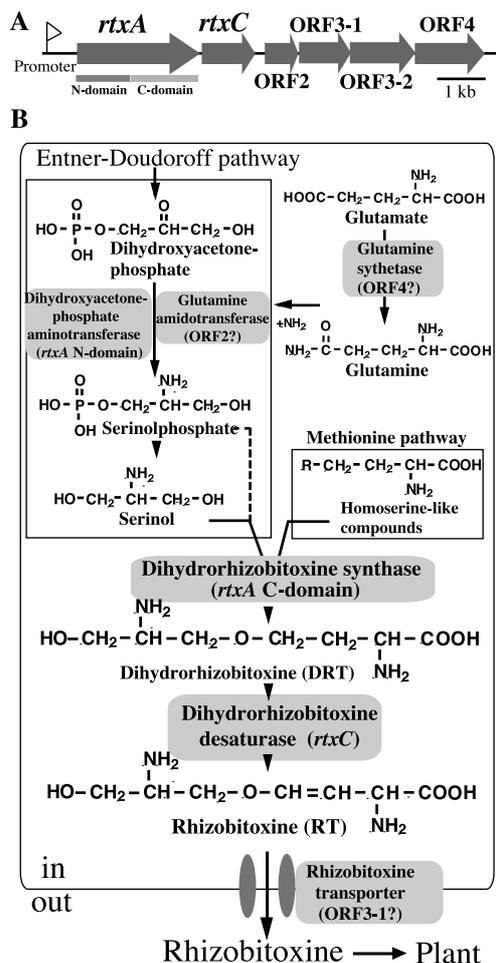


Fig. 3. Rhizobitoxine biosynthesis operon (A) and proposed pathway for rhizobitoxine biosynthesis (B) in *Bradyrhizobium elkanii*.

ular, the nodulation competitiveness of *B. elkanii* USDA94 was significantly enhanced by rhizobitoxine production (As for nodulation competitiveness, see below). Duodu *et al.*¹⁰ also reported a positive role of rhizobitoxine in the symbiosis between *B. elkanii* USDA61 and *Vigna radiata* (mungbean). 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 car-

ried out by Parker *et al.* using *Amphicarpaea*⁶⁷). 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.

Rhizobitoxine biosynthesis genes and pathway

The genes involved in rhizobitoxine biosynthesis have been identified in two *B. elkanii* strains, USDA61⁷¹) and USDA94⁹⁶). Ruan *et al.*⁷¹) 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*. Yasuta *et al.*⁹⁶) 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 ORFs downstream of the *rtxA* gene in a single transcriptional unit (RT operon), as shown in Fig. 3A (Sugawara, unpublished results). A large deletion mutant of *B. elkanii*, USDA94Δ*rtx*::Ω1, which lacks *rtxA*, *rtxC*, and the downstream ORFs, does not produce rhizobitoxine, dihydrorhizobitoxine, or serinol. The latter two compounds have been found to be coproduced with rhizobitoxine and are regarded as intermediates^{50,63,96}). Complementation analyses with cosmids containing an insertional mutagenesis within the putative RT 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 (Fig. 3). Recently, ORFs downstream of *rtxC* were found to be likely to mediate the incorporation of the amido-N of glutamine into serinol phosphate and to be involved in rhizobitoxine secretion (Sugawara *et al.* unpublished results) (Fig. 3).

Large amounts of dihydrorhizobitoxine are generally coproduced with rhizobitoxine^{48,63}). However, no conclusive data have been reported on the biological effects of dihydrorhizobitoxine *in planta*. Okazaki *et al.*⁵⁷) demonstrated that dihydrorhizobitoxine production does not affect the 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*.

Studies with a rhizobitoxine-producing pathogen,

Burkholderia (formerly *Pseudomonas*) *andropogonis*, have suggested L-aspartate, L-homoserine, and L-threo-hydroxythreonine as possible intermediates in rhizobitoxine biosynthesis⁵²). However, the proposed biosynthetic pathway needs additional yet-unidentified precursor(s), and genes involved in rhizobitoxine synthesis have not been isolated from *B. andropogonis*.

ACC deaminase in rhizobia: alternative strategy for decreasing ethylene levels

ACC deaminase degrades ACC (the immediate precursor of ethylene) into ammonia and α -ketobutyrate (Fig. 2). Historically, ACC deaminase has been found and well characterized in plant-growth-promoting rhizobacteria (PGPR)^{18,76}. The ACC deaminase of PGPRs likely reduces the amount of ACC in associated roots and promotes root elongation via the decrease in ethylene production¹⁷.

