รายงานวิจัยฉบับสมบูรณ์

การศึกษาการควบคุมการตอบสนองต่อ โลหะและสภาวะ oxidative stress โดยยีน *irr* และ *rirA* ของเชื้อแบคทีเรีย *Agrobacterium tumefaciens*

โดย ดร. รจนา สุขชวลิต

เดือน เมษายน พ.ศ. 2553

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สถาบันวิจัยจุฬาภรณ์

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รหัสโครงการ TRG5180009

ชื่อโครงการ การศึกษาการควบคุมการตอบสนองต่อ โลหะและสภาวะ oxidative stress โดยยีน *irr* และ *rirA* ของเชื้อแบคทีเรีย *Agrobacterium tumefaciens*

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ระยะเวลาโครงการ 2 ปี

Agrobacterium tumefaciens เป็นแบคทีเรียที่ก่อโรคในพืช เหล็กเป็นธาตุที่จำเป็นต่อการเจริญเติบโตของแบคทีเรีย แต่หากมีปริมาณมากเกินความจำเป็นก็จะเป็นอันตรายต่อเซลล์ได้เช่นกัน โดยสามารถก่อให้เกิดสารอนุมูลอิสระ การรักษาสมดุลของเหล็กภายในเซลล์จึงมีความสำคัญอย่างยิ่งต่อการเจริญเติบโต การอยู่รอด และในระหว่างการก่อพยาธิสภาพในพืช การควบคุมระดับเหล็กภายในเซลล์ของแบคทีเรียในกลุ่ม alphaproteobacteria ถูกควบคุมโดยโปรตีน RirA (rhizobial iron regulator) และ โปรตีน Irr (iron response regulator) โปรตีน RirA และ โปรตีน Irr ทำหน้าที่ร่วมกันในการควบคุมยีนที่เกี่ยวกับการนำเหล็กเข้าเซลล์ โดยโปรตีน RirA ทำหน้าที่เป็นตัวกด (repressor) การแสดงออกของยีนในสภาวะเหล็กสูง เพื่อป้องกันไม่ให้มีเหล็กเกินภายในเซลล์ ในขณะที่โปรตีน Irr จะถูกทำลายในสภาวะเหล็กสูง โปรตีน Irr จึงทำหน้าที่ได้ในสภาวะที่มีเหล็กต่ำโดยเป็นตัวกระตุ้น (activator) ยีนที่นำเหล็กเข้าเซลล์ พบว่าแบคทีเรียกลายพันธุ์ที่สูญเสียยีน *rirA* จะมีภาวะเหล็กเกินภายในเซลล์ ซึ่งทำให้มีความไวต่อสาร H_2O_2 เพิ่มขึ้น และมีความสามารถในการก่อพยาธิสภาพในพืชน้อยลง ในขณะที่แบคทีเรียกลายพันธุ์ที่สูญเสียยีน *irr* มีความสามารถในการก่อพยาธิสภาพในพืชเช่นเดียวกับแบคทีเรียสายพันธุ์ปกติ และมีความต้านทานต่อสาร H_2O_2 เพิ่มขึ้น ทั้งนี้เนื่องจากโปรตีน Irr ทำหน้าที่เป็นตัวตัวกด (repressor) การแสดงออกของยีน *mbfA* ซึ่งเป็นยีนที่ช่วยป้องกันแบคทีเรียจากสาร H_2O_2 นอกจากโปรตีน Irr จะสามารถทำงานในสภาวะเหล็กต่ำแล้ว ในสภาวะที่มีแมงกานีสโปรตีน Irr ทำหน้าที่เป็นตัวกดการแสดงออกของยีน(repressor) ได้ดียิ่งกว่าสภาวะเหล็กต่ำ ซึ่งยังไม่เคยมีการรายงานมาก่อน

คำหลัก: *rirA, irr*, metal response, oxidative stress, virulence

Abstract

Project Code: TRG5180009

Project Title: Study of *Agrobacterium tumefaciens irr* and *rirA* genes: their physiological roles in controlling the response to metals and oxidative stress.

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Project Period: 2 years

Agrobacterium tumefaciens causes crown gall tumor disease on dicotyledonous plants. Iron is an essential micronutrient for most bacteria. However, high levels of intracellular iron are toxic since iron can cause cellular damage by catalyzing the production of harmful hydroxyl radicals. Iron homeostasis is critical for bacterial survival and is therefore tightly regulated, mostly at the transcription level, in response to iron availability. RirA (rhizobial iron regulator) and Irr (iron response regulator) are iron regulators found exclusively in alphaproteobacteria. *A. tumefaciens* has an effective regulation of iron acquisition to ensure cellular iron demand. This is reflected by the fact that siderophore synthesis and transport genes are co-regulated by RirA and Irr. Siderophore synthesis and transport genes are turned on via derepression through RirA and activation through Irr that help to increase efficiency for iron acquisition under iron deprivation. Iron regulation is crucial for bacterial survival and infection. Loss of the iron-responsive transcriptional regulator could be lethal to bacteria. Mutation in the *A. tumefaciens rirA* gene reduced the bacterial ability to cause tumor on tobacco leaves. The attenuated virulence in the *rirA* mutant most likely resulted from the increased sensitivity to oxidants and the decreased ability to induce virulence genes (*virB* and *virE*). Nonetheless, the *irr* mutant strain had no apparent defects in tumorigenesis on tobacco leaves. The *irr* mutant strain showed increased resistance to H_2O_2 killing but had reduced catalase activity. MbfA was highly expressed in the *irr* mutant strain and its role in the H_2O_2 protection was demonstrated. Generation of H_2O_2 is an important mechanism that plant use to inhibit invading bacteria during infection The fact that *irr* mutant strain was more resistant to H_2O_2 killing, perhaps this ability may, at least in part, contribute to the fully virulent phenotype. Importantly, Irr was found to respond to both iron and manganese in controlling its target genes.

Keywords: *rirA, irr*, metal response, oxidative stress, virulence

สารบัญ

หน้า

หน้าสรุปโครงการ (Executive Summary)

ความสำคัญและที่มาของปัญหา

Iron is an essential metal for the growth of most bacteria because of its importance in many biological processes. Although iron is abundant in nature, its availability to living cells is limited due to its low solubility under aerobic conditions and physiological pH values. Most iron is present in the ferric Fe^{3+} form which is insoluble and biologically inaccessible. Therefore, bacteria have evolved highly efficient iron uptake mechanisms to acquire iron [1]. Under iron starvation, bacteria produce and secrete high-affinity iron binding molecules, such as siderophores, to scavenge iron from the environment. The ferri-siderophore complexes are then taken up into bacteria via specific iron transport systems. Once ferri-siderophore complex is inside the cell, iron is released by reduction of ferric Fe^{3+} to ferrous Fe^{2+} form which is the relevant biological cofactor. Although essential, excess intracellular free Fe^{2+} is toxic due to its ability to catalyze the production of highly deleterious hydroxyl radicals via the Fenton reaction [2]. The generated reactive hydroxyl radicals can damage DNA, proteins, and lipids. The balance between acquiring enough iron to grow and avoiding iron-induced toxicity is adjusted by modulating iron transport, storage and consumption. In many bacteria, this regulation is mediated by the Fur (ferric uptake regulator) protein [1]. Iron regulation by Fur is particularly well studied in γ-proteobacteria, such as *Escherichia coli*, providing the classic model of Fur regulation [1]. Fur functions as a transcriptional repressor of iron uptake systems and iron-regulated genes when sufficient iron is available. In the absence of the cofactor Fe^{2+}, Fur no longer binds to the regulated promoters, leading to derepression of iron uptake systems under the iron-deficient conditions. Fur has also been reported to regulate genes involved in acid tolerance, production of toxins and virulence factors, and defense against oxidative stress.

Agrobacterium tumefaciens is a Gram-negative, member of α-proteobacteria, soil-born phytopathogen that causes crown gall tumor disease on dicotyledonous plants. Withholding of iron and oxidative burst are plant defense mechanisms to inhibit bacterial invasion [3,4]. In order to survive and cause disease, phytopathogens have to combat both iron deprivation and oxidative threat. Therefore, the microbial abilities of iron sequestering and balancing as well as defending against oxidative damage would indeed determine its pathogenesis on host plant.

Over the past decade, it has emerged that the metal specificity and function of Fur-like proteins from members of α-proteobacteria such as *Rhizobium leguminosarum* and

Sinorhizobium meliloti, are different than in the model γ-proteobacteria [5-7]. Fur is not the major iron regulator. The function of Fur is more limited and has changed to control manganese transport instead. These observations led to rename Rhizobial Fur-like proteins as Mur (manganese uptake regulator). Other two iron responsive regulators, RirA (rhizobial iron regulator) and Irr (iron response regulator), have evolved to play dominant roles in controlling iron homeostasis in members of α-proteobacteria. The RirA protein from the Rrf2 family, not related to Fur, had been shown to replace typical Fur function in regulation of iron-responsive genes under iron-replete conditions in rhizobia [8,9]. The RirA regulon includes genes for the synthesis (*vbs*) and uptake (*fhu*) of the siderophore vicibactin, genes involved in heme uptake (*hmu* and *tonB*), genes that probably participate in the transport of Fe^{3+} (*sfu*), a putative ferri-siderophore ABC transporter (*rrp1*), a gene specifies an ECF RNA polymerase δ factor (*rpoI*), genes for the synthesis of Fe-S clusters (*suf*), an iron response regulator (*irr*) and *rirA* itself [10]. In contrast to Fur and RirA, Irr functioned only under iron limitation. The presence of iron caused repression of *irr* transcription [11] and degradation of Irr protein in a heme-dependent mechanism [12]. Irr, a member of the Fur family, is both a transcriptional activator and repressor of many genes involved in iron transport (*fbpA*, *irpA*), storage (*mbfA*, *bfr*) and metabolism (*hemA*, *hemB*, *suf*, *fssA*, *fdx)*. Moreover, Irr also controlled genes involved in the TCA cycle, energy metabolism and oxidative stress response [13,14]. It has been shown that some genes such as *suf*, *irp*, and *bfr*, are under the dual control of both Irr and RirA [10]. The overlap of Irr and RirA regulons suggests that iron regulation in α-proteobacteria is distinct and more complex than in other bacteria.

A. tumefaceins genome contains genes annotated as iron-responsive transcriptional regulator *fur* (Atu0354), *irr* (Atu0153) and *rirA* (Atu0201) [15]. From these three genes, only *fur* gene was recently characterized by our group [16]. *A. tumefaciens fur* controlled the manganese transport operon *sitABCD* and was not the major regulator of iron transport and metabolism, however, *fur* was required for bacterial survival under iron limiting conditions [16]. Further characterization of iron-sensing regulators, *irr* and *rirA*, from *A. tumefaciens* would help to understand the distinct iron regulation and metals homeostasis in this phytopathogen, as well as the impact of these regulators on bacterial survival and its virulence during plant-pathogen interaction.

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วัตถุประสงค์ของโครงการ

1. สร้างแบคทีเรียกลายพันธุ์ *Agrobacterium tumefaciens* ที่สูญเสียยีน *irr* หรือ *rirA*
2. ทดสอบความต้านทานต่อสาร oxidants และโลหะของ *irr* หรือ *rirA* mutant strains เปรียบเทียบกับ wild-type strain
3. วิเคราะห์ปริมาณเอนไซม์ SOD และ catalase ของ *irr* หรือ *rirA* mutant strains เปรียบเทียบกับ wild-type strain
4. ศึกษาบทบาทของยีน *irr* หรือ *rirA* ต่อ promoter activity ของยีนที่เกี่ยวข้องกับ iron homeostasis และ oxidative stress response
5. ทดสอบความสามารถในการก่อพยาธิสภาพในพืชของ *irr* หรือ *rirA* mutant strains เปรียบเทียบกับ wild-type strain

ระเบียบวิธีวิจัย

Construction of *irr* and *rirA* mutant strains.

Mutants will be generated by insertional inactivation of the *irr* or *rirA* gene on the chromosome by homologous recombination. The *A. tumefaciens irr* or *rirA* fragment will be cloned into the plasmid pKNOCK-Km and will be transferred to *A. tumefaciens* by conjugation. The mutant will be selected on LB agar plates containing kanamycin, and confirmed by Southern blot hybridization.

Metals and oxidants resistance of *Agrobacterium* mutant strains.

Cells from an overnight culture (10^8) will be subcultured into 10 ml of LB medium and grown at 28^{o}C with shaking for 4 h. Exponential phase cells (10^8) will be diluted and spotted on LB plates containing varying concentrations of either metals ($FeCl_3$, $MnCl_2$, $ZnCl_2$, $CdCl_2$, and $NiCl_3$) or oxidants (H_2O_2, menadione, and tBOOH: *tert*-butyl hydroperoxide). Plates will be incubated at 28 ^{o}C for 24 h.

Complementation assay.

The full-length of wild-type *irr* or *rirA* gene will be cloned into the expression vector and transferred into mutant strains. Sensitivity to metals and oxidants will be tested. If mutation of interested gene is responsible for observed phenotypes in the mutant strain, the reverse phenotype would be observed in mutant strain expressing the functional protein.

Construction of promoter-*lacZ* fusions and β-galactosidase activity assay.

To identify genes regulated by Irr or RirA, promoter regions of some iron-responsive genes (eg. *hemA*, *hemB*, *fhuA*, *bfr*, *rbr*, *suf*) will be separately cloned into a promoter-less vector, pUFR027*lacZ*. The resultant plasmids will be transferred into wild-type and mutant strains. Bacteria grown overnight in LB medium will be sub-cultured into fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) will be treated with 50 μM of $FeCl_3$, or or 200 μM of the iron chelator 2,2'-dipyridyl for 30 min. Cells will be harvested and used for β-galactosidase activity assay. Levels of β-galactosidase activity from wild-type and mutant backgrounds would reveal the mechanisms by which regulators, Irr or RirA control their targeted genes.

Determination of SOD and catalase levels.

Bacterial lysates will be prepared from log phase cells by sonication. To visualize SOD activity on gel, non-denaturating electrophoresis will be performed and follow by staining with nitroblue tetrazolium/riboflavin photochemical stain. To visualize catalase activity on native gel, after gel electrophoresis, the gel will be incubated with horse-radish peroxidase, follow by reacting with H_2O_2 and staining with diamino benzidine. Catalase activity will appear as colorless bands.

Tumor formation assay.

The virulence of mutant strains will be determined and compared to those of wild-type strain. *A. tumefaciens* strains containing pCMA1 plasmid will be used to infect tobacco (*Nicotiana tabacum*) leaves according to the method described previously. Cells from an overnight culture in MG/L medium (10^8) will be subcultured into 10 ml of MG/L medium and grown at 28°C with shaking for 4 h. The cells will be harvested and washed with 10 ml of MSO medium. The cells suspension will be adjusted with MSO medium to an OD_{600} of 0.01. Tobacco leaves will be cut to small pieces and soaked in the bacterial suspension for 20 min at room temperature. After removing excessive bacterial suspension, the tobacco leaves will be placed onto MSO agar containing 300 μM acetosyringone and incubated at 28°C in the dark for 2 days. The tobacco leaves will then be transferred on to MSO agar containing 200 μg/ml timentin and incubated at 28°C in the dark. After 2 weeks, the tumors on the tobacco leaves will be monitored.

