Rhizobitoxine production in *Agrobacterium tumefaciens* C58 by *Bradyrhizobium elkanii rtxACDEFG* genes

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Bradyrhizobium elkanii; rhizobitoxine; *Agrobacterium tumefaciens*; ethylene; 1-aminocyclopropane-1-carboxylate synthase.

Abstract

We examined the genetic basis and transfer for production of rhizobitoxine, an inhibitor of ethylene biosynthesis in plants, directed by the *rtx* genes of *Bradyrhizobium elkanii*. Comparison with genome sequences of *Bradyrhizobium japonicum* and *Xanthomonas oryzae* suggests that the *rtx* genes extend from the previously identified *rtxAC* genes through four additional genes *rtxDEFG*. Reverse transcription-PCR analysis showed that the *rtxACDEFG* genes are expressed as an operon. Mutational analysis indicated that *rtxDEG* mutants reduced rhizobitoxine biosynthesis, while the *rtxA* gene is essential for its synthesis. Introduction of the *rtxACDEFG* into *Agrobacterium tumefaciens* resulted in strong expression of *rtxACDEFG* and production of RtxA protein, but no rhizobitoxine was detectable. Addition of *O*-acetylhomoserine, a precursor of rhizobitoxine, to the *Agrobacterium* derivative, however, fostered production of rhizobitoxine in culture. The diluted culture supernatant inhibited the activities of β-cystathionase and 1-aminocyclopropane-1-carboxylate synthase, indicating that *A. tumefaciens* carrying *rtxACDEFG* genes excreted biologically active rhizobitoxine.

Introduction

Agrobacterium-mediated transformation is widely used in plant molecular genetics and its applications. In particular, efficient systems of genetic transformation are required for plant functional genomics and molecular breeding to improve traits (Tanaka *et al.*, 2005; Sun *et al.*, 2006). Ethylene, a gaseous plant hormone, is generated by plant tissues inoculated with *Agrobacterium*, and often inhibits *Agrobacterium*-mediated transformation (Davis & Moore, 1992; Ezura *et al.*, 2000; Hang *et al.*, 2005; Seong *et al.*, 2005). This inhibition is markedly reduced by the application of aminoethoxyvinylglycine, an inhibitor of 1-aminocyclopropane-1-carboxylate synthase (Ezura *et al.*, 2000; Hang *et al.*, 2005).

Bradyrhizobium elkanii produces rhizobitoxine, 2-amino-4-(2-amino-3-hydroxypropoxy)-*trans*-3-butenoic acid, to enhance nodulation by lowering ethylene biosynthesis in host plants (Yuhashi *et al.*, 2000; Sugawara *et al.*, 2006), similar to the mechanism of rhizobial 1-aminocyclopropane-1-carboxylate deaminase (Ma *et al.*, 2002, 2003; Hoa *et al.*, 2004; Nukui *et al.*, 2006). Rhizobitoxine is a structural analogue of aminoethoxyvinylglycine and inhibits 1-aminocyclopropane-1-carboxylate synthase (Yasuta *et al.*, 1999). At least *rtxAC* genes are required for the enzymes in rhizobitoxine biosynthesis in *B. elkanii*: dihydrorhizobitoxine synthase and desaturase (Yasuta *et al.*, 2001; Okazaki *et al.*, 2004b). The functions of additional genes downstream from *rtxAC* are still unknown.

The present work aimed to determine which genes are involved in rhizobitoxine biosynthesis in *B. elkanii*, and to test whether the introduction of these genes into *Agrobacterium tumefaciens* strain C58 would produce rhizobitoxine.

Materials and methods

Bacteria and growth conditions

Table 1 lists the bacterial strains and plasmids used in this work. *Bradyrhizobium elkanii* was grown at 30 °C in Tris-YMRT medium (Minamisawa *et al.*, 1990). *Agrobacterium tumefaciens* and *Escherichia coli* were grown at 30 and 37 °C, respectively, in Luria–Bertani (LB) medium (Sambrook *et al.*, 1989) with appropriate antibiotics.