The ability to synthesize rhizobitoxine is confined to the slow-growing *B. elkanii* among rhizobia so far. Thus, Yasuta *et al.*⁹⁷) addressed the question as to whether fast-growing rhizobia possess another inhibitory factor for ethylene biosynthesis of host plants, because it could enhance nodulation⁵⁶). They sought potential enzymes and com-

pounds for reducing ethylene biosynthesis from the entire genome of the fast-growing *M. loti* MAFF303099 presented in a database³²), and identified ACC deaminase gene as a candidate⁸⁸). Indeed, ACC deaminase was verified to enhance nodulation and competitiveness to the host legume by using a disruption mutant of ACC deaminase gene (*mlr5932*)⁸⁸). Recently, genes encoding ACC deaminase have been also found in other rhizobia, such as *Rhizobium leguminosarum* and *Bradyrhizobium japonicum*^{33,44,45}). ACC deaminase from *Rhizobium leguminosarum* bv. *viciae* has been confirmed to enhance nodulation of *Pisum sativum* as well⁴⁴).

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.

Table 2. Rhizobial strategy for lowering ethylene in host plants

Rhizobia	Strain	Gene ^a	Expression ^b	Activity ^c	Plant showing nodulation enhancement ^d	Reference
<i>Bradyrhizobium elkanii</i>	USDA94	<i>rtxA, rtxC</i>	σ^{70}	Free/Symbiosis	<i>Macropodium atropurpureum</i> cv. Siratro	57, 58, 96, 98
	USDA61	<i>rtxA</i>		Free/Symbiosis	<i>Vigna radiata, Amphicarpaea edgeworthii</i>	10, 67, 71
<i>Bradyrhizobium japonicum</i>	USDA110	Fragmented <i>rtxA, rtxC</i>	None	Not detected		20, 33
	USDA110	<i>acdS</i> (blr0241)	σ^{70} /LRP	(Free/Symbiosis)		33
<i>Mesorhizobium loti</i>	MAFF303099	<i>acdS</i> (<i>mlr5932</i>)	σ^{54} /UAS	Symbiosis	<i>Lotus japonicus</i> MG20	32, 88
	R7A	<i>accD</i>	σ^{54} /UAS	(Symbiosis)		83
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	128C53K	<i>acdS</i>	σ^{70} /LRP	Free/(Symbiosis)	<i>Pisum sativum</i> L cv. Sparkle	44, 45
<i>Rhizobium radiobacter</i> ^e	d3	<i>acdS</i>	σ^{70} /LRP			87

^a Parenthesis indicates gene number of RHIZOBASE in Kazusa DNA research institute^{32,33}). *rtxAC* genes are rhizobitoxine biosynthesis genes, while *acdS* and *accD* genes encode ACC deaminase protein.

^b Mode of gene expression predicted by DNA sequences. σ^{70} means $-35/-10$ promoter. LRP (Leucine-responsive regulatory protein) indicates a negative regulatory system with LRP box. UAS and σ^{54} indicate upstream activating sequence (NifA-binding site) and consensus sequences of σ^{54} promoter ($-24/-12$ promoter).

^c Activities of rhizobitoxine biosynthesis and ACC deaminase in free-living cells (Free) and bacteroids (Symbiosis). Data in parenthesis are deduced from promoter structures.

^d Plant species showing nodulation enhancement by rhizobitoxine production or ACC deaminase activity. The nodulation enhancement was verified by the parent strain and disruption mutant of rhizobia.

^e Formerly, *Agrobacterium tumefaciens*⁷³)

Distribution and expression of genes involved in the synthesis of rhizobitoxine and ACC deaminase in rhizobia

The distributions of genes involved in the synthesis of rhizobitoxine and ACC deaminase are summarized in Table 2. The *rtx* genes involved in rhizobitoxine biosynthesis have been found in *B. elkanii* USDA94⁹⁶, USDA61⁷¹) and *B. japonicum* USDA110^{20,33}). The fact that all strains of *B. elkanii* isolated from soybean nodules produce rhizobitoxine in culture and *in planta*^{46,47}) indicates that *B. elkanii* commonly carry *rtx* genes. On the other hand, the situation regarding the existence and function of *rtx* genes in *B. japonicum* is complex. Genome sequencing revealed the presence of *rtx* genes in the *B. japonicum* USDA110 chromosome, although this bacterium does not produce rhizobitoxine^{46,47}). Perhaps partial disruption of *rtx* genes has caused them to lose the ability to biosynthesize rhizobitoxine⁹⁶). In addition, some strains of *B. japonicum*, such as USDA122, lack the entire *rtx* region (Minamisawa *et al.* unpublished results).