แผนการดำเนินงานวิจัยตลอดโครงการ

	Year 1		Year 2	
	1st half	2nd half	1st half	2nd half
1. Construction of an *A. tumefaciens rirA* and *irr* knockout mutants and plasmid construction	←	→		
2. Characterization of *rirA* and *irr* mutants		←	→	
- Determination of oxidant and metal resistance				
- SOD and catalase activity assays				
- Promoter-*lacZ* activity assays				
- Virulence assay				
3. Preparation of manuscripts				← →

ผลงานวิจัยและรายละเอียดวิธีวิจัย

1. Construction of *A. tumefaciens rirA* and *irr* mutant

Disruption of *rirA* gene (Atu0201) (Wood, Setubal et al. 2001) was performed by the method described previously (Kitphati, Ngok-Ngam et al. 2007). The internal DNA fragment of *rirA* coding region was amplified by PCR with primers BT1201-5'GGCTTACGGCGTATCTGAGC3' and BT1202-5'CGTCCTCAAAGCACTCAGCC3', using Pfu DNA polymerase and genomic DNA isolated from wild-type NTL4 as the template. The 188 bp PCR product was cloned into the unique *Sma*I site of the pKNOCK-Km suicide plasmid (2 Kb in size) (Alexeyev 1999) which is unable to replicate in *A. tumefaciens*, generating the recombinant plasmid, pKNOCKrirA. The cloned DNA region was confirmed by automated DNA sequencing. The pKNOCKrirA was transferred into wild-type NTL4 by conjugation (Cangelosi, Best et al. 1991). The single homologous recombinants were selected on LB agar plates containing 25 μg ml^{-1} chloramphenicol and 30 μg ml^{-1} kanamycin. Correct integration of the pKNOCKrirA into the *rirA* locus was confirmed by Southern Blot analysis. Chromosomal DNA samples isolated from wild-type NTL4 and the *rirA* mutant were digested with *Bst*EII. The DNA fragments were separated on a 1% agarose gel and blotted onto a nylon membrane. The hybridization probe was a PCR-amplified 188 bp fragment from *rirA* gene using primers BT1201 and BT1202. The probe was radioactively labelled using a random priming kit (Amersham Pharmacia Biotech) and [α-^{32}P]dCTP. Wild-type NTL4 gave an expected hybridizing band of 1.35 Kb. In contrast, a hybridizing band of 3.5 Kb was obtained from the *rirA* mutant, confirming that pKNOCKrirA had correctly integrated into the *rirA* gene (Figure 1A). The *rirA* mutant was named NTLrirA.

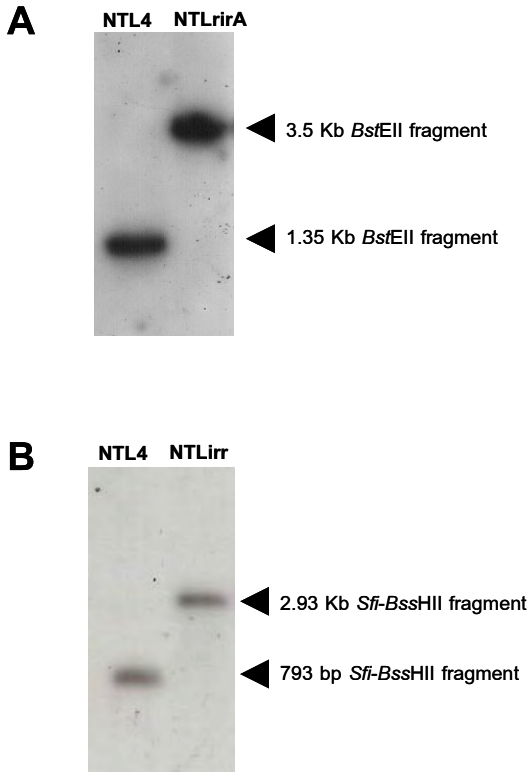
The *A. tumefaciens irr* gene was inactivated by integration of the suicide plasmid into the chromosomal *irr* gene. The 223 bp DNA fragment of *irr* internal region was amplified by PCR using primers BT696-5'GCCAGCGCGTTGCTTTGGGT3' and BT697-5'AAGAAGTGATGGTGATCCG3' designed from the sequence of a putative *irr* gene (Atu0153) (Wood, Setubal et al. 2001), Pfu DNA polymerase (Promega) and genomic DNA isolated from wild-type NTL4 as the template. The PCR product was cloned into pKNOCK-Gm (1.6 Kb in size) (Alexeyev 1999), a non-replicative plasmid in *Agrobacterium*, digested at the unique *Sma*I site. The insert's nucleotide sequence was confirmed by automated DNA sequencing. The resultant plasmid,

pKNOCKirr, was then transferred to *A. tumefaciens* NTL4 by conjugation (Cangelosi, Best et al. 1991). The single homologous recombinants were selected on LB agar containing 25 μg ml^{-1} chloramphenicol and 90 μg ml^{-1} gentamicin. Southern blot analysis was performed to confirm the *irr* mutant. Chromosomal DNA samples isolated from wild-type NTL4 and the *irr* mutant were digested with *Sfi*I and *Bss*HII. The DNA fragments were separated on a 1% agarose gel and blotted onto a nylon membrane. The blot was hybridized to 223 bp coding region of *irr* probes which were prepared by PCR amplification of NTL4 genomic DNA using primers BT696 and BT697 and radioactively labeled using a random priming kit (Amersham Pharmacia Biotech) and [α-^{32}P]dCTP. A single hybridizing band of 0.79 Kb was detected from wild-type NTL4, as expected from the genomic sequence. A hybridizing band of 2.39 Kb was obtained from the *irr* mutant, confirming that pKNOCKirr had correctly integrated into the chromosomal *irr* gene (Figure 1B). The *irr* mutant was named NTLirr.

2. Cloning of full-length *rirA* and *irr* genes

The full-length of wild-type *rirA* and *irr* genes were amplified from *A. tumefaciens* NTL4 genomic DNA by PCR with specific primers for rirA gene (BT1701-5'GCGGTCAACAAGGCTGTTCG3' and BT1205-5'CGATCAGGCTGCCGGAAGTG3') and for *irr* gene (BT694-5'GTTTGGAACGGGATTGCATG3' and BT695-5'AACGTCAACCGCGCTTGCGA3'), using Pfu DNA polymerase. The PCR products were cloned into the unique *Sma*I site of an expression vector, pBBR1MCS-4 (Kovach, Elzer et al. 1995), creating the recombinant plasmids pRirA and pIrr. The cloned DNA region was confirmed by automated DNA sequencing. The plasmids pRirA and pIrr will be used for *rirA* and *irr* mutant complementation experiments.

Figure1. Southern blot analysis of *A. tumefaciens* **wild-type NTL4, NTLrirA and NTLirr mutants.** Numbers to the right indicate the molecular size of positively hybridized bands.

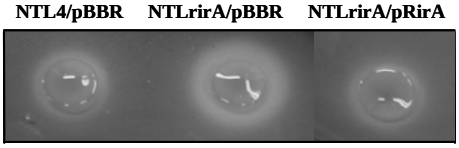


3. Characterization of *rirA* mutant

3. 1 Siderophore production

Siderophores are ferric-ion-chelating molecules synthesized and secreted by bacteria growing under low-iron stress. In many bacteria, synthesis of siderophores is negatively regulated by iron and the Fur (ferric uptake regulator) protein. Several previous studies had clearly shown that RirA evolved to replace typical Fur functions in controlling iron uptake systems (Todd, Wexler et al. 2002; Viguier, O Cuiv et al. 2005). Siderophore production was analysed using a chrome azural S (CAS) agar plate (Schwyn and Neilands 1987). Solid CAS medium was made by adding 10 ml of CAS stock (Schwyn and Neilands 1987) to 100 ml of YEM medium (Vincent 1970) containing 1.5% agar. Overnight cultures grown in LB medium (5 μl at an OD_{600} of 0.1) were spotted onto a YEM+CAS plate containing 200 μM 2,2'-dipyridyl and incubated at 28°C for 2 days. Siderophore production is indicated by the presence of an orange halo zone around the bacteria. This occurs because siderophores produced by bacteria remove iron from the original blue CAS-Fe^{3+} complex contained in the plate, resulting in a change in color of the dye. As shown in Fig. 2, the NTLrirA mutant strain harbouring plasmid vector (NTLrirA/pBBR) produced more siderophores than wild-type strain (NTL4/pBBR) as indicated by a larger halo zone surrounding NTLrirA/pBBR compared to a halo zone surrounding NTL4/pBBR. Furthermore, complementation of the mutant strain by expressing functional *rirA* gene on the plasmid pRirA (NTLrirA/pRirA) could restore the siderophore production to a similar level as that of wild-type NTL4/pBBR (Fig. 2). This confirmed that the overproduction of siderophores in NTLrirA was due to the loss of *rirA* gene.

Figure 2. Detection of siderophores. Analysis of siderophore production was performed using siderophore indicator CAS agar plates. NTL4/pBBR and NTLrirA/pBBR strains are the wild-type and the *rirA* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTLrirA/pRirA is the *rirA* mutant expressing functional RirA from the plasmid pRirA.



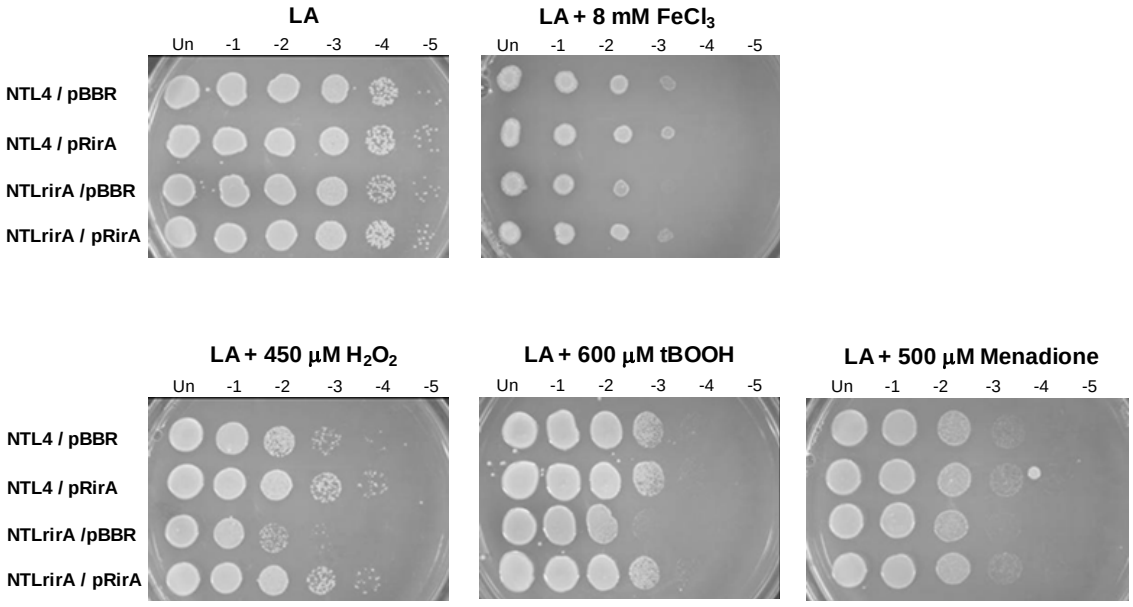
3.2 *fhuA-lacZ* fusion assay

The role of *A. tumefaciens rirA* in controlling iron uptake was further supported by monitoring expression of an outer membrane siderophore receptor *fhuA* gene (Atu4022), from the *fhuA-lacZ* transcriptional fusion plasmid (pP*fhuA-lacZ*) in NTL4 and NTLrirA strains. The putative *fhuA* promoter region was amplified from *A. tumefaciens* NTL4 genomic DNA with primers BT1095-5'CGTAGCTCGAATGTATCCGC3' and BT1096-5'CGCGACATAACCTTTCACCG3' using Pfu DNA polymerase (Promega). The 414 bp PCR product was cloned into the unique *Hin*dIII site (end-gap fill with Klenow enzyme) of the promoter probe vector pUFR027*lacZ*, a derivative of pUFR027. The resultant recombinant plasmid was named pP*fhuA-lacZ* and was transferred into wild-type NTL4 and the NTLrirA mutant. Bacteria grown overnight in LB medium were sub-cultured into fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were treated with 50 μM $FeCl_3$ or 200 μM Dipy for 1 h. Cells were harvested and used for a β-galactosidase activity assay. The levels of β-galactosidase activity from NTLrirA/pP*fhuA-lacZ* grown under iron-replete (860 ± 20 U mg of protein^{-1}) and iron-depleted (1,350 ± 35 U mg of protein^{-1}) conditions were higher than those of wild-type NTL4/pP*fhuA-lacZ* (570 ± 15 and 700 ± 25 U mg of protein^{-1}, respectively). These data demonstrated that *A. tumefaciens* RirA was the repressor of iron uptake systems and provided experimental verification of predicted members of the RirA regulon (Rodionov, Gelfand et al. 2006).

3.3 Determination of oxidant and metal resistance

Cells grown on LB agar plates were washed once and then were 10-fold serially diluted in fresh LB medium. An aliquot (10 μl) of each dilution was spotted onto an LB agar plate containing 450 μM H_2O_2, 600 μM *tert*-butyl hydroperoxide (tBOOH), 500 μM menadione (MD), or 8 Mm $FeCl_3$. Plates were incubated at 28^{o}C for 2 days. The results in Fig. 3 show that the NTLrirA mutant was more sensitive to oxidants and iron than wild-type NTL4. Moreover, the hypersensitive phenotype of the NTLrirA mutant could be complemented by overexpression of the functional *rirA* gene on the plasmid pRirA. This indicated that oxidants and iron hypersensitive phenotype of the NTLrirA mutant was due to the loss of *rirA* gene.

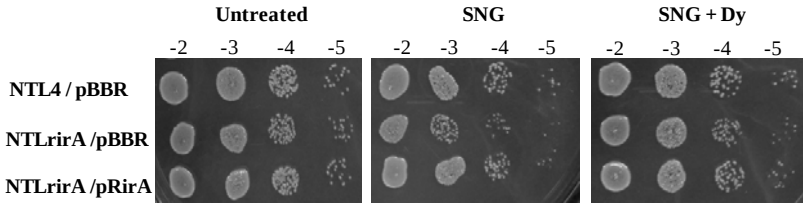
Figure 3. Sensitivity to oxidants and metal. Cells were diluted and spotted on LB agar plates containing 8 Mm $FeCl_3$, 450 μM H_2O_2, 600 μM *tert*-butyl hydroperoxide (tBOOH), or 500 μM menadione. Plates were incubated at 28°C for 48 h. Cells spotted on an LB agar plate (LA) were used as a control. 10-fold serial dilutions are marked above each column.