DNA manipulation

DNA preparation, ligation, transformation of *E. coli*, PCR, and hybridization were carried out as described previously (Sambrook *et al.*, 1989; Minamisawa, 1990; Isawa *et al.*,

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Reference or source
Bacterial strains		
Bradyrhizobium elkanii		
USDA94	Wild-type strain	H.H. Keyser†
USDA94Δ*rtxA*	USDA94, *rtxA*::del/ins *aph* cassette; Km^r	This study
USDA94Δ*rtxD*	USDA94, *rtxD*::del/ins *aph* cassette; Km^r	This study
USDA94Δ*rtxE*	USDA94, *rtxE*::del/ins *aph* cassette; Km^r	This study
USDA94Δ*rtxG*	USDA94, *rtxG*::del/ins *aph* cassette; Km^r	This study
Agrobacterium tumefaciens		
C58C1Rif^R	C58 chromosomal background; pTiC58 cured, harbors pTiAch5 carrying a pBR322 insertion in *vir* reagion (pGV2260) avirulent strain; Rif^r Ap^r Tc^r	Deblaere *et al*. (1985)
Escherichia coli		
DH5α	*recA*, cloning strain	Toyobo Inc.
BL21 (DE3)	Lacks *lon* and *ompT* proteases	Novagen
Plasmids		
pRK2013	ColE1 replicon carrying RK2 transfer genes; Km^r	Figurski & Helinsk (1979)
pBlueScript II SK(+)	Cloning vector; Ap^r	Takara Shuzo Co.
pSUP202	pBR325 carrying *oriT* from RP4; Ap^r Cm^r Tc^r	Simon *et al*. (1983)
pSUP-SS	pSUP202 carrying *aadA* gene; Ap^r Cm^r Sp^r Sm^r Tc^r	This study
pUC4-KIXX	Plasmid carrying *aph* cassette; Ap^r Km^r	Pharmacia
pHP45Ω	Plasmid carrying *aadA* gene; Sp^r Sm^r Ap^r	Fallay *et al*. (1987)
pBBR1MCS-2	Broad-host-range plasmid; Km^r	Kovach *et al*. (1995)
pBBR::P*lac*RT	pBBR1MCS-2 carrying *rtxACDEFG;* Km^r	This study
pLAFR1	Broad-host-range cosmid; IncP Tc^r	Friedman *et al*. (1982)
pRTF1	pLAFR1 carrying *rtxACDEFG* and flanking regions from *B. elkanii* USDA94	Yasuta *et al*. (2001)
pET-20b (+)	Vector for expression by T7 RNA polymerase; Ap^r	Novagen
pET-rtxA-His	pET-20b (+) carrying *rtxA* for expression RtxA and His-Tag fusion protein; Ap^r	This study
pBS-*rtxA*	pBlueScript II SK(+) carrying a 9.0-kb AvrII and KpnI fragment including *rtxA*; Ap^r	This study
pBS-*rtxD*	pBlueScript II SK(+) carrying a 5.0-kb HindIII and ScaI fragment including *rtxD*; Ap^r	This study
pBS-*rtxE*	pBlueScript II SK(+) carrying a 4.2-kb BamHI and KpnI fragment including *rtxE*; Ap^r	This study
pBS-*rtxG*	pBlueScript II SK(+) carrying a 5.0-kb SspI and NheI fragment including *rtxG*; Ap^r	This study
pBS-Δ*rtxA*	pBS-*rtxA* derivatives containing HindIII-NdeI fragment deletion and *aph* cassette insertion; Ap^r Km^r	This study
pBS-Δ*rtxD*	pBS-*rtxD* derivatives containing EcoRI-AscI fragment deletion and *aph* cassette insertion; Ap^r Km^r	This study
pBS-Δ*rtxE*	pBS-*rtxE* derivatives containing Eco47III-FseI fragment deletion and *aph* cassette insertion; Ap^r Km^r	This study
pBS-Δ*rtxG*	pBS-*rtxG* derivatives containing PstI-HindIII fragment deletion and *aph* cassette insertion; Ap^r Km^r	This study
pSUP-Δ*rtxA*	pSUP-SS carrying a 8.5-bp XbaI-KpnI fragment from pBS-Δ*rtxA*; Km^r Sp^r Sm^r	This study
pSUP-Δ*rtxD*	pSUP-SS carrying a 5.6-bp XbaI-KpnI fragment from pBS-Δ*rtxD;* Km^r Sp^r Sm^r	This study
pSUP-Δ*rtxE*	pSUP-SS carrying a 4.7-bp XbaI-KpnI fragment from pBS-Δ*rtxE*; Km^r Sp^r Sm^r	This study
pSUP-Δ*rtxG*	pSUP-SS carrying a 5.2-bp AscI-XbaI fragment from pBS-Δ*rtxG*; Km^r Sp^r Sm^r	This study

*Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; Rif^r, rifampicin resistant; Sm^r, streptomycin resistant; Sp^r, spectinomycin resistant; Tc^r, tetracycline resistant.
†US Department of Agriculture, Beltsville, MD.

1999). Plasmid introduction into *A. tumefaciens* was performed as described by Shen & Forde (1989). Deletion mutants of *rtx* genes were constructed according to *rtxC* deletion mutant construction (Okazaki *et al*., 2004b), except for pSUP-SS usage as a suicide vector. pSUP-SS was constructed by insertion of the aminoglycoside adenyltransferase (*aadA*) gene into the EcoRI site of pSUP202 (Table 1). Total RNA was prepared and subjected to reverse transcription (RT)-PCR as described previously (You *et al*., 2005; Saito & Minamisawa, 2006).

Western blotting analysis

rtxA was PCR-amplified using rtxA-1 and rtxA2-3 primers (Table 2). After digestion by NdeI and BamHI, the resultant *rtxA* fragment was ligated into expression vector pET-20b

Table 2. Primers used in this study

Primers	Sequence	Position*
p1F	5′-TCGAGTTGAGCATTCGAGC-3′	12546–12564
p2F	5′-GGATCGGCTCATCAGCAAC-3′	12903–12922
pR	5′-GGTCACTTCCTTGAACTCAC-3′	13341–13322
acF	5′-AACTGTCGACCAGACACTCA-3′	15408–15427
acR	5′-GTTCCAGGTCGTGTTCTTC-3′	16024–16006
cdF	5′-ATCGACGCTTGTATGAGCAAT-3′	16737–16757
cdR	5′-ATTATGCAGGGAGTTCACTTGC-3′	17397–17376
deF	5′-ACGGCTTTTGGAGAGGCGTGT-3′	17560–17580
deR	5′-ACGATCGAGCTCCCCACT-3′	18211–18194
efF	5′-CGGCATCGTATTTTCAGCGG-3′	17816–17835
efR	5′-CGTCGACGATTACACAGTTTCG-3′	18669–18648
fgF	5′-TGCGTCTCGCTGATCTCCGACA-3′	19662–19683
fgR	5′-CATAAACTCAAACTCGAACC-3′	20329–20310
BBR-Km3	5′-CTGTCCGGTGCCCTGAATG-3′	
BBR-Km4	5′-CTAGGGTCACGACGAGAT-3′	
C58sigA-F	5′-CATGTATCTGCGCGAAATG-3′	
C58sigA-R	5′-CTTCAGTTCCTTGTAACGAC-3′	
rtxA-1†	5′- GGCACTCCATATGCTGCTCGACC TCGCATCGC-3′	13294–13315
rtxA-2.3‡	5′-GGAATTCTTGTCGTCGTCGTCGATT GCGGAAAGCGCC-3′	15706–15687

*Nucleotide sequence position in accession no. AB062279.
†The NdeI restriction site is underlined.
‡The EcoRI restriction site is underlined.

(+) (Novagen, Darmstadt, Germany). The His-tagged RtxA protein produced in *E. coli* BL21 (DE3) was purified using Ni-NTA agarose (QIAGEN, Hilden, Germany). Rabbit antiserum against the RtxA protein was prepared by Sigma Genosys, Japan (Ishikari, Hokkaido, Japan). Cells of *B. elkanii* and *A. tumefaciens* were washed with 50 mM potassium phosphate buffer (pH 6.8). Cell-free extract was prepared by centrifugation (12 000 ***g***, 10 min, 4 °C) after sonication. Western blotting analysis was performed as described by Sambrook *et al.* (1989).