The rhizobitoxine genes of *B. elkanii* USDA94 are located in the symbiosis locus of the genome and are preceded by nodulation genes⁹⁶). However, the rhizobitoxine operon in *B. elkanii* USDA94 is likely to have no regulatory system, because it is expressed constitutively (Sugawara, unpublished data), which is supported by the presence of a σ^{70} promoter (-35/-10 general promoter) and the production of rhizobitoxine in both free-living cells and bacteroids (Table 2). The synthesis of rhizobitoxine depends on the presence of amino acids such as homoserine⁹⁶), which suggests that most rhizobitoxine production occurs in the rhizosphere and in plant tissues and little if any occurs in oligotrophic environments such as bulk soils.

Genes encoding ACC deaminase have been found in *B. japonicum*, *M. loti*, and *R. leguminosarum* (Table 2). ACC deaminase genes in *M. loti* strains MAFF303099 and R7A are preceded by a NifA-dependent σ^{54} promoter and thus appear to be expressed only in the symbiotic state^{83,88}). Indeed, the ACC deaminase gene of *M. loti* MAFF303099 was drastically upregulated in bacteroids compared with the level of its transcription in free-living cells⁸⁸). ACC deaminase activity was detected exclusively in bacteroids of wild type of *M. loti* MAFF303099⁸⁸). On the other hand, the ACC deaminase gene in *B. japonicum* and *R. leguminosarum* possesses a σ^{70} promoter with an LRP (leucine-responsive regulatory protein) box^{33,44}) (Table 2). Since the promoter structure of σ^{70} /LRP was involved in ACC induction of ACC deaminase gene in *R. leguminosarum* and *Entrobacter cloacae*^{44,45}), ACC is probably an inducer of the gene in

B. japonicum chromosome (Table 2). Recently, proteome analysis of *B. japonicum* bacteroids demonstrated that ACC deaminase is one of major proteins in bacteroids rather than free-living cells²⁹), suggesting that ACC in host plants might induce the ACC deaminase gene in rhizobia. Interestingly, a pathogen of *Rhizobium radiobacter* (formerly, *Agrobacterium tumefaciens*) strain d3 harbored *acdS* gene preceded by σ^{70} /LRP as well⁸⁷) (Table 2).

Mechanisms for nodulation enhancement

Rhizobitoxine is secreted outside rhizobial cells, probably by a rhizobitoxine transporter (Fig. 3), and delivered to the plant. In fact, rhizobitoxine has been detected in leaves as well as in nodules of soybeans inoculated with *B. elkanii*⁴⁸). Moreover, ethylene evolution is blocked in legume root systems inoculated with *B. elkanii*⁹⁸). Therefore, the explanation of how rhizobitoxine decreases endogenous levels of ethylene in legume roots appears straightforward. A model has been proposed for decreasing and adjusting endogenous ethylene levels in plant roots by rhizobial ACC deaminase. This was deduced by a diffusion model that ACC in plant roots is up-taken and decomposed in PGPR *P. putida*^{17,18}). So far there is no direct evidence for decreased ethylene evolution after bacterial inoculation that can be attributed to ACC deaminase.

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^{21,23,80}). 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 manner²). In petunia and tomato, transformation with a mutant *Arabidopsis* ethylene receptor gene (*etr1-1*) conferred reduced sensitivity to ethylene⁹²).

To address the mechanism by which ethylene inhibits nodulation, Nukui *et al.*⁵⁵) transformed *L. japonicus* B-129 'Gifu'²⁶) with a mutated ethylene receptor gene *Cm-ERS1/H70A*: A point mutation was introduced into the melon ethylene receptor *Cm-ERS1*⁷²) 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