3.4 Sensitivity to streptonigrin

The previous data showed that the NTLrirA mutant had derepression of iron uptake systems. To determine whether the NTLrirA mutant has increased in the levels of intracellular iron, streptonigrin sensitivity test was performed. Streptonigrin (SNG) is an iron-activated antibiotic. Increased intracellular iron concentrations have been shown to correlate with an increased sensitivity to SNG (White and Yeowell 1982). The relative intracellular iron levels in NTL4 and the NTLrirA mutant were assessed by SNG sensitivity assays. The results in Fig. 4 show that the NTLrirA/pBBR mutant was 10-fold more sensitive to 40 μg ml^{-1} SNG treatment than wild-type NTL4/pBBR. This suggests that the NTLrirA mutant had intracellular free iron levels higher than that of wild-type NTL4. The expression of the functional *rirA* gene in the NTLrirA mutant (NTLrirA/pRirA) could restore resistance to SNG to the wild-type (NTL4/pBBR) levels. Moreover, the addition of 2,2'-dipyridyl, a cell membrane-permeable iron chelator that chelates intracellular iron, could protect the NTLrirA mutant from SNG killing. This further supported the conclusion that loss of *rirA* led to iron overload conditions in the NTLrirA mutant.

Figure. 4. Sensitivity to streptonigrin. Cells were untreated and treated with 40 μg ml^{-1} streptonigrin in the absence (SNG) or presence of 100 μM 2,2'-dipyridyl (SNG+Dy) at 28°C for 3 h. Cells were then diluted and spotted on LB agar plates (LA). Ten-fold serial dilutions are indicated above each column. NTL4/pBBR and NTLrirA/pBBR strains are the wild-type and the *rirA* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTLrirA/pRirA is the *rirA* mutant expressing functional RirA from the plasmid pRirA.

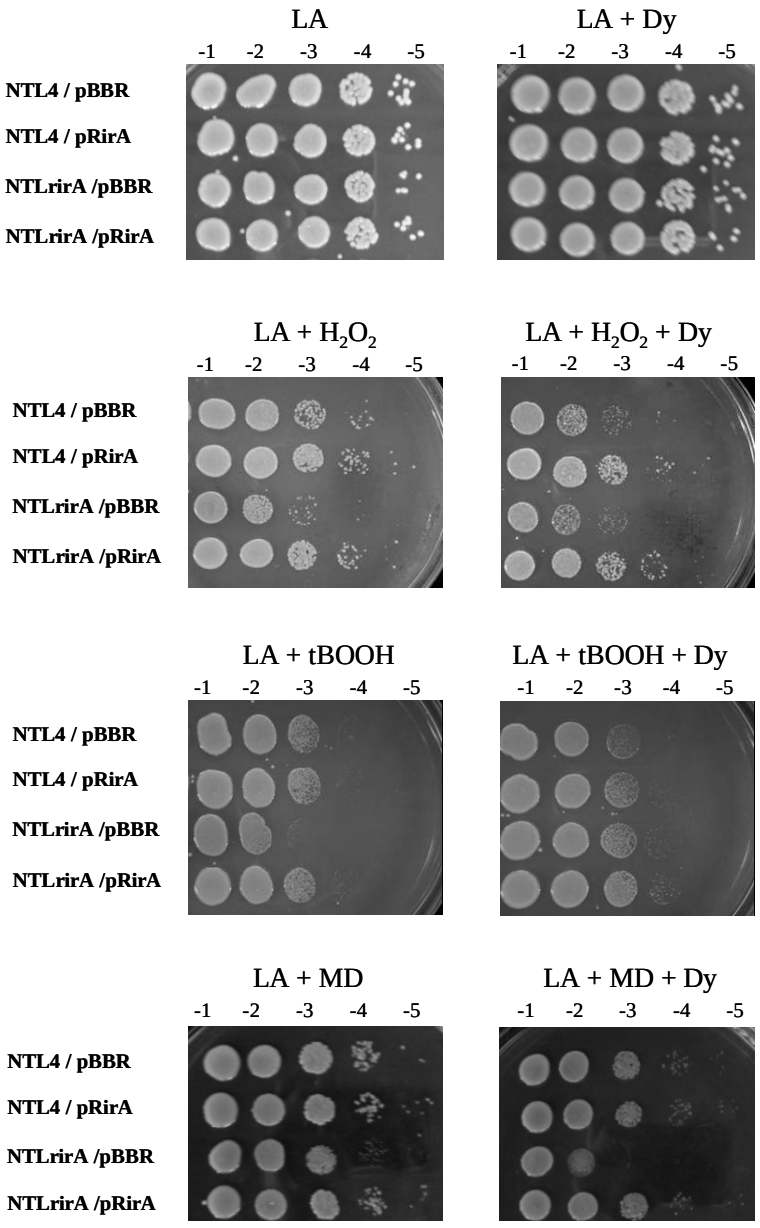


3.5 Sensitivity to oxidants in the presence of an iron chelator

The NTLrirA mutant was more sensitive to oxidants, including H_2O_2, *tert*-butyl hydroperoxide (tBOOH) and a superoxide generator, menadione (MD), than wild-type NTL4. NTLrirA/pBBR was 10-fold more sensitive than NTL4/pBBR to 450 μM H_2O_2, 600 μM tBOOH and 450 μM MD (Fig. 5).

Interestingly, the addition of an iron chelator 2,2'-dipyridyl (Dy) in the medium could also reverse the hypersensitive phenotype of the NTLrirA to H_2O_2 and tBOOH, but not to MD. The growth of NTLrirA/pBBR was similar to that of NTL4/pBBR on LA + 450 μM H_2O_2 + 100 μM Dy and LA+ 600 μM tBOOH + 100 μM Dy plates (Fig. 2). The data implied that increased sensitivities to H_2O_2 and tBOOH toxicity in the NTLrirA mutant were most likely due to the iron overload condition. This condition likely resulted from derepression of the expression of iron uptake systems. This is consistent with exposure to peroxides, leading to the Fenton reaction and generation of highly reactive oxygen species, which are responsible for the phenotype of the NTLrirA mutant with an increased sensitivity to peroxides. In contrast, the hypersensitivity of the NTLrirA mutant to MD could not be reversed by the addition of an iron chelator (Fig. 2), suggesting that mechanisms other than the iron overload condition mediated MD toxicity in the NTLrirA mutant.

Figure. 5. Sensitivity to oxidants. Cells were diluted and spotted on LB agar plates containing 450 μM H_2O_2, 600 μM *tert*-butyl hydroperoxide (tBOOH), or 450 μM menadione (MD), in the absence or presence of 100 μM 2,2'-dipyridyl (Dy). Plates were incubated at 28°C for 48 h. Ten-fold serial dilutions are marked above each column. Cells spotted on an LB agar plate (LA) were used as a control. NTL4/pBBR and NTLrirA/pBBR strains are the wild-type and the *rirA* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTL4/pRirA and NTLrirA/pRirA strains are the wild-type and the *rirA* mutant, respectively, expressing functional RirA from the plasmid pRirA.



3.6 Sod activity gel assay

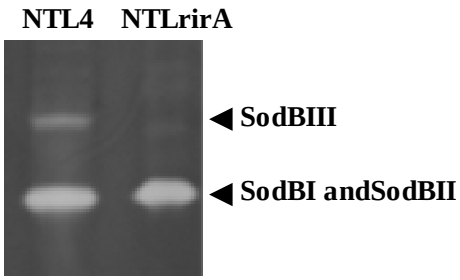
The NTLrirA mutant showed an increase in the sensitivity to a superoxide generator, menadione. A possible mechanism was that the NTLrirA mutant might have a defect in enzymes involved in the superoxide detoxification. To test this hypothesis, a Sod activity gel assay was performed using cell lysates from wild-type NTL4 and the NTLrirA mutant (Fig. 6). Crude bacterial lysates were prepared using bacterial suspensions in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM PMSF, a protease inhibitor. Cell suspensions were lysed by brief sonication followed by centrifugation at 12,000 *g* for 10 min. Cleared lysates were used for total protein determination and Sod activity gel assay. Protein concentrations were determined using the Bradford BIO-RAD protein assay. To visualize Sod activity on gel, 25 μg proteins from lysate samples were separated on a 10% non-denaturing gel followed by staining with nitroblue tetrazolium-riboflavin photochemical stain (Beauchamp and Fridovich 1971).

A. tumefaciens contains three superoxide dismutases, namely SodBI, SodBII and SodBIII (Saenkham, Eiamphungporn et al. 2007). All Sod enzymes have iron-cofactors. These *sods* exhibit differential expression patterns, and their gene products are found in different cellular locations. SodBI is the major cytoplasmic Sod enzyme and its gene is constitutively expressed throughout all growth phases, whereas *sodBII* is scarcely expressed under normal conditions. *sodBII* has a MD-inducible expression pattern and is regulated by a superoxide sensor and transcription regulator SoxR (Saenkham, Eiamphungporn et al. 2007). SodBII is a cytoplasmic enzyme. *sodBIII* is highly expressed during the stationary phase of growth and the enzyme is located in the periplasmic space. At present, the regulators of *sodBI* and *sodBIII* genes have not been identified.

The results in Fig. 6 clearly showed that SodBIII could not be detected in the NTLrirA mutant. SodBI and SodBII migrated to the same position on a Sod activity gel, which contributed to the major visible band (Saenkham, Eiamphungporn et al. 2007). These data indicated that the levels of SodBIII and not SodBI or SodBII are affected by inactivation of *rirA*. Therefore, the lower levels of

superoxide dismutase (SodBIII) were partly responsible for the menadione-sensitive phenotype of the NTLrirA mutant.

Figure 6. Sod activity gel. Equal amounts of protein (25 μg) from cell lysates of NTL4 and NTLrirA were loaded and separated on a 10% non-denaturing gel. Superoxide dismutase isozymes were visualized by activity staining.



3.7 Virulence assay

A. tumefaciens induces the formation of crown gall tumors by transferring T-DNA from the bacterium's tumor-inducing (Ti) plasmid into host plant cells (Ziemienowicz 2001). The effect of *rirA* inactivation on the virulence of *A. tumefaciens* was evaluated by the analysis of tumor formation on tobacco leaf pieces infected with wild-type and *rirA* mutant strains containing tumor-inducing plasmid pCMA1 according to the method described previously (Kitphati, Ngok-Ngam et al. 2007).

Cells were grown on LB agar plates at 28°C for 2 days. Cells were washed with hormone-free MS liquid medium, and the cell concentration was adjusted to an OD_{600} of 0.01 in 20 ml of hormone-free MS medium. The cell suspensions were co-cultivated with a ~0.5 cm square leaf of tobacco (30 leaf squares for each bacterial strain) at room temperature for 20 min. Tobacco leaf pieces incubated in hormone-free MS medium without bacterial cells were used as a negative control. The tobacco leaf pieces were transferred onto hormone-free MS agar plates containing 300 μM acetosyringone and incubated at 28°C in the dark for 2 days. The tobacco leaf pieces were then transferred onto hormone-free MS agar plates containing 200 μg ml^{-1} timentin and incubated at

28°C in the dark. The tumors on each leaf piece were observed after 14 days. The experiments were repeated twice.

The mutant NTLrirA/pCMA1 showed significantly less virulence than the wild-type strain NTL4/pCMA1 (Fig. 7). The tumors that formed on tobacco leaf pieces infected with NTLrirA/pCMA1 were much fewer and smaller than those caused by NTL4/pCMA1. Furthermore, the attenuated virulence of the NTLrirA/pCMA1 mutant could be complemented by pRirA, as shown by the fact that the tumor-inducing ability of NTLrirA/pCMA1/pRirA was completely restored to NTL4/pCMA1 levels (Fig. 4). In contrast, NTLrirA/pCMA1/pBBR1MCS-4 could not complement the reduced-virulence phenotype of the mutant (data not shown). These data confirmed that the loss of *rirA* led to the defect in *A. tumefaciens* virulence.

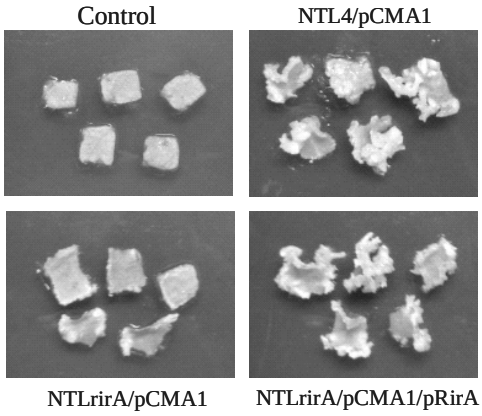


Figure 7. Virulence assay. Tumor formation was determined on tobacco leaf squares infected with *A. tumefaciens* wild-type NTL4 or mutant NTLrirA containing the pCMA1 plasmid (NTL4/pCMA1 and NTLrirA/pCMA1, respectively). The NTLrirA mutant was complemented by expression of functional RirA from the plasmid pRirA (NTLrirA/pCMA1/pRirA). Control: tobacco leaf squares without infection. Representative leaf pieces (from n=30) are shown.

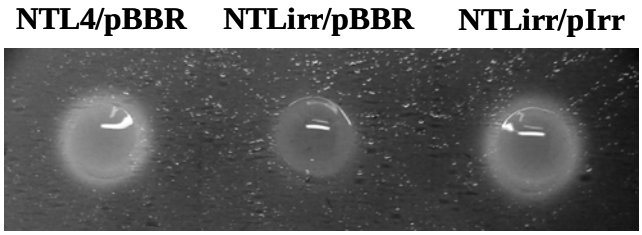
4. Characterization of *irr* mutant

4.1 Determination of role of Irr in the iron uptake systems

Under iron-deficient conditions, many bacteria produce and secrete siderophores to sequester ferric ion from the environments (Andrews, Robinson et al. 2003). The siderophore synthesis gene cluster in *A. tumefaciens* consisted of two divergently transcribed operons, one consisting of the genes from Atu3675 through Atu3685, and the other consisting of Atu3673 through Atu3670 (Rondon, Ballering et al. 2004). This siderophore synthesis gene cluster was predicted to be regulated by RirA according to the presence of IRO motifs in the promoter regions of those genes (Rodionov, Gelfand et al. 2006). It was confirmed that RirA has a negative effect on siderophore production and transport genes since the *A. tumefaciens rirA* mutant (NTLrirA) showed an increase in siderophore production and derepression of siderophore receptor genes (*fhuA* and *irp6A*) (Ngok-Ngam, Ruangkiattikul et al. 2009).

It has been shown that Irr positively affected the iron regulation of siderophore production in *B. abortus* (Martinez, Ugalde et al. 2006). In order to investigate whether siderophore biosynthesis was affected in the NTLirr mutant, cells were spotted onto a YEM+CAS plate containing 200 μM 2,2'-dipyridyl as previously described (Ngok-Ngam, Ruangkiattikul et al. 2009). The NTLirr/pBBR strain had significantly decreased production of siderophores compared to wild-type strain, NTL4/pBBR, as indicated by an apparently smaller halo zone surrounding NTLirr/pBBR colonies compared to the halo zone surrounding NTL4/pBBR (Fig. 8). A decrease in siderophore production in the NTLirr mutant could be restored by providing a functional *irr* gene on the plasmid pIrr, as observed in the complemented strain NTLirr/pIrr. These data indicated that *A. tumefaciens* Irr has a positive effect on the siderophore biosynthesis under iron limitation similar to that reported in *B. abortus*.