Determination of rhizobitoxine-related compounds

Agrobacterium tumefaciens strains were precultured in LB medium containing kanamycin (50 μg mL^{-1}) until the stationary phase. Washed cells were suspended in 10 mL of LB medium at an OD of 2.0 at 660 nm. After shaking for 5 days, rhizobitoxine and its intermediates were quantified using liquid chromatography-MS (Minamisawa *et al.*, 1990; Yasuta *et al.*, 2001). Rhizobitoxine bioassays were based on the inhibition of β-cystathionase and 1-aminocyclopropane-1-carboxylate synthase (Ruan & Peters, 1991; Yasuta *et al.*, 1999).

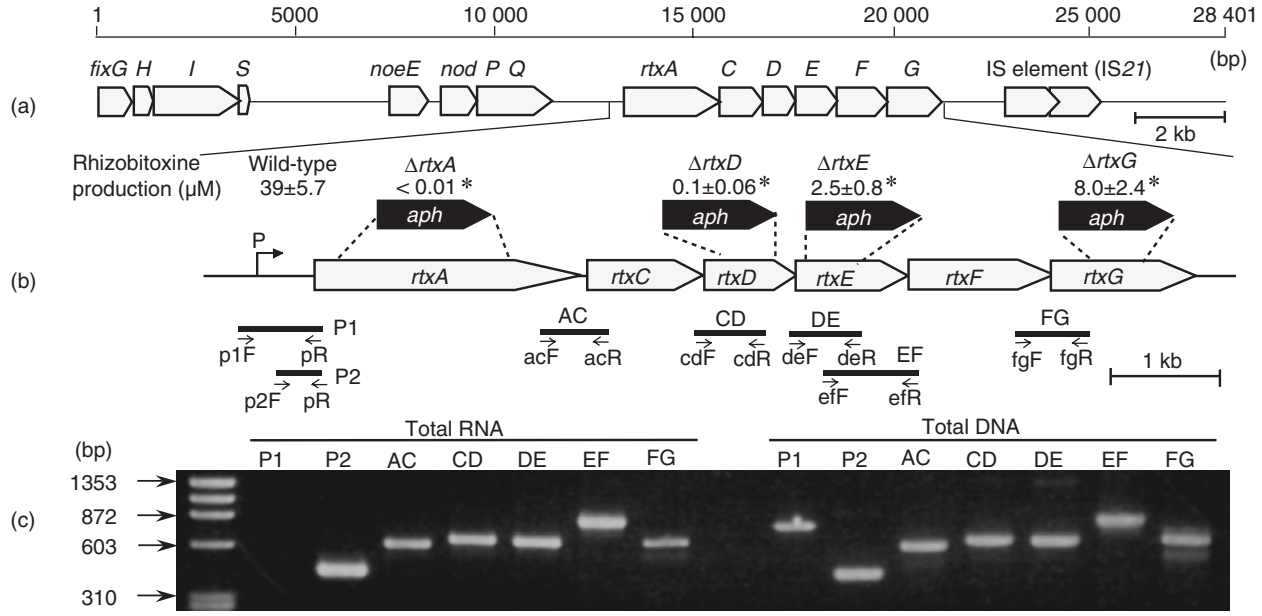

Fig. 1. Organization, function, and transcription of the *rtx* gene cluster in *Bradyrhizobium elkanii*. (a) Map in and around the gene cluster (DDBJ accession no. AB062279) (Yasuta *et al.*, 2001). (b) Position of *rtx* gene deletion and rhizobitoxine production (mean ± SD) of the deletion mutants grown in Tris-YMRT medium without antibiotics for 14 days. Horizontal black bars represent the locations of fragments amplified by RT-PCR using primers (arrows; Table 2). P indicates the putative −35/−10 promoter. (c) PCR amplification of intergenic transcripts. P1: negative control including primer p1F upstream from P. (Reannotated *rtxDEFG* genes have DDBJ accession no. AB062279.) The amino acid sequences of RtxD, RtxE, RtxF, and RtxG were homologous to glutamine amidotransferase class I in *Burkholderia cenocepacia* (accession no. AAHL01000021.1, BLAST score 165), permeases of the drug/metabolite transporter superfamily in *Yersinia pestis* (accession no. NZ_AAKS01000045.1, BLAST score 64), biotin carboxylase in *Y. pestis* (accession no. AE017140.1, BLAST score 217), and glutamine synthetase in *Legionella pneumophila* (accession no. NC_002942.5, BLAST score 364). *Statistically significant (*t*-test, $P \leq 0.01$) between rhizobitoxine production of the mutant and wild-type stain.