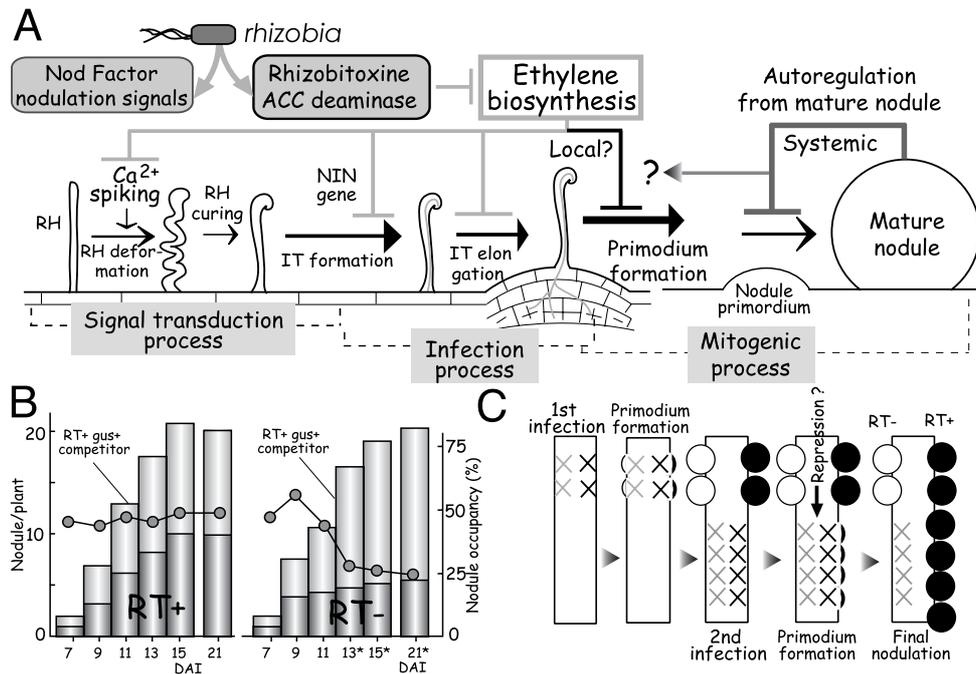


Fig. 4. Schematic presentations for a mechanism of ethylene inhibition of nodulation (A) and enhancement of nodulation competitiveness by rhizobitoxine (RT) production (B and C). (A) RH, root hair; IT, infection thread. In addition to nodulation signals (Nod factors, Fig. 1), rhizobia produce rhizobitoxine and/or ACC deaminase to reduce local ethylene levels by targeting ethylene biosynthesis in the host plants. High levels of endogenous ethylene would negatively regulate signal transduction, infection, and mitogen processes during rhizobia-legume interactions. In addition to the inhibition of nodulation by ethylene, nodule numbers in legumes already infected with rhizobia are strictly regulated by unknown factors from mature nodules (autoregulation) to maintain mutual symbiosis by both partners. (B) Time-course of nodule numbers (per plant) in competitive nodulation by a rhizobitoxine (RT)-producing strain (RT+) and non-producer (RT-) as compared with a reference strain (RT+/gus+ competitor)⁵⁸. Line graphs indicate the nodule occupancy percentage during nodulation. (C) A proposed model for rhizobitoxine enhancement of competitive nodulation. Black and white (or gray) indicate the rhizobitoxine (RT) producing strain (RT+) and non-producer (RT-), respectively. The cross, swelling and circle show infection focus, nodule primordium and mature nodule, respectively. The rhizobitoxine-producing (RT+) strain may locally overcome the repression of primordia formation at a stage of secondary infection, resulting in higher nodule occupancy of the RT+ strain.

without the transgene. In addition, the amount of transcripts of *NIN*, a gene governing formation of infection threads⁷⁴, 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 processes⁵⁵ (Fig. 4A).

So far, ethylene inhibition of nodulation has been studied by using chemical treatments (Table 1), ethylene-insensitive mutants⁶⁸, and transformants with reduced ethylene sensitivity⁵⁵. These studies have revealed the existence of several target processes that ethylene inhibits: signal transduction at the beginning of nodulation, the infection process, and mitogenic processes (Fig. 4A). In particular, the marked enhancement of primordium formation observed in later stages of nodulation⁵⁵ suggests that the mitogenic processes are deeply involved in ethylene inhibition of nodule

formation (Fig. 4A).

The inhibition of nodulation by ethylene is thought to be independent of feedback regulation during nodulation termed as “autoregulation”^{6,35,54} for the following reasons: (i) two hypernodulated mutants, *summ* of *Medicago truncatula*⁶⁹ and *har1* of *L. japonicus*⁹³, showed normal sensitivity to ethylene, although a hyperinfected *sickle* mutant of *M. truncatula* is insensitive to ethylene⁶⁸; (ii) the number of mature nodules on the *L. japonicus* transformant showing reduced ethylene sensitivity remained restricted, although the transformant had dramatically increased numbers of nodule primordia⁵⁵.