Figure 8. Detection of siderophores. Analysis of siderophore production was performed using siderophore indicator CAS agar plates. NTL4/pBBR and NTLirr/pBBR strains are the wild-type and the *irr* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTLirr/pIrr is the *irr* mutant expressing functional Irr from the plasmid pIrr.



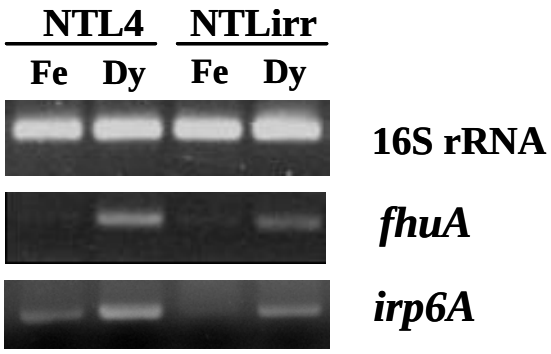
The positive regulatory role of Irr on the ferric iron transport was further demonstrated in *B. japonicum* (Small, Puri et al. 2009). The induction of ferric siderophore receptor genes (blr3555, blr3904, blr4504, bll4920, and bll7968) under low iron conditions was abolished in an *irr* mutant. *fhuA* and *irp6A* are siderophore receptor genes in *A. tumefaciens* that previously were shown to be negatively controlled by RirA under iron-replete conditions (Ngok-Ngam, Ruangkiattikul et al. 2009). In addition to the presence of IRO motifs, the ICE motifs were also found in the promoter regions of *fhuA* and *irp6A* (Rodionov, Gelfand et al. 2006). Therefore, it is likely that these genes are also regulated by Irr. The reverse transcriptase PCR (RT-PCR) analysis was performed to determine the role of Irr in controlling ferric siderophore receptor genes.

Bacteria grown overnight in LB medium were sub-cultured into 20 ml of fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were treated with 50 μM $FeCl_3$ or 250 μM 2,2'-dipyridyl for 15 min before harvesting by centrifugation at 6,000 rpm for 5 min. Total RNA was extracted from untreated and treated cells using the modified hot phenol method as described previously (Ngok-Ngam, Ruangkiattikul et al. 2009). The RNA samples were treated with DNase I using the DNA-*free*™ Kit (Ambion), according to the manufacturers'

protocols. Reverse transcription (converting mRNA to cDNA before PCR) was accomplished using SuperScript™ II Reverse Transcriptase (Invitrogen) with random hexamer primers (BioDesign, Thailand). Reverse transcribed RNA samples (0.1 μg) from each condition were used in the PCR reaction. Control reactions, where reverse transcriptase was omitted, were run in parallel to ensure that there was no DNA contamination. Positive controls were performed using genomic DNA isolated from wild-type NTL4. Gene-specific primers for *fhuA* (BT1346 , 5'-GGTGACGAAGGGTATTGGCG3' and BT1096, 5'-CGCGACATAACCTTTCACCG3'), *irp6A* (BT2734, 5'-CACCGTCAAGGATGTGACCG3' and BT2735, 5'-TCATGCCGCCGAAAGTCG3'), and 16S rRNA (BT1421, 5'-GAATCTACCCATCTCTGCG3' and BT1422, 5'-AAGGCCTTCATCACTCACGC3') were used for PCR reactions using the Taq PCR Master Mix Kit (Qiagen). PCR reactions were carried out with an initial denaturation step at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min. RT-PCR products were visualized through gel electrophoresis on a 2% agarose gel and ethidium bromide staining. The 16S rRNA, a housekeeping gene, was used as a loading control and to quantitate the amount of RNA in RT-PCRs.

In wild-type NTL4, the *fhuA* and *irp6A* RT-PCR products were largely increased under iron-depleted conditions (Dy) (Fig. 9). Inactivation of *irr* resulted in reduction in the magnitude of *fhuA* and *irp6A* induction. The *fhuA* and *irp6A* RT-PCR products from iron-depleted NTLirr samples were apparently lower than those from iron-depleted NTL4 samples (Fig. 9). The results indicated that *A. tumefaciens* Irr also plays a positive regulatory role in the induction of ferric siderophore receptor genes under iron limitation.

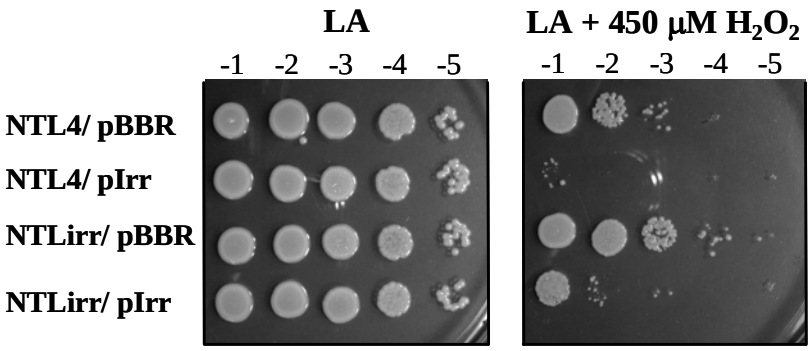
Figure 9. Reverse transcriptase PCR (RT-PCR) analysis was used to monitor the mRNA levels for *fhuA*, *irp6A*, and 16S rRNA. RNA samples were isolated from wild-type NTL4 and mutant NTLirr cultures treated with 50 μM $FeCl_3$ (Fe) or 250 μM 2,2'-dipyridyl (Dy) for 15 min.



4.2 Role of Irr in the peroxide stress response

The role of Irr in the oxidative stress response has been reported. The *B. abortus irr* mutant strain showed an increased resistance to H_2O_2 killing as a result of elevated levels of catalase activity (Martinez, Ugalde et al. 2006). To address the role of Irr in the response of *A. tumefaciens* to peroxide stress, H_2O_2 sensitivity test was performed as previously described (Ngok-Ngam, Ruangkiattikul et al. 2009). The mutant NTLirr/pBBR was about 10-fold more resistant than wild-type NTL4/pBBR to 450 μM H_2O_2 (Fig. 3). Interestingly, survival of cells exposed to H_2O_2 was negatively affected by increased levels of Irr. Overproduction of Irr in strains NTL4/pIrr (10^2-fold) and NTLirr/pIrr (10^2-fold), enhanced sensitivity to H_2O_2 compared with the parental strains NTL4/pBBR and NTLirr/pBBR, respectively (Fig. 10). These data suggested that *A. tumefaciens* Irr has a negative regulatory role in the peroxide stress response. Moreover, high levels of Irr enhanced sensitivity of cells to H_2O_2 killing, therefore, Irr levels must be expressed at suitable levels to ensure survival under peroxide stress.

Figure 10. H_2O_2 sensitivity test. Cells were diluted and spotted on LB agar plates containing 450 μM H_2O_2. Plates were incubated at 28°C for 48 h. Ten-fold serial dilutions are marked above each column. Cells spotted on an LB agar plate (LA) were used as a control. NTL4/pBBR and NTLirr/pBBR strains are the wild-type and the *irr* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTL4/pIrr and NTLirr/pIrr strains are the wild-type and the *irr* mutant, respectively, expressing functional Irr from the plasmid pIrr.



4.3 Catalase activity assay

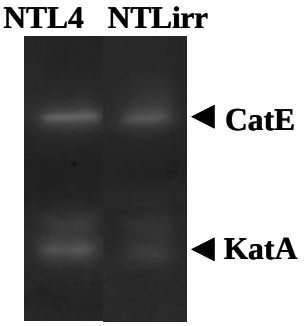
Catalase is the enzyme that degrades H_2O_2. *A. tumefaciens* has two catalases named KatA and CatE, which were shown to play major protective roles against H_2O_2 toxicity (Prapagdee, Eiamphungporn et al. 2004). KatA is a bifunctional catalase-peroxidase and is H_2O_2 inducible, whereas CatE is a growth phase regulated monofunctional catalase. Catalase activity gel staining assay was used to determine whether the H_2O_2-hyperresistant phenotype of NTLirr was resulted from an increase in the levels of catalases. Bacteria grown overnight in LB medium were sub-cultured into fresh LB medium to give an OD_{600} of 0.1 and grown at 28°C for 24 h. Cells were harvested and resuspended in 50 mM sodium phosphate buffer (PB), pH 7.0, containing 1 mM protease inhibitor PMSF. Cells were disrupted by brief sonication followed by centrifugation at 12,000 *g* for 10 min. Cell lysates were collected and protein concentrations were determined using the Bradford BIO-RAD protein assay. Proteins (25 μg) were separated on 7.5% non-denaturing gel

and catalase isozymes were visualized by activity staining as described previously (Gregory and Fridovich 1974). The gel was soaked in PB containing 50 μg ml^{-1} of horseradish peroxidase (Sigma) for 45 min at room temperature, and was then soaked in PB containing 5 mM H_2O_2 for 10 min. After, briefly washing twice with distilled water, the gel was stained with freshly prepared PB containing 0.5 mg ml^{-1} diamino benzidine until the background became dark. Catalase isozymes appeared as colorless bands against a dark brown background.

KatA and CatE activities were determined using lysates prepared from NTL4 and NTLirr cells. Surprisingly, in contrast to what observed in *B. abortus*, the catalase activity gel staining assay showed that the NTLirr mutant had lower levels of both KatA and CatE than wild-type NTL4 (Fig. 11). This indicated that the H_2O_2-hyperresistant phenotype of NTLirr was not due to the increased levels of H_2O_2-degrading enzyme catalases, and that other Irr-mediated mechanism(s) was responsible for H_2O_2 resistance.

Figure 11. Catalase activity gel staining assay

Cell lysates were prepared from NTL4 and NTLirr. Proteins (25 μg) were separated on 7.5% non-denaturing gel and catalase isozymes were visualized by activity staining (Gregory and Fridovich 1974).

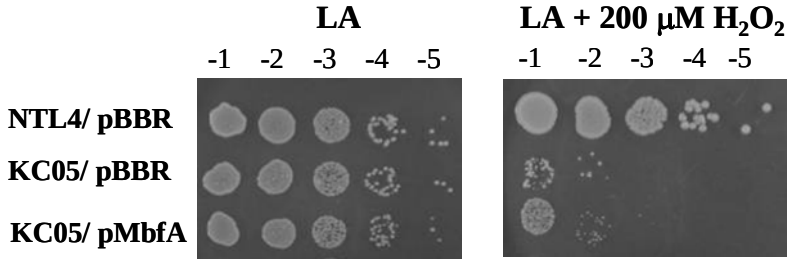


4.4 Role of MbfA in the peroxide protection of A. tumefaciens

Rubrerythrin (Rbr) proteins play a role in peroxide stress protection in an anaerobic sulphate-reducing bacterium *Desulfovibrio vulgaris* and an archaeon *Pyrococcus furiosus* (Lumppio *et al.*, 2001; Weinberg *et al.*, 2004). It has been shown that Rbr from *D.vulgaris* was able to partially rescue a catalases-deficient strain (*katG* and *katE* double mutation) of *E. coli* from H_2O_2 killing with 25% increase in survival (Lumppio *et al.*, 2001). The MbfA protein is a rubrerythrin-like membrane-bound ferritin. The high-level constitutive expression of *mbfA* in NTLirr raised the question of whether the overproduction of MbfA contributed to the H_2O_2-hyperresistant phenotype of NTLirr. The ability of MbfA to protect *A. tumefaciens* against H_2O_2 killing was assessed in wild-type NTL4 and the catalases-deficient strain (KC05, *katA* and *catE* double mutation) (Prapagdee *et al.*, 2004).

The catalases-deficient strain, KC05/pBBR, was highly sensitive to 200 μM H_2O_2 than wild-type, NTL4/pBBR (Fig. 12). The KC05 strain complemented with functional KatA (KC05/pKatA) showed similar levels of H_2O_2 resistance to NTL4/pBBR (Prapagdee *et al.*, 2004). While complementation of KC05 by overproduction of MbfA, KC05/pMbfA, could partially reverse the H_2O_2-hypersensitive phenotype of KC05/pBBR (Fig. 12). These data suggested that MbfA plays a role in H_2O_2 resistance but to a lesser extent than catalase which directly degrades H_2O_2.

Figure 12. Role of MbfA in the peroxide protection of *A. tumefaciens*. Wild-type (NTL4) and the catalase-deficient, *katA* and *catE* double mutant (KC05) contain a plasmid vector (pBBR) or express functional MbfA (pMbfA). Cells were spotted on LA plates containing 200 μM H_2O_2.



4.5 Virulence assay

The effect of *irr* inactivation on the virulence of *A. tumefaciens* was evaluated by the analysis of tumor formation on tobacco leaf pieces infected with wild-type and *irr* mutant strains containing tumor-inducing plasmid pCMA1 according to the method described previously (Kitphati, Ngok-Ngam et al. 2007). The results showed that the mutant NTLirr/pCMA1 had ability to cause tumor as well as the wild-type strain NTL4/pCMA1 (Fig. 13).

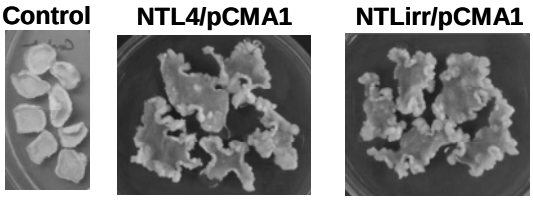


Figure 13. Virulence assay. Tumor formation was determined on tobacco leaf squares infected with *A. tumefaciens* wild-type NTL4 or mutant NTLirr containing the pCMA1 plasmid (NTL4/pCMA1 and NTLirr/pCMA1, respectively). Control: tobacco leaf squares without infection. Representative leaf pieces (from n=30) are shown.

ภาคผนวก

- ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ 1 เรื่อง

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Roles of *Agrobacterium tumefaciens* RirA in Iron Regulation, Oxidative Stress Response, and Virulence▽

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The analysis of genetics and physiological functions of *Agrobacterium tumefaciens* RirA (rhizobial iron regulator) has shown that it is a transcription regulator and a repressor of iron uptake systems. The *rirA* mutant strain (NTLrirA) overproduced siderophores and exhibited a highly constitutive expression of genes involved in iron uptake (*fhuA*, *irp6A*, and *fbpA*) compared to that of the wild-type strain (NTL4). The deregulation in the iron control of iron uptake in NTLrirA led to iron overload in the cell, which was supported by the observation that the NTLrirA mutant was more sensitive than wild-type NTL4 to an iron-activated antibiotic, streptonigrin. The NTLrirA mutant was more sensitive than the parental strain to oxidants, including hydrogen peroxide, organic hydroperoxide, and a superoxide generator, menadione. However, the addition of an iron chelator, 2,2′-dipyridyl, reversed the mutant hypersensitivity to H_2O_2 and organic hydroperoxide, indicating the role of iron in peroxide toxicity. Meanwhile, the reduced level of superoxide dismutase (SodBIII) was partly responsible for the menadione-sensitive phenotype of the NTLrirA mutant. The NTLrirA mutant showed a defect in tumorigenesis on tobacco leaves, which likely resulted from the increased sensitivity of NTLrirA to oxidants and the decreased ability of NTLrirA to induce virulence genes (*virB* and *virE*). These data demonstrated that RirA is important for *A. tumefaciens* during plant-pathogen interactions.