Results and discussion

Characterization of *rtx* genes in *B. elkanii*

Recently, *rtx* gene homologues have been found as gene clusters in genome sequences of *Bradyrhizobium japonicum* USDA110 (Kaneko *et al.*, 2002) and *Xanthomonas oryzae* (Ochiai *et al.*, 2005; Sugawara *et al.*, 2006). The present study reannotated the *B. elkanii* sequences downstream from the *rtxAC* genes and found four genes designated *rtxDEFG* (Fig. 1a). Consequently, the six genes probably form a successive *rtx* gene cluster as *rtxACDEFG* (Fig. 1a). The transcripts of the intergenic regions between the *rtxACDEFG* genes were consistently detected (Fig. 1b and c). However, fragment P1, which encompasses a putative promoter (P), could not be amplified on the RNA level, although all fragments were detected on the DNA level (Fig. 1c). These results suggest that *rtxACDEFG* genes formed a transcriptional unit from the putative $-35/-10$ promoter to *rtxG*.

Deletion mutants of *rtxA, rtxD, rtxE*, and *rtxG* in *B. elkanii* were constructed using the nonpolar *aph* cassette (Okazaki *et al.*, 2004b). The *rtxA* mutant synthesized no rhizobitoxine, while *rtxD*, *rtxE*, and *rtxG* mutants produced significantly less rhizobitoxine than the wild type (Fig. 1b). This indicates that *rtxDEFG* is also involved in rhizobitoxine biosynthesis in *B. elkanii* (Yasuta *et al.*, 2001; Okazaki *et al.*, 2004b).

Transfer of *rtx* genes into *A. tumefaciens*

Bradyrhizobium elkanii rtxACDEFG was then transferred into *A. tumefaciens*. pBBR::P*lac*RT was constructed containing *rtxACDEFG* genes in a broad-host-range vector (pBBR1MCS-2) under a *lac* promoter (Fig. 2a), and was then introduced into *A. tumefaciens* strain C58C1Rif^R. The transconjugant by pBBR::P*lac*RT expressed *rtxACDEFG* genes even in *A. tumefaciens*, whereas these transcripts were not detected in *A. tumefaciens* C58C1Rif^R containing the vector (Fig. 2b). Western blot analysis revealed the RtxA protein (88 kDa) in *A. tumefaciens* C58C1Rif^R (pBBR::P*lac*RT) carrying the *rtxA* gene; its size was identical to the predicted size of RtxA in wild-type USDA94 (Fig. 2c). On the other hand, no RtxA-specific band was detected in C58C1Rif^R containing the vector. Therefore, *rtxA* encoding key enzymes for rhizobitoxine biosynthesis (Yasuta *et al.*, 2001) was translated in *A. tumefaciens*.