Enhancement of nodulation competitiveness

When mixed populations of diverse rhizobial strains exist in soil, certain strains can selectively infect and form nod-

ules on host legumes. The ability of a certain strain to form nodules in a multi-strain environment is referred to as nodulation competitiveness. Nodulation competitiveness has practical importance in agriculture: rhizobial inoculants that are superior for efficient symbiotic nitrogen fixation are often nodulated poorly in fields, owing to the presence of better-competing populations of inferior rhizobia in soils⁸¹).

Recent studies have revealed that both rhizobitoxine production by *B. elkanii* USDA94 and ACC deaminase activity in *M. loti* MAFF303099 significantly enhance their nodulation competitiveness on host legumes rather than nodulation by single inoculation^{58,88,98}. Enhancement of nodulation competitiveness by rhizobitoxine in siratro (*Macroptilium atropurpureum*) occurs at a late stage during nodulation (Fig. 4B), but does not affect cell growth in the rhizosphere⁵⁸. All other known rhizobial factors that affect nodulation competitiveness act in the rhizosphere and during the initial interaction with legumes, such as production of antibiotics or bacteriocin, cell surface characteristics, and motility^{85,86}).

Although it is still unclear how rhizobitoxine production and ACC deaminase activity affect nodulation competitiveness, a possible explanation is that they block ethylene biosynthesis locally at the infection site and cancel the negative effects of ethylene on nodulation during the mitogenic processes. According to the result of *B. elkanii* USDA94 in Fig. 4B, a rhizobitoxine-producing strain may locally overcome the repression of primordia formation at a stage of secondary infection, resulting in higher nodule occupancy of the RT+ strain (Fig. 4C). However, many uncertainties remain (Fig. 4C). For example: Does the nodulation enhancement occur locally? Do many nodulation foci of RT-strain remain? Is secondary primordium formation repressed exclusively by the systemic autoregulation? Does ethylene inhibition on nodulation fluctuate during nodulation and/or legume growth stages? Are there different effects and mechanisms between rhizobitoxine and ACC deaminase?

Coevolution of ethylene-mediated interactions between rhizobia and legumes

The symbiosis between legumes and rhizobia has endured since the origin of ancient Leguminosae⁸. Cumulative evidence has shown that most economically important legumes possess a mechanism for the regulation of nodule formation by ethylene (Table 1, Fig. 4), although most varieties of soybeans are not subjected to such ethylene regulation. Recent studies in turn have indicated that rhizobial species have evolved relevant strategies to cope with ethyl-

ene, that is, rhizobitoxine production and the expression of ACC deaminase. Ethylene-decreasing strategies have been found at least in three genera of rhizobia (Table 2), which implies that this strategy has been beneficial for rhizobia as they have coevolved with host legume species.

Parker and Peters⁶⁷ presented an interesting observation relevant to the evolution of symbiotic compatibility. A Japanese population of the legume *Amphicarpaea edgeworthii* formed few or no nodules with North American bradyrhizobial strains that did not produce detectable levels of rhizobitoxine. However, all North American *A. bracteata* lineages formed efficient nodules with Japanese *Bradyrhizobium* strains that produce rhizobitoxine and which were isolated from *A. edgeworthii*. They speculated that *A. edgeworthii* has adapted to an environment in which rhizobitoxine-producing rhizobia are prevalent and that long-term geographic isolation might lead to changes in symbiotic compatibility.

Incompatible symbioses between *B. elkanii* and modern cultivars of soybean appear to be an alternative example of coevolution. Rhizobitoxine-producing *B. elkanii* induce foliar chlorosis in modern cultivars of soybean^{11,95}, however, these soybeans are compatible with *B. japonicum*, which does not produce rhizobitoxine. It is possible that, as compatibility with *B. japonicum* strains has been bred into modern cultivars of soybean, they have concomitantly become sensitive to rhizobitoxine-inducing chlorosis. Interestingly, *B. japonicum* USDA110 possesses partially collapsed *rtx* genes⁹⁶, suggesting that an ancestor of *B. japonicum* that had the ability to produce rhizobitoxine. *B. japonicum* probably lost the ability to produce rhizobitoxine during the co-evolutionary process with soybeans, because rhizobitoxine plays no beneficial role but has the harmful effect of chlorosis induction on soybeans.

Exploitation of rhizobial strategies

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 melons¹². 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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