The regulation of intracellular iron concentrations is a critical task for bacteria. Acquiring enough iron to grow is as crucial as preventing iron overload and its consequential toxicity. The Fur (ferric uptake regulator) proteins from many bacteria have been shown to play a major role in controlling genes involved in iron transport, storage, consumption, and the overall maintenance of intracellular iron homeostasis (2). Under high-iron conditions, Fur binds to its corepressor ferrous ion (Fe^{2+}). The Fe^{2+}-Fur complex binds to the promoter region of iron uptake genes, thus shutting down iron uptake and preventing iron overload toxicity. When iron is scarce, Fur exists in the apo form and no longer can repress iron uptake genes, and iron uptake resumes (2). However, Fur-mediated iron regulation does not occur in all bacteria.

Rhizobia are symbiotic soil bacteria that form N_2-fixing nodules on the roots of leguminous plants. These bacteria have a high demand for iron during symbiosis, since nitrogenase and other iron-containing proteins are required for N_2 fixation. Rhizobia have atypical regulation of iron homeostasis. The Fur-like protein has been shown to play only a minor role in the regulation of iron uptake. Instead, Fur physiologically functions in response to manganese by repressing the transcription of the *sitABCD* operon, which encodes a Mn^{2+} uptake system, under manganese-replete conditions (7, 9, 10, 18, 19). The role of Fur-like proteins seems to be restricted to the regulation of the manganese uptake gene, and thus Fur was renamed Mur (7, 9). The RirA (rhizobial iron regulator) protein evolved to carry out typical Fur functions in the regulation of iron-responsive genes for maintaining iron homeostasis in rhizobia (8, 29). The RirA protein belongs to the Rrf2 family of transcription regulators. The RirA homologues are found exclusively in members of alphaproteobacteria, including the rhizobia of *Rhizobium*, *Sinorhizobium*, and *Mesorhizobium*, the human pathogen *Bartonella*, the animal pathogen *Brucella*, and the phytopathogen *Agrobacterium*.

RirA is an Fe-S protein. RirA acts as a repressor of many iron-responsive genes under iron-replete conditions. The RirA regulon includes genes for the synthesis (*vbs*) and uptake (*fhu*) of the siderophore vicibactin, genes involved in heme uptake (*hmu* and *tonB*), genes encoding hemin-binding proteins (*hbp*), genes that probably participate in the transport of Fe^{3+} (*sfu*), a putative ferri-siderophore ABC transporter (*rrp1*), a gene that specifies an extracytoplasmic-function RNA polymerase δ factor (*rpoI*), genes for the synthesis of Fe-S clusters (*suf*), an iron response regulator (*irrA*), and *rirA* itself (4, 28). The iron-responsive operator (IRO) motif (TGA-N9-TCA) has been identified as a DNA-binding site for RirA. Computational analysis has been used to search for IRO motifs in the genomes of alphaproteobacteria, leading to the identification of target genes in the RirA regulon (20).

Agrobacterium tumefaciens, a gram-negative member of the alphaproteobacteria, is a soil-borne plant pathogen that causes crown gall tumor disease in dicotyledonous plants. The pathogenesis involves the attachment of *A. tumefaciens* to the wounded plant cells and the subsequent transfer of a segment

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
A. tumefaciens		
NTL4	Wild-type strain, a Ti plasmid-cured derivative of strain C58	S. K. Farrand
NTLrirA	*rirA* mutant, derivative of NTL4 in which *rirA* was disrupted by pKNOCKrirA, Km^r	This study
E. coli		
DH5α	*supE* Δ*lac*(ϕ80d*lacZ*ΔM15) *hsdR recA endA gyrA thi relA*	12
BW20767	*leu*-63::IS*10 recA1 creC510 hsdR17 endA1 zbf*-5 *uidA*(ΔMlu1)::*pir*$^+$ *thi* RP4-2-tet::Mu-1kan::Tn*7*	16
Plasmids		
pGEM-T-easy	Cloning vector, Ap^r	Promega
pKNOCK-Km	Suicide vector, Km^r	1
pKNOCKrirA	pKNOCK-Km containing a 188-bp fragment of the *rirA* coding region, Km^r	This study
pBBR1MCS-4	Expression vector, Ap^r	15
pRirA	Full-length *rirA* coding region cloned into pBBR1MCS-4, Ap^r	This study
pSodBIII	Full-length *sodBIII* coding region cloned into pBBR1MCS-4, Ap^r	21
pCMA1	pTiC58*traM*::*nptII*, Cb^r, Km^r	S. K. Farrand
pSM243cd	*virB*::*lacZ* fusion, Cb^r, Km^r	S. K. Farrand
pSM358cd	*virE*::*lacZ* fusion, Cb^r, Km^r	S. K. Farrand

[a] Km^r, kanamycin resistance; Ap^r, ampicillin resistance; Cb^r, carbenicillin resistance.

of its tumor-inducing (Ti) plasmid into plant cells (36). The virulence (*vir*) genes located on the Ti plasmid are important primarily for tumorigenesis. The *vir* genes are induced in response to acidic pHs (approximately 4.8 to 5.5) and phenolic compounds, such as acetosyringone (AS), that are released by wounded plant cells. Plants generate high levels of reactive oxygen species, such as H_2O_2 and superoxide radicals, which are used as an important initial defense mechanism to inhibit bacterial invasion and proliferation. Pathogenic bacteria need to carry out antioxidant responses in order to survive in host plants and ultimately cause disease. Catalase (Kat) and superoxide dismutase (Sod) are key antioxidant enzymes for degrading H_2O_2 and superoxides, respectively. These enzymes have been shown to be virulence factors that are involved in the tumorigenesis of *A. tumefaciens* (21, 35). Iron is an essential metal for bacterial growth and also is required as a cofactor for Kat and Sod. However, excessive amounts of iron can be toxic due to its ability to induce the production of highly deleterious hydroxyl radicals via the Fenton reaction (13). Controlling intracellular iron levels and modulating antioxidant responses therefore are crucial tasks for pathogenic bacteria during infection. The iron-dependent transcriptional regulator has been reported to play a key role in both adjusting intracellular iron levels and controlling oxidative stress responses during host plant-pathogen interactions (11, 26). The role of RirA in iron regulation and oxidative stress response has not been studied in *A. tumefaciens*. Here, the physiological functions of *A. tumefaciens* RirA in iron regulation and oxidative stress response are shown, and its regulatory roles in bacterial stress survival and virulence are demonstrated.

MATERIALS AND METHODS

Bacterial growth conditions. Bacterial strains and plasmids are listed in Table 1. *A. tumefaciens* strains were grown aerobically in Luria-Bertani (LB) medium at 28°C with shaking at 150 rpm, supplemented with 100 μg ml^{-1} carbenicillin (Cb), 25 μg ml^{-1} chloramphenicol (Cm), 90 μg ml^{-1} gentamicin (Gm), 30 μg ml^{-1} kanamycin (Km), or 10 μg ml^{-1} tetracycline (Tc) as required. *Escherichia coli* strains were used for routine DNA cloning experiments and grown aerobically in LB medium at 37°C, supplemented with 100 μg ml^{-1} Ap, 30 μg ml^{-1} Gm, 30 μg ml^{-1} kanamycin (Km), or 15 μg ml^{-1} Tc as required.

Molecular techniques. General molecular techniques were performed using standard protocols (22). Plasmid DNA was isolated using the QIAprep kit (Qiagen). DNA restriction and modifying enzymes were purchased from Promega, Fermentas, or New England Biolabs and used according to the suppliers' recommendations. PCR products and restriction fragments were purified using PCR clean-up and gel extraction kits (Qiagen). Sequencing was carried out on an ABI 310 automated DNA sequencer (Applied Biosystems) using a BigDye terminator cycle sequencing kit (PE Biosystems). Plasmids (50 to 100 ng) were transferred into *A. tumefaciens* strains by electroporation (6). The primers used are listed in Table 2.

Construction of *A. tumefaciens rirA* mutant. The disruption of the *rirA* gene (Atu0201) (34) was performed by the method described previously (14). The internal DNA fragment of the *rirA* coding region was amplified by PCR with primers BT1201 and BT1202, using *Pfu* DNA polymerase and genomic DNA isolated from wild-type NTL4 as the template. The 188-bp PCR product was cloned into the unique SmaI site of the pKNOCK-Km suicide plasmid (2 kb in size) (1), which is unable to replicate in *A. tumefaciens*, generating the recombinant plasmid pKNOCKrirA. The cloned DNA region was confirmed by automated DNA sequencing. pKNOCKrirA was transferred into wild-type NTL4 by conjugation (6). The single homologous recombinants were selected on LB agar plates containing 25 μg ml^{-1} chloramphenicol and 30 μg ml^{-1} kanamycin. The correct integration of pKNOCKrirA into the *rirA* locus was confirmed by Southern blot analysis.

Cloning of full-length *rirA*. The full length of the wild-type *rirA* gene was amplified from wild-type NTL4 genomic DNA by PCR using primers BT1701 and BT1205 and *Pfu* DNA polymerase. The PCR products were cloned into the expression vector pBBR1MCS-4 (15), which had been digested with SmaI. The cloned DNA region was confirmed by automated DNA sequencing. The resulting plasmid, named pRirA, was used for *rirA* mutant complementation experiments.

Siderophore detection. Siderophore production was analyzed using a chrome azural S (CAS) agar plate (24). Solid CAS medium was made by adding 10 ml of CAS stock (24) to 100 ml of YEM (yeast extract-mannitol) medium (31) containing 1.5% agar. Overnight cultures grown in LB medium (5 μl at an optical density at 600 nm [OD_{600}] of 0.1) were spotted onto a YEM-CAS plate containing 200 μM 2,2′-dipyridyl (Dy) and incubated at 28°C for 2 days. Siderophore production is indicated by the presence of a halo zone around the bacteria. This occurs because siderophores produced by bacteria remove iron from the original green CAS-Fe^{3+} complex contained in the plate, resulting in a change in the color of the dye.

TABLE 2. Primers used in this study

Gene-primer name and purpose	Sequence (5′→3′)	PCR product size (bp)
Gene inactivation		
rirA-BT1201	GGCTTACGGCGTATCT GAGC	188
rirA-BT1202	CGTCCTCAAAGCACTCAGCC	
Complementation		
rirA-BT1701	GCGGTCAACAAGGCTG TTCG	593
rirA-BT1205	CGATCAGGCTGCCGGA AGTG	
RT-PCR		
16S rRNA-BT1421	GAATCTACCCATCTCTGCGG	280
16S rRNA-BT1422	AAGGCCTTCATCACTCACGC	
fhuA-BT1346	GGTGACGAAGGGTATT GGCG	202
fhuA-BT1096	CGCGACATAACCTTTCACCG	
fbpA-BT2728	CTGCGGATGTGCTGCT GACG	190
fbpA-BT2729	GTCACCCGCTCCTTCGAAGC	
irp6A-BT2734	CACCGTCAAGGATGTG ACCG	232
irp6A-BT2735	CTTCATGCCGCCGAAA GTCG	
sodBI-BT534	CAGCACTACAACCACGTC	198
sodBI-BT535	TTCAAGCTTGCCGTTCTT	
sodBII-BT641	GCGCAGTCCGGCATATTCA	200
sodBII-BT640	ACCAGCCACGCCCAGCCCG	

RNA extraction and reverse transcriptase PCR (RT-PCR) analysis. Bacteria grown overnight in LB medium were subcultured into 20 ml of fresh LB medium to give an OD_{600} of 0.1. Exponential-phase cells (OD_{600} of 0.5 after incubation for 4 h) were treated with 50 μM $FeCl_3$, 250 μM Dy, 50 μM $MnCl_2$, or 200 μM menadione (MD) for 15 min. Cells were harvested by centrifugation at 6,000 rpm for 5 min. Total RNA was extracted from untreated and treated cells using the modified hot phenol method (25). Briefly, the cell pellet was suspended in 300 μl of 0.3 M sucrose and 10 mM sodium acetate (NaOAc). Lysis buffer (300 μl) containing 2% sodium dodecyl sulfate and 10 mM NaOAc was added, and the mixture was incubated at 65°C for 5 min with gentle mixing. The hot phenol (300 μl), maintained at 65°C, was added. After incubation at 65°C for 5 min with occasional mixing, the phases were separated by centrifugation at 12,000 rpm for 10 min. The aqueous phase was reextracted twice with an equal volume of hot phenol as described above, followed by extraction with an equal volume of chloroform. RNA was precipitated by adding 10% of the volume of 3 M NaOAc and two volumes of absolute ethanol. After overnight incubation at −20°C, the RNA was pelleted by centrifugation at 12,000 rpm for 15 min and washed once with 1 ml of 70% ethanol. The RNA pellet was dried and suspended in diethylpyrocarbonate (DEPC)-treated sterile distilled water.

The RNA samples were treated with DNase I using the DNA-free kit (Ambion) according to the manufacturer's protocols. Reverse transcription (converting mRNA to cDNA before PCR) was accomplished using SuperScript II RT (Invitrogen) with random hexamer primers (BioDesign, Thailand). Reverse-transcribed RNA samples (0.1 μg) from each condition were used in the PCR. Control reactions, where RT was omitted, were run in parallel to ensure that there was no DNA contamination. Positive controls were performed using genomic DNA isolated from wild-type NTL4. Gene-specific primers for *fhuA*, *irp6A*, *fbpA*, *sodBI*, *sodBII*, and 16S rRNA (Table 2) were used for separate PCRs using the *Taq* PCR master mix kit (Qiagen). PCRs were carried out with an initial denaturation step at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C (*sodBI* and *sodBII*) or 58°C (*fhuA*, *irp6A* and *fbpA*) for 30 s, and extension at 72°C for 30 s, with a final extension step at 72°C for 5 min. RT-PCR products were visualized through gel electrophoresis on a 2% agarose gel and ethidium bromide staining.

Sensitivity to SNG. Cells grown in LB medium for 24 h were washed once with fresh LB medium, and their concentration was adjusted to give an OD_{600} of 0.1. Cells were treated with streptonigrin (SNG) at a concentration of 40 μg ml^{-1} in the absence or presence of 100 μM Dy. SNG was prepared as a stock solution at 10 mg ml^{-1} in dimethyl sulfoxide. Control cells (untreated) received equivalent amounts of dimethyl sulfoxide. The cells were incubated at 28°C with shaking for 3 h and were diluted (10-fold serial dilutions). An aliquot (10 μl) of each dilution was spotted onto an LB agar plate and incubated at 28°C for 2 days. Each strain was tested in duplicate, and the experiment was repeated at least twice to ensure the reproducibility of the results.

Sensitivity to oxidants. Cells grown in LB medium for 24 h were washed once and then were 10-fold serially diluted in fresh LB medium. An aliquot (10 μl) of each dilution was spotted onto LB agar plates containing 450 μM H_2O_2, 600 μM *tert*-butyl hydroperoxide (tBOOH), or 450 μM MD in the absence or presence of 100 μM Dy. Plates then were incubated at 28°C for 2 days. Each strain was tested in duplicate, and the experiment was repeated at least twice to ensure the reproducibility of the results.

Sod activity gel assay. Crude bacterial lysates were prepared using bacterial suspensions in 50 mM sodium phosphate buffer, pH 7.0, containing 1 mM phenylmethylsulfonyl fluoride, a protease inhibitor. Cell suspensions were lysed by brief sonication followed by centrifugation at 12,000 × *g* for 10 min. Cleared lysates were used for total protein determination and Sod activity gel assays. Protein concentrations were determined using the Bradford Bio-Rad protein assay. To visualize Sod activity on the gel, 25 μg protein from lysate samples was separated on a 10% nondenaturing gel, followed by staining with nitroblue tetrazolium-riboflavin photochemical stain (5).

Tumor formation assay. *A. tumefaciens* strains containing pCMA1 plasmid were used to infect tobacco (*Nicotiana tabacum*) leaves according to the method described previously (14, 17).

***vir* gene induction assay.** Overnight cultures of wild-type NTL4 and mutant NTLrirA strains containing pSM243cd (*virB*::*lacZ*) or pSM358cd (*virE*::*lacZ*) in LB medium were washed and adjusted to an OD_{600} of 0.5 in 10 ml of induction broth, pH 5.5 (6), in the absence or presence of 50 μM acetosyringone (AS). In some experiments, cells were grown in media with 50 μM AS in the presence of either 50 μM $FeCl_3$ or an iron chelator, 50 μM Dy. Cells were further incubated at 28°C with shaking for 24 h. Cells were harvested, and β-galactosidase activity was measured as previously described (14). β-Galactosidase activity is presented in units per milligram of protein.

RESULTS AND DISCUSSION

***A. tumefaciens* RirA is the repressor of iron uptake systems, and loss of RirA leads to intracellular iron overload.** The *A. tumefaciens* genome contains an *rirA* homologue (Atu0201), $rirA_{At}$ (34). The $rirA_{At}$ gene is 471 bp long and is flanked upstream by an *fbpA* gene (Atu0202) encoding a putative iron ABC transporter periplasmic binding protein. The *rirA* and *fbpA* genes are transcribed in the opposite direction. Downstream of the $rirA_{At}$ gene is a gene encoding a conserved hypothetical protein (Atu0200) similar to ammonia monooxygenase and a gene cluster (Atu0199, Atu0198, Atu0197, and Atu0196) encoding a putative proline/glycine/betaine ABC transport system. The protein encoded by $rirA_{At}$ has a predicted molecular mass of 17.1 kDa and shows high sequence similarity to RirA proteins from *Rhizobium leguminosarum* (88% identity) and *Sinorhizobium meliloti* (85% identity). While *R. leguminosarum rirA* is preceded by an *iolA* gene encoding a semialdehyde decarboxylase (29), there is an *fbpA* gene located immediately upstream of *S. meliloti rirA* that is similar to that observed in *A. tumefaciens*. The *S. meliloti fbpA* gene was found to be regulated by *rirA* (8). Unlike in *A. tumefaciens*, a cluster of genes (*dppA-dppF*) involved in the uptake of a heme precursor is found immediately downstream of *rirA* in those two rhizobia.

Several studies have clearly shown that RirA evolved to carry out typical Fur functions in controlling iron uptake systems (29, 30). The inactivation of *rirA* led to the deregulation of iron uptake, which typically was indicated by the overexpression of siderophore synthesis and transport genes. We

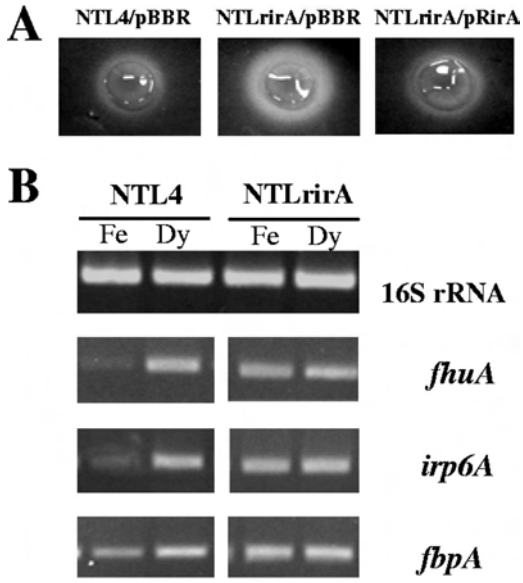

FIG. 1. (A) Analysis of siderophore production was performed using siderophore indicator CAS agar plates. NTL4/pBBR and NTLrirA/pBBR strains are the wild type and the *rirA* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTLrirA/pRirA is the *rirA* mutant expressing functional RirA from the plasmid pRirA. Cells were spotted onto YEM-CAS agar plates containing 200 μM Dy and incubated at 28°C for 2 days. The halo zone surrounding the bacteria indicates the production of siderophores. (B) RT-PCR was used to monitor the mRNA levels for *fhuA*, *irp6A*, *fbpA*, and 16S rRNA. RNA samples were isolated from wild-type NTL4 and mutant NTLrirA cultures treated with 250 μM Dy or 50 μM $FeCl_3$ (Fe) for 15 min.

have shown that the inactivation of *A. tumefaciens fur* has no effect on the siderophore synthesis and iron transport genes, suggesting that *fur* is not the major regulator of iron uptake genes (14). An *A. tumefaciens rirA* mutant (NTLrirA) was constructed as described in Materials and Methods. First, the effect of *rirA* inactivation on siderophore production was investigated. As shown in Fig. 1A, the NTLrirA mutant strain harboring a plasmid vector (NTLrirA/pBBR) produced more siderophores than the wild-type strain (NTL4/pBBR), as indicated by a larger halo zone surrounding NTLrirA/pBBR than that surrounding NTL4/pBBR. Furthermore, the complementation of the mutant strain by the expression of the functional *rirA* gene on the plasmid pRirA (NTLrirA/pRirA) could restore the siderophore production to levels similar to that of wild-type NTL4/pBBR (Fig. 1A). This confirmed that the overproduction of siderophores in NTLrirA was due to the loss of the *rirA* gene.

The role of *A. tumefaciens rirA* in controlling iron uptake was further investigated by monitoring the expression of *fhuA* (Atu4022), *irp6A* (Atu3391), and *fbpA* (Atu0202) genes encoding iron ABC transporter periplasmic binding proteins by using RT-PCR analysis. RNA samples were isolated from NTL4 and NTLrirA cells grown under iron-replete (LB plus 50 μM $FeCl_3$) and iron-depleted (LB plus 250 μM Dy) conditions. Under iron-replete conditions, the expression of *fhuA*, *irp6A*, and *fbpA* was repressed compared to that of wild-type NTL4 under iron-depleted conditions (Fig. 1B). The iron-mediated repression of these genes was lost in the NTLrirA mutant. The amounts of RT-PCR products from iron-replete NTLrirA samples were as large as those from iron-depleted NTLrirA samples. These data demonstrated that *A. tumefaciens rirA* was the repressor of iron uptake systems and that the inactivation of *rirA* led to a constitutive high-level expression of genes involved in the process. In addition, the data provided experimental verification of the predicted members of the RirA regulon (20). The iron-dependent regulation of *fbpA* may be more complex than those of *fhuA* and *irp6A*. In the wild-type NTL4 under iron-replete conditions, the expression of *fbpA* was not fully repressed compared to those of *fhuA* and *irp6A* (Fig. 1B). There might be another mechanism to activate *fbpA* expression under iron-replete conditions.

The derepression of iron uptake in the NTLrirA mutant led us to ask whether the NTLrirA mutant experienced intracellular iron overload. SNG is an iron-activated antibiotic. Increased intracellular iron concentrations have been shown to correlate with an increased sensitivity to SNG (14, 26, 32). The relative intracellular iron levels in NTL4 and the NTLrirA mutant were assessed by SNG sensitivity assays. The results in Fig. 2 show that the NTLrirA/pBBR mutant was 10-fold more sensitive to 40 μg ml^{-1} SNG treatment than wild-type NTL4/pBBR. This suggests that the NTLrirA mutant had intracellular free iron levels that were higher than that of wild-type NTL4. The expression of the functional *rirA* gene in the NTLrirA mutant (NTLrirA/pRirA) could restore resistance to SNG to wild-type (NTL4/pBBR) levels. Moreover, the addition of Dy, a cell membrane-permeable iron chelator that chelates intracellular iron, could protect the NTLrirA mutant from SNG killing. This further supported the conclusion that the loss of *rirA* led to iron overload conditions in the NTLrirA mutant.

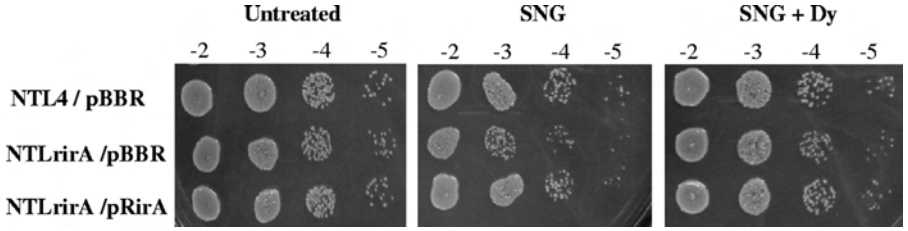

FIG. 2. Sensitivity to SNG. Cells were left untreated or were treated with 40 μg ml^{-1} SNG in the absence (SNG) or presence of 100 μM Dy (SNG+Dy) at 28°C for 3 h. Cells then were diluted and spotted onto LB agar plates (LA). Tenfold serial dilutions are indicated above each column. NTL4/pBBR and NTLrirA/pBBR strains are the wild type and the *rirA* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTLrirA/pRirA is the *rirA* mutant expressing functional RirA from the plasmid pRirA.

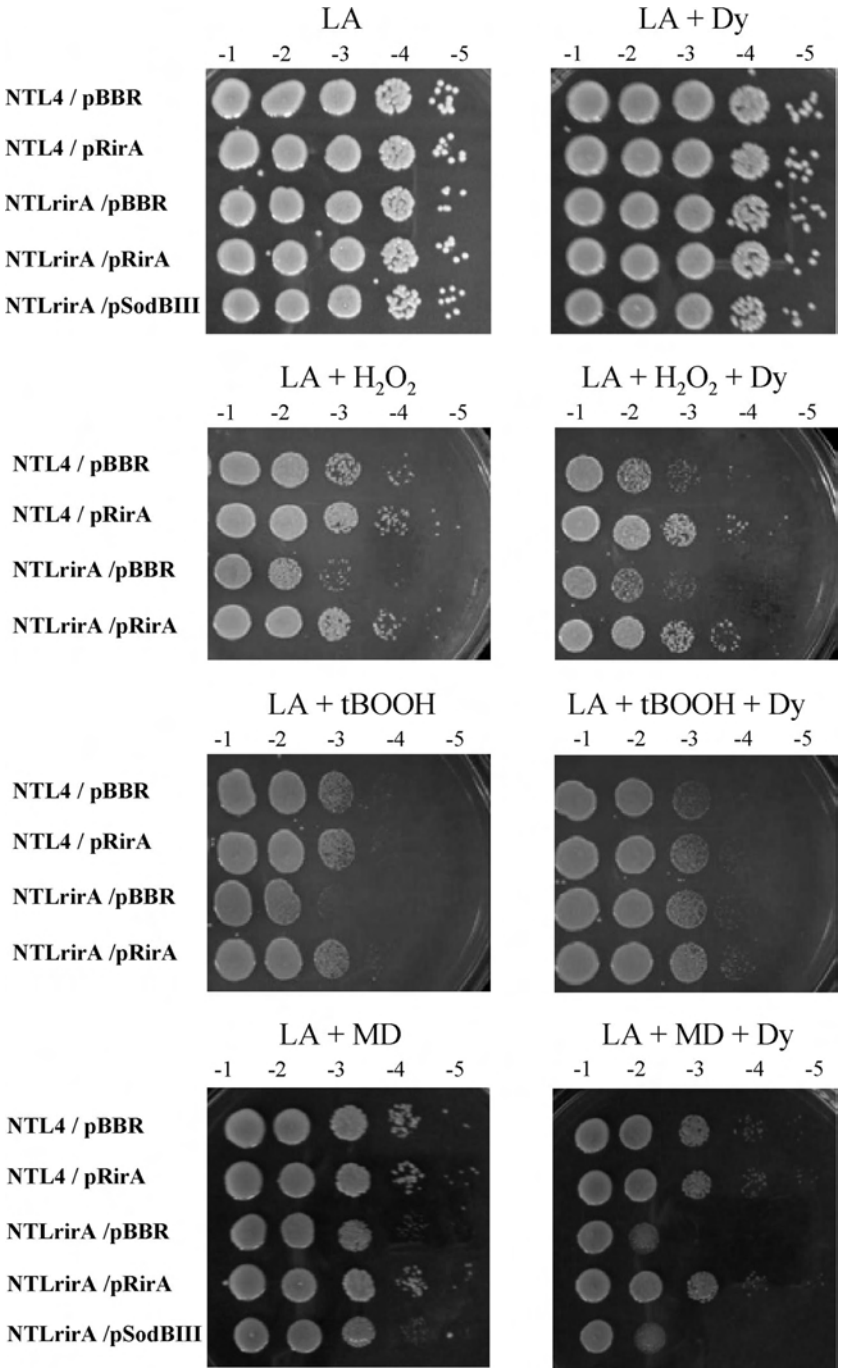

FIG. 3. Sensitivity to oxidants. Cells were diluted and spotted on LB agar plates (LA) containing 450 μM H_2O_2, 600 μM tBOOH, or 450 μM MD in the absence or presence of 100 μM Dy. Plates were incubated at 28°C for 48 h. Tenfold serial dilutions are marked above each column. Cells spotted on an LB agar plate were used as a control. NTL4/pBBR and NTLrirA/pBBR strains are the wild type and the *rirA* mutant, respectively, expressing the plasmid vector pBBR1MCS-4. NTL4/pRirA and NTLrirA/pRirA strains are the wild type and the *rirA* mutant, respectively, expressing functional RirA from the plasmid pRirA. NTLrirA/pSodBIII is the *rirA* mutant expressing SodBIII from the plasmid pSodBIII.

Role of RirA in oxidant resistance. An oxidative burst is the first-line plant defense response against microbial infection (33). Therefore, the ability of pathogens to respond to oxidants is important for survival and successful infection. We tested whether the inactivation of *rirA* affected the sensitivity of *A. tumefaciens* to oxidants including H_2O_2, tBOOH, and a superoxide generator, MD. The results in Fig. 3 showed that the NTLrirA mutant was more sensitive to oxidants than wild-type NTL4. NTLrirA/pBBR was 10-fold more sensitive than NTL4/pBBR to 450 μM H_2O_2, 600 μM tBOOH, and 450 μM MD. The hypersensitive phenotype of NTLrirA to oxidants could be complemented by expressing a functional *rirA* gene on the

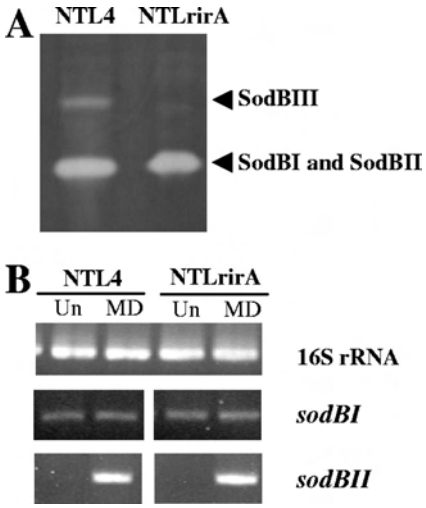

FIG. 4. (A) Sod activity gel. Equal amounts of protein (25 μg) from cell lysates of NTL4 and NTLrirA were loaded and separated on a 10% nondenaturing gel. Sod isozymes were visualized by activity staining as described in Materials and Methods. Arrowheads indicate the positions of SodBI, SodBII, and SodBIII activity. (B) RT-PCR analysis of *sodBI* and *sodBII* transcripts. RNA samples were isolated from wild-type NTL4 and mutant NTLrirA cultures left untreated (Un) or treated with 200 μM MD for 15 min.

plasmid pRirA (NTLrirA/pRirA). Interestingly, the addition of the iron chelator Dy to the medium also could reverse the hypersensitive phenotype of NTLrirA to H_2O_2 and tBOOH but not to MD. The growth of NTLrirA/pBBR was similar to that of NTL4/pBBR on LB agar plates containing 450 μM H_2O_2 and 100 μM Dy or 600 μM tBOOH and 100 μM Dy (Fig. 3). The data implied that increased sensitivities to H_2O_2 and tBOOH toxicity in the NTLrirA mutant most likely were due to the iron overload condition. This condition likely resulted from the derepression of the expression of iron uptake genes (Fig. 1). This is consistent with exposure to peroxides, leading to the Fenton reaction and the generation of highly reactive oxygen species, which are responsible for the phenotype of the NTLrirA mutant with an increased sensitivity to peroxides. In contrast, the hypersensitivity of the NTLrirA mutant to MD could not be reversed by the addition of an iron chelator (Fig. 3), suggesting that mechanisms other than the iron overload condition mediated MD toxicity in the NTLrirA mutant. A possible mechanism is a defect in the NTLrirA mutant in enzymes involved in the superoxide detoxification.

To test this hypothesis, an Sod activity gel assay was performed using cell lysates from wild-type NTL4 and the NTLrirA mutant (Fig. 4A). *A. tumefaciens* contains three Sods, namely SodBI, SodBII, and SodBIII (21). All Sod enzymes have iron cofactors. These *sod* genes exhibit differential expression patterns, and their gene products are found in different cellular locations. SodBI is the major cytoplasmic Sod enzyme, and its gene is constitutively expressed throughout all growth phases, whereas *sodBII* is scarcely expressed under normal conditions. *sodBII* has an MD-inducible expression pattern and is regulated by a superoxide sensor and transcription regulator, SoxR (21). SodBII is a cytoplasmic enzyme. *sodBIII* is highly expressed during the stationary phase of growth, and the enzyme is located in the periplasmic space. At present, the regulators of *sodBI* and *sodBIII* genes have not been identified. The results in Fig. 4A clearly showed that SodBIII could not be detected in the NTLrirA mutant. SodBI and SodBII migrated to the same position on an Sod activity gel, which contributed to the major visible band (21). The RT-PCR analysis was performed in order to measure the expression levels of *sodBI* and *sodBII* in the NTLrirA mutant. The results showed that *sodBI* RT-PCR products obtained from the NTLrirA mutant were not different from those from wild-type NTL4 (Fig. 4B). We also tested whether the MD-induced expression of *sodBII* was affected by *rirA* inactivation. The *sodBII* RT-PCR products were detected only in MD-treated samples; moreover, the amounts of *sodBII* RT-PCR products were similar in wild-type NTL4 and mutant NTLrirA. These data indicated that the levels of SodBIII and not SodBI or SodBII are affected by the inactivation of *rirA*. Next, we determined whether the reduction in SodBIII levels was responsible for the MD-sensitive phenotype of the NTLrirA mutant. The MD-sensitive phenotype of the NTLrirA mutant could not be fully complemented by the overexpression of *sodBIII* alone, as observed for NTLrirA/pSodBIII (Fig. 3). This observation could be explained by a previous observation that SodBIII has a minor role in protecting *A. tumefaciens* from superoxide stress (21). The inactivation of *sodBIII* alone has no effect on the MD resistance levels. However, the contribution of *sodBIII* to MD resistance is revealed only in the double *sod* mutant strains (21). It is likely that in addition to reductions in SodBIII levels, other not-yet-identified RirA-regulated genes also participated in the hypersensitivity of the NTLrirA mutant to MD.

RirA is required for induction of virulence genes and tumorigenesis in *A. tumefaciens*. *A. tumefaciens* induces the formation of crown gall tumors by transferring T-DNA from the bacterium's Ti plasmid into host plant cells (36). The effect of *rirA* inactivation on the virulence of *A. tumefaciens* was evaluated by the analysis of tumor formation on tobacco leaf pieces infected with wild-type and *rirA* mutant strains containing Ti plasmid pCMA1. The mutant NTLrirA/pCMA1 showed significantly less virulence than the wild-type strain NTL4/pCMA1 (Fig. 5A). The tumors that formed on tobacco leaf pieces infected with NTLrirA/pCMA1 were much fewer and smaller than those caused by NTL4/pCMA1. Furthermore, the attenuated virulence of the NTLrirA/pCMA1 mutant could be complemented by pRirA, as shown by the fact that the tumor-inducing ability of NTLrirA/pCMA1/pRirA was completely restored to NTL4/pCMA1 levels (Fig. 5A). In contrast, NTLrirA/pCMA1/pBBR1MCS-4 could not complement the reduced-virulence phenotype of the mutant (data not shown). These data confirmed that the loss of *rirA* led to the defect in virulence.

There are reports that the attenuated virulence of *A. tumefaciens* mutants results from defects in *vir* gene induction (3, 27). Thus, we sought to determine whether a mutation in *rirA* affected *vir* gene induction. The β-galactosidase activity was measured in wild-type and mutant strains containing plasmid pSM243cd (*virB*::*lacZ*) or pSM358cd (*virE*::*lacZ*) grown in induction broth (pH 5.5) and in the absence or presence of 50 μM AS. There was no β-galactosidase activity from cells grown in the absence of AS, whereas β-galactosidase activity was largely increased when cells were grown in the presence of 50 μM AS. This was consistent with the fact that *vir* genes are

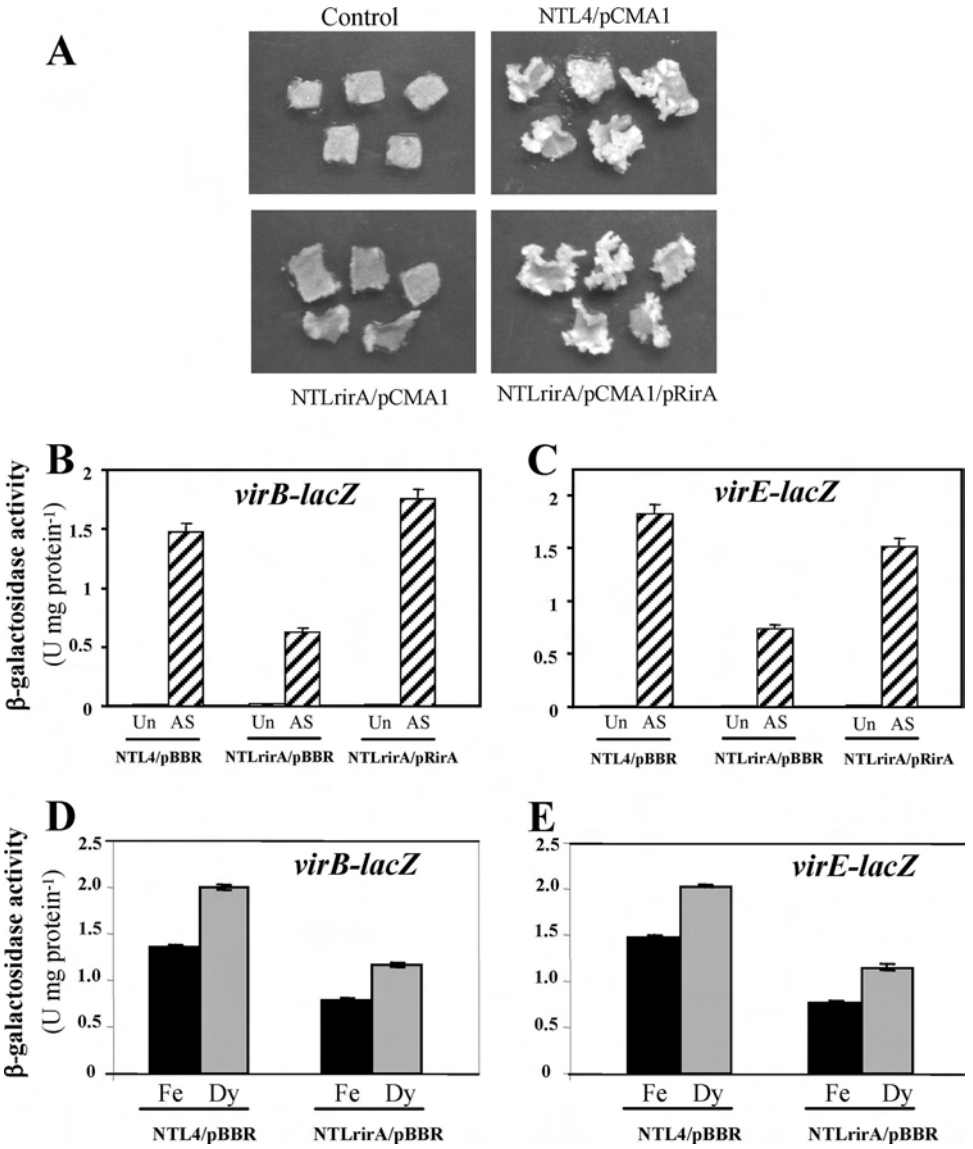

FIG. 5. Virulence assay. (A) Tumor formation was determined on tobacco leaf squares infected with *A. tumefaciens* wild-type NTL4 or mutant NTLrirA containing the pCMA1 plasmid (NTL4/pCMA1 and NTLrirA/pCMA1, respectively). The NTLrirA mutant was complemented by the expression of functional RirA from the plasmid pRirA (NTLrirA/pCMA1/pRirA). The controls are tobacco leaf squares without infection. Representative leaf pieces ($n = 30$) are shown. (B) *virB* gene induction. (C) *virE* gene induction. Wild-type (NTL4/pBBR), *rirA* mutant (NTLrirA/pBBR), and complementation (NTLrirA/pRirA) strains containing pSM243cd (*virB*::*lacZ*) or pSM358cd (*virE*::*lacZ*) were grown in induction broth (pH 5.5) in the absence (Un) or presence of 50 μM AS at 28°C for 24 h. β-Galactosidase activity was measured. Also shown are the induction of *virB* (D) and *virE* (E) in induction broth (pH 5.5) containing 50 μM AS and in the presence of 50 μM $FeCl_3$ (Fe) or 50 μM Dy.

induced in response to phenolic compounds (36). The inactivation of *rirA* resulted in a reduction in the magnitude of *virB* and *virE* gene induction by AS (Fig. 5B and C), as shown by the β-galactosidase levels from NTLrirA/pBBR containing pSM243 or pSM358, which were lower than those from NTL4/pBBR containing pSM243 or pSM358. The reduced induction of *virB* and *virE* by AS could be restored in the complemented NTLrirA/pRirA strains containing pSM243 or pSM358 (Fig. 5B and C, respectively). These data indicated that RirA played a role in the induction of *vir* gene expression. The inactivation of *rirA* caused a reduction in *virB* and *virE* induction but did not abolish the induction. This implied that the RirA protein could have an indirect role in modulating the level of *vir* gene induction. One possible explanation for the impairment of *vir* gene induction by AS in the NTLrirA mutant is related to the levels of intracellular iron. The NTLrirA mutant had intracellular free iron levels that were higher than that of wild-type NTL4 (Fig. 2). We observed that the induction levels of *vir* genes by AS from cells grown in iron-replete medium were lower than those from cells grown in iron-depleted medium. The induction of *virB* (Fig. 5D) and *virE* (Fig. 5E) by AS in both wild-type NTL4 and the NTLrirA mutant could be enhanced when cells were grown in the presence of the iron chelator Dy. The impairment of *virB* and *virE* induction in the

NTLrirA mutant could be partially restored by the addition of an iron chelator, as shown by the induction levels from NTLrirA/pBBR cells grown under iron-depleted conditions (Dy) and from NTL4/pBBR cells grown under iron-replete conditions (Fe). However, under iron-depleted conditions (Dy), the β-galactosidase levels from NTLrirA/pBBR were lower than those from NTL4/pBBR. These results suggested that mechanisms other than the iron overload condition also contributed to the impairment of *vir* gene induction by AS in the NTLrirA mutant.

The host plant environment is low in iron (23). *A. tumefaciens* might sense a low-iron environment as a signal for the induction of virulence genes upon entry to the host plant. During plant-pathogen interactions, an oxidative burst is the first line of plant defense against microbial infection. The NTLrirA mutant showed increased sensitivity to oxidants compared to that of wild-type NTL4 (Fig. 3). Hence, both the increased sensitivity of NTLrirA to oxidants and the decreased ability of NTLrirA to induce virulence genes likely contributed to the attenuated virulence of the NTLrirA mutant on tobacco leaves.

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The *Agrobacterium tumefaciens* Irr protein controls target genes in response to iron and manganese concentrations

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Summary

The physiological function of *Agrobacterium tumefaciens* Irr (iron response regulator) was investigated. Irr functioned under iron limitation to negatively control heme biosynthesis and positively affect siderophore biosynthesis. The *A. tumefaciens irr* mutant strain, NTLirr, exhibited derepression of *hemA* gene and had a reduction in siderophore production. Furthermore, Irr was involved in the oxidative stress response. The NTLirr mutant showed increased resistance to H_2O_2 killing but had reduced catalase activity, suggesting the existence of other Irr-mediated peroxide resistant mechanism(s). The promoter-*lacZ* fusion analysis revealed derepression of an oxidative stress-response gene, rubrerythrin-like membrane bound ferritin (*mbfA*), in the NTLirr mutant. In wild-type NTL4, expression of *mbfA-lacZ* was reduced in the iron-depleted medium and was further strongly repressed in the presence of manganese. Inactivation of the *irr* gene in the NTLirr strain resulted in high-level constitutive, metal-independent expression of *mbfA-lacZ*. The role of MbfA in the peroxide protection was demonstrated. Overproduction of MbfA partially protected a catalase-deficient strain of *A. tumefaciens* against H_2O_2 killing. In conclusion, we showed that Irr was crucial for regulating iron-responsive genes and oxidative stress response in *A. tumefaciens*. Moreover, another important finding of this study was that Irr mediated its maximum repression activity on target genes in the presence of manganese.

INTRODUCTION

Iron is an essential micronutrient for most bacteria. However, high levels of intracellular iron are toxic since iron can cause cellular damage by catalyzing the production of harmful hydroxyl radicals via the Fenton reaction (Imlay *et al.*, 1988). Iron homeostasis is critical for bacterial survival and is therefore tightly regulated in response to iron availability. The classical iron regulatory model is derived from extensive studies in γ-proteobacterium including *Escherichia coli* and *Pseudomonas aeruginosa* and is mediated by the ferric uptake regulator (Fur) (Andrews *et al.*, 2003). Under high iron conditions, Fur complexes with Fe(II) and represses iron uptake genes in order to prevent excessive amounts of intracellular iron. When intracellular iron is inadequate, Fur is in an apo form and can no longer bind to iron uptake genes, leading to turning on the iron uptake systems to satisfy the cellular iron demand.

The α-proteobacteria contain both beneficial and harmful bacteria, including nitrogen-fixing symbionts *Bradyrhizobium*, *Rhizobium*, and *Sinorhizobium*, the human pathogen *Bartonella*, the animal pathogen *Brucella*, and the phytopathogen *Agrobacterium*. Iron regulation in α-proteobacteria is rather distinct compared to other bacteria. The Fur-like proteins from some members of α-proteobacteria have been shown to have the different physiological function (Chao *et al.*, 2004; Diaz-Mireles *et al.*, 2004; Kitphati *et al.*, 2007). Instead, Fur senses and responds to Mn(II) by functioning as a repressor of manganese uptake operon *sitABCD*, consequently, Fur was renamed Mur (manganese uptake regulator). Furthermore, Mur has been shown to bind the 7-N7-7 inverted repeats, instead of the conventional Fur box (GATAATGATAATCATTATC), within the *sitABCD* promoter region called the Mur-responsive sequence (MRS1[TGCAATT-N7-AATTGCA] and MRS2 [TGCAAAT-N7-AATCGCA]) (Diaz-Mireles *et al.*, 2005).

The protein from the Rrf2 family named RirA (rhizobial iron regulator) evolved to adopt typical Fur functions in the regulation of iron-responsive genes under iron-replete conditions for controlling iron homeostasis in some members of α-proteobacteria (Todd *et al.*, 2002; Chao *et al.*, 2005; Ngok-ngam *et al.*, 2009). Orthologs of RirA are present exclusively in *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Brucella*, *Bartonella* and *Agrobacterium* (Rodionov *et al.*, 2006). RirA is an Fe-S protein. The RirA proteins were shown to controls a wide range of genes involved in iron transport (e.g. *fbp*, *sfu*), iron metabolism (*suf*), synthesis and uptake of siderophores (e.g. *vbs*, *fhu*, *irp6*), heme uptake (e.g. *hmu*, *shmR*, *hbp*) and transcription factors (e.g. *rhrA*, *irr*, *rirA*) (Chao *et al.*, 2005; Todd *et al.*, 2005; Todd *et al.*, 2006; Battisti *et al.*, 2007; Ngok-ngam *et al.*, 2009). The iron-responsive operator (IRO:

TGA-N9-TCA) motif was identified as a DNA binding site for RirA (Yeoman *et al.*, 2004; Rodionov *et al.*, 2006). Moreover, RirA likely senses iron in the form of Fe-S clusters whereas Fur senses iron directly (Todd *et al.*, 2006).

There is an additional iron-responsive transcriptional regulator named Irr (iron response regulator) that found to be restricted to α-proteobacteria (Rodionov *et al.*, 2006). Irr is a member of the Fur family, first identified and best characterized in *Bradyrhizobium japonicum* (Hamza *et al.*, 1998). In contrast to Fur and RirA, Irr functioned under iron-limiting conditions and had roles in positive modulating iron uptake and negative regulating iron control of heme biosynthesis (*hemB*). Iron strongly affected Irr protein stability. Irr sensed intracellular iron concentrations by responding to heme availability, which led to Irr degradation and regulation of target genes (Yang *et al.*, 2006b).

The iron control element (ICE: TTTA-N9-TAAA) motif was identified as a DNA-binding site for Irr *in vitro* and *in vivo* (Nienaber *et al.*, 2001; Yang *et al.*, 2006b; Sangwan *et al.*, 2008). However, not all Irr regulated genes contain ICE motif in their promoter regions including *hemB* which was first reported to be regulated by Irr. It is likely that Irr regulates its target genes via mechanism other than direct binding to the ICE motif. This is supported by the observation in *Bartonella quintana* that Irr interacted with a novel DNA motif called H-box (5'-TTTTTACTACAGAT) (Battisti *et al.*, 2007; Parrow *et al.*, 2009).

Iron regulation in *B. japonicum* is rather different from even close relative *R. leguminosarum* in that *B. japonicum* does not contain the *rirA* gene (Rodionov *et al.*, 2006). Its regulation of iron metabolism is, at least in part, still modulated by Fur in cooperation with Irr (Rudolph *et al.*, 2006). In *R. leguminosarum* that Fur becomes Mur, RirA exists to play a major role in controlling iron homeostasis. Moreover, RirA functions in collaboration with Irr to regulate some iron-responsive genes (*suf*, *rrp1* and *rirA*).The dual regulation mediated by RirA and Irr was experimentally demonstrated (Todd *et al.*, 2006). Accordingly, those genes contain both IRO and ICE motifs in their promoter regions. Genome analysis of members of α-proteobacteria, searching for IRO and ICE motifs, led to prediction of candidate genes in the RirA and Irr regulons (Rodionov *et al.*, 2006). It seems that the dual regulation also likely occurs in *Sinorhizobium*, *Mesorhizobium*, *Brucella*, *Bartonella* and *Agrobacterium*.

Iron and oxidative stress are closely linked. In many bacteria, the iron regulator not only controls the iron-responsive genes but also controls genes involved in the oxidative stress response (Touati *et al.*, 1995; Horsburgh *et al.*, 2001b; Ernst *et al.*, 2005). The role of Irr in response to oxidative conditions has also been described. In addition to iron, reactive oxygen species such as H_2O_2 could induce *B. japonicum* Irr degradation in a heme-dependent

manner leading to derepression of heme biosynthesis (Yang *et al.*, 2006a). There has been reported that loss of *irr* gene affected the cell's ability to respond to oxidative conditions. The *Brucella abortus irr* mutant had increased resistance to H_2O_2 that was a result of increased levels of H_2O_2-degrading enzyme catalase (Martinez *et al.*, 2006). It was found that the *B. abortus irr* mutant had increased intracellular heme content which in turn contributed to higher levels of heme-containing catalase enzyme. These findings in *B. japonicum* and *B. abortus* showed that Irr responded to oxidative stress through regulating heme biosynthesis.

Agrobacterium tumefaciens causes crown gall tumor disease on dicotyledonous plants. Inevitably, iron restriction and oxidative burst are vital environmental stresses for phytopathogens during infection. Plants have ability to capture iron (Mila *et al.*, 1996; Karamanoli and Lindow, 2006) and have an initial defense mechanism by inducing generation of reactive oxygen species (Wojtaszek, 1997), to inhibit bacterial proliferation and invasion. In order to survive and cause disease, phytopathogens have to successfully combat both iron deprivation and oxidative threat. At present, iron regulation in *A. tumefaceins* is not fully understood. *A. tumefaceins* genome contains genes annotated as the iron-responsive transcriptional regulator including *fur* (Atu0354), *rirA* (Atu0201), and *irr* (Atu0153) (Wood *et al.*, 2001). *A. tumefaciens fur*-like gene controlled the manganese transport operon *sitABCD* and was not the major regulator of iron transport and metabolism, thus original annotated gene *fur* was actually *mur* (manganese uptake regulator) (Kitphati *et al.*, 2007). *A. tumefaciens rirA* was the repressor of iron uptake systems. Mutation in the *rirA* gene led to derepression of iron uptake and an increase in free intracellular iron concentrations, consequently, the *rirA* mutant strain had increased sensitivity to peroxide killing via the Fenton reaction (Ngok-ngam *et al.*, 2009). Importantly, both *mur* and *rirA* were required for the full virulence of *A. tumefaciens* (Kitphati *et al.*, 2007; Ngok-ngam *et al.*, 2009). However, functional role of *A. tumefaciens irr* gene has not been characterized.

Here, the physiological function of *A. tumefaciens irr* gene was determined. The important roles of *irr* for controlling iron-responsive genes and oxidative stress responses are demonstrated. Furthermore, we reveal that Irr mediates its maximum repression activity on *rbr* promoter in the presence of manganese.

Results

The iron response regulator in A. tumefaciens

Primary sequence of *A. tumefaciens* Irr (Irr_{At}) protein has high identity to Irr from *R. leguminosarum* (84%) and *S. meliloti* (81%). Irr_{At} has moderate levels of identity to Irr from *B. abortus* (60%), *B. quintana* (53%) and *B. japonicum* (53%). Irr degradation occurs in the presence of iron and is heme dependent (Qi *et al.*, 1999). Base on studies in *B. japonicum* (Yang *et al.*, 2005), there are two heme binding sites for iron-induced Irr degradation (Fig. 1). The first site, named heme regulatory motif (HRM), binds ferric heme containing amino acid GCPWHD residues. The second site, which is conserved in most Irr proteins, binds ferrous heme consisting of three consecutive histidine residues. The Irr_{At} has the conserved three consecutive histidine residues but does not contain the HRM motif (Fig. 1). It was shown that even though *B. abortus* Irr does not have the HRM motif and the second motif contains only two histidines (HQH), the *B. abortus* Irr was able to bind heme and degraded in the presence of iron (Martinez *et al.*, 2005).

A. tumefaciens *Irr regulates heme biosynthesis via repression of* hemA

In order to assess the functional role of *irr* gene in *A. tumefaciens*, an *irr* mutant strain (NTLirr) was constructed. The regulatory role of Irr was first reported in the regulation of the heme biosynthesis (Hamza *et al.*, 1998). Under iron-limiting conditions, Irr repressed *hemB* expression to prevent the accumulation of protoporphyrin IX (PPIX) intermediates from exceeding iron supply. Loss of Irr led to derepression of *hemB*, resulting in the accumulation of PPIX under iron-depleted conditions and the *B. japonicum irr* mutant cells exhibited pink fluorescence under UV light (Hamza *et al.*, 1998). The *irr* mutant of *R. leguminosarum* also accumulated PPIX, however, this was due to derepression of *hemA* instead of *hemB* (Todd *et al.*, 2006). Accordingly, the ICE motif for Irr binding was found in the promoter region of *R. leguminosarum hemA* (Rodionov *et al.*, 2006).

The NTLirr mutant strain harboring plasmid vector (NTLirr/pBBR) showed brownish-red colonies on iron-depleted medium and exhibited pink fluorescence under UV light (R. Sukchawalit, unpublished). It was found that wild-type NTL4 harboring plasmid vector (NTL4/pBBR) and a complemented strain (NTLirr/pIrr) did not show the fluorescent colony phenotype. The results indicated that the pigmented and fluorescent phenotype of NTLirr was due to the loss of *irr* gene. Like *R. leguminosarum*, heme biosynthesis in *A. tumefaciens* seemed to be regulated by Irr via negative controlling expression of *hemA*, which was implied by the presence of ICE motif in the promoter region of *A. tumefaciens hemA* (Atu2613)

(Rodionov *et al.*, 2006). The expression of *hemA* was determined using RT-PCR analysis. Expression of 16S rRNA (a housekeeping gene) was used as a control that was expressed at constant levels and amounts of 16S rRNA RT-PCR products obtained from all samples were similar (Fig. 2A). In wild-type NTL4, the *hemA* RT-PCR products from iron-depleted sample (Dy) were lower than those from iron-replete sample (Fe) (Fig. 2A). Inactivation of *irr* resulted in the derepression of *hemA*. In iron-depleted samples, the levels of *hemA* RT-PCR products from NTLirr were higher than those from wild-type NTL4 (Fig. 2A). The results confirmed that Irr_{At} regulates heme biosynthesis via repression of *hemA*.

Irr positively affects siderophore biosynthesis and transport genes under iron limitation

Under iron-deficient conditions, many bacteria produce and secrete siderophores to sequester ferric ion from the environment (Andrews *et al.*, 2003). The siderophore synthesis gene cluster in *A. tumefaciens* composed of two divergently transcribed operons, one consisting of the genes from Atu3675 through Atu3685, and the other consisting of Atu3673 through Atu3670 (Rondon *et al.*, 2004). This siderophore synthesis gene cluster was predicted to be regulated by RirA according to the presence of IRO motifs in the promoter regions of those genes (Rodionov *et al.*, 2006). It was confirmed that RirA has a negative effect on siderophore production and transport genes since the *A. tumefaciens rirA* mutant strain (NTLrirA) showed an increase in siderophore production and derepression of siderophore receptor genes (*fhuA* and *irp6A*) (Ngok-ngam *et al.*, 2009).

It has been reported that Irr positively affected the iron regulation of siderophore production in *B. abortus* (Martinez *et al.*, 2006). In order to test whether siderophore biosynthesis was affected in the NTLirr mutant, detection of siderophore production was done. Cells were spotted onto a YEM+CAS plate containing 200 μM 2,2'-dipyridyl (an iron-limiting condition). The *irr* mutant strain (NTLirr/pBBR) had significantly decreased production of siderophores compared to wild-type strain (NTL4/pBBR) as indicated by an apparently smaller halo zone surrounding NTLirr/pBBR colonies compared to the halo zone surrounding NTL4/pBBR colonies (Fig. 2B). A decrease in siderophore production in the NTLirr mutant could be restored in the complemented strain NTLirr/pIrr. These data indicated that Irr_{At} has a positive effect on the siderophore production under iron limitation similar to that reported in *B. abortus.* Although the siderophore synthesis genes were not predicted to be in the Irr regulon of *A. tumefaciens* (Rodionov *et al.*, 2006), a potential ICE motif, TTTCAGGCAGCCGAATA, at position 129 bp upstream of the putative ATG start codon of Atu3675 is present. RT-PCR analysis was performed to determine transcriptional

levels of Atu3675 gene in wild-type NTL4 and in the NTLirr mutant. It appeared that expression of Atu3675 was induced under iron-limiting conditions in both NTL4 and NTLirr (Fig. 2A). However, Atu3675 RT-PCR products from iron-depleted sample (Dy) of NTLirr were lower than those from wild-type NTL4. These results revealed that Irr was required for the full induction of Atu3675 expression and further supported that Irr_{At} is a positive regulator of the siderophore biosynthesis under iron-limiting conditions.

The positive regulatory role of Irr on the ferric siderophore receptor genes was demonstrated in *B. japonicum* (Small *et al.*, 2009). *fhuA* (Atu4022) and *irp6A* (Atu3391) are siderophore receptor genes in *A. tumefaciens* that previously were shown to be negatively controlled by RirA under iron-replete conditions (Ngok-ngam *et al.*, 2009). In addition to the presence of IRO motifs, the ICE motifs were also found in the promoter regions of *A. tumefaciens fhuA* and *irp6A* (Rodionov *et al.*, 2006). Therefore, it is likely that these genes are also regulated by Irr_{At}. The RT-PCR analysis was performed to determine the role of Irr in controlling ferric siderophore receptor genes. In wild-type NTL4, the *fhuA* and *irp6A* RT-PCR products were largely increased under iron-depleted conditions (Dy) (Fig. 2A). Inactivation of *irr* resulted in reduction in the magnitude of *fhuA* and *irp6A* induction. The *fhuA* and *irp6A* RT-