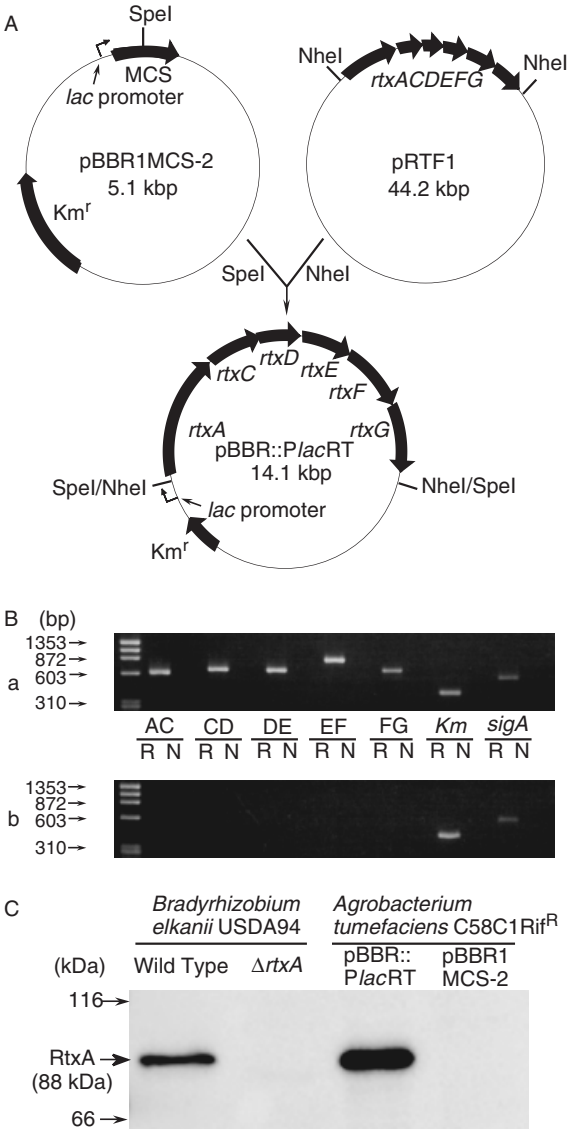

Fig. 2. (A) Construction of the *rtxACDEFG* plasmid for *Agrobacterium tumefaciens* and (B, C) expression of *rtx* genes in the *A. tumefaciens* transconjugant. (A) An NheI fragment (9.0 kb) containing *Bradyrhizobium elkanii rtxACDEFG* genes was ligated into the SpeI site of the broad-host-range plasmid pBBR1MCS-2, resulting in pBBR::P*lac*RT. (B) Analysis of RT–PCR for intergenic regions of *rtx* genes in *A. tumefaciens* C58C1Rif^R containing (a) pBBR::P*lac*RT and (b) pBBR1MCS-2. R and N indicate RT-PCR and PCR without RT reaction, respectively. Lane labelled N lacked amplification products, indicating no contamination of genomic DNA in the RNA preparation. Km and *sigA* indicate positive expression controls for the kanamycin resistance gene in plasmid pBBR1MCS-2 and the *sigA* gene in *A. tumefaciens* C58, respectively. (C) Western blot analysis using RtxA antiserum.

Rhizobitoxine production in *A. tumefaciens*

The above results prompted an examination of rhizobitoxine production by *A. tumefaciens* C58C1Rif^R carrying *rtxACDEFG*. However, C58C1Rif^R (pBBR::P*lac*RT) grown in LB medium without additives did not produce rhizobitoxine, although a large amount of serinol (a rhizobitoxine precursor) was detected (Table 3).

Yasuta *et al.* (2001) suggested that rhizobitoxine is synthesized from serinol and *O*-acetylhomoserine in the

Table 3. Determination of rhizobitoxine, dihydrorhizobitoxine, and serinol in culture supernatants of *Agrobacterium tumefaciens* C58C1Rif^R carrying pBBR::P*lac*RT (*rtxACDEFG* genes) and the vector pBBR1MCS-2*

Plasmid	pBBR1MCS-2 (Vector)			pBBR::P*lac*RT (*rtxACDEFG*)				
							% Inhibition of enzyme activity†	
addition	Rhizobitoxine (μM)	DRT (μM)	Serinol (μM)	Rhizobitoxine (μM)	DRT (μM)	Serinol (μM)	β-cystathionase	1-aminocyclopropane-1-carboxylate synthase
None	< 0.03	< 0.01	2.3 ± 0.3	< 0.03	< 0.01	355 ± 30	ND	ND
Homoserine (5 mM)	< 0.03	< 0.01	1.0 ± 0.2	< 0.03	5.3 ± 0.5	606 ± 173	–	–
O-acetylhomoserine (5 mM)	< 0.03	< 0.01	0.8 ± 0.1	1.2 ± 0.1	31 ± 3	534 ± 41	21	6
Homoserine (25 mM)	< 0.03	< 0.01	1.1 ± 0.1	0.2 ± 0.3	12 ± 10	949 ± 155	–	–
O-acetylhomoserine (25 mM)	< 0.03	< 0.01	1.3 ± 0.1	14.4 ± 1.2	250 ± 24	572 ± 25	66	42

*Rhizobitoxine, DRT and serinol were quantified by liquid chromatography-MS. These values are shown as mean ± SD ($n = 3$).

†The 20% diluted culture supernatant with 5 and 25 mM *O*-acetylhomoserine by C58C1Rif^R (pBBR::P*lac*RT) was subjected to two bioassays based on β-cystathionase and 1-aminocyclopropane-1-carboxylate synthase (see text). The percentage of enzyme inhibition was expressed as mean of triplicate determinations.

ND, Not detected; DRT, dihydrorhizobitoxine.

methionine biosynthetic pathway. There are two different pathways of methionine biosynthesis: via *O*-acetylhomoserine catalyzed by MetX and via *O*-succinylhomoserine by MetA in bacteria (Hacham *et al*., 2003). A search for methionine biosynthesis genes in *A. tumefaciens* C58 genome sequences (Wood *et al*., 2001) suggested that the bacterium does not possess *metX* for homoserine *O*-acetyltransferase but carries *metA* to encode homoserine *O*-succinyltransferase. This suggests that strain C58 provided no *O*-acetylhomoserine as a rhizobitoxine precursor.

When 5 or 25 mM *O*-acetylhomoserine was supplied in the growing medium, rhizobitoxine was successfully produced by *A. tumefaciens* C58C1Rif^R (pBBR::P*lac*RT) (Table 3). In the presence of *O*-acetylhomoserine, the *A. tumefaciens* derivative also produced a large amount of dihydrorhizobitoxine, which is a precursor of rhizobitoxine and shows very low inhibition for β-cysthathionase and 1-aminocyclopropane-1-carboxylate synthase (Yasuta *et al*. 2001; Okazaki *et al*. 2004b). On the other hand, the cells in the growing medium with homoserine produced much less amounts of rhizobitoxine and dihydrorhizobitoxine than those with *O*-acetylhomoserine at 5 or 25 mM (Table 3). These results support the genomic insight that *A. tumefaciens* strain C58 is able to convert homoserine into *O*-succinylhomoserine by MetA but not into *O*-acetylhomoserine.

In addition, the activities of two enzymes were markedly inhibited in 20% diluted culture supernatant with 5 and 25 mM *O*-acetylhomoserine by C58C1Rif^R (pBBR::P*lac*RT) as compared with the activities for C58C1Rif^R (pBBR1MCS-2) (Table 3). These results indicate that *A. tumefaciens* C58C1Rif^R (pBBR::P*lac*RT) excreted biochemically active rhizobitoxine in culture in the presence of *O*-acetylhomoserine.

Towards metabolic engineering of plant-associated bacteria

Rhizobitoxine production by *A. tumefaciens* C58C1Rif^R (pBBR::P*lac*RT) reached 14 μM in culture supplemented with 25 mM *O*-acetylhomoserine (Table 3); this level is expected to reduce ethylene emission and enhance *Agrobacterium*-mediated transformation as effectively as aminoethoxyvinylglycine treatment (10 μM) (Ezura *et al*., 2000). To attain the final goal, we should improve *O*-acetylhomoserine metabolic fitness in *A. tumefaciens*, overcome incompatibility with binary vector such as pIG121Hm (Ezura *et al*., 2000; Sun *et al*., 2006), and maximize rhizobitoxine production where *Agrobacterium*-mediated transformation takes place (Ezura *et al*., 2000).

Bradyrhizobium elkanii produces rhizobitoxine to enhance nodulation via inhibition of ethylene biosynthesis in host plants (Yuhashi *et al*., 2000; Okazaki *et al*., 2004a; Sugawara *et al*., 2006). The present work is a first step in utilizing that strategy as a natural source for constructing an efficient plant transformation system. In addition, this work uniquely grafted six genes related to the complex rhizobitoxine biosynthetic pathway (Yasuta *et al*., 2001; Okazaki *et al*., 2004b) into a different metabolic background (*Agrobacterium*) as a trial of metabolic engineering of plant-associated bacteria (Strohl, 2001).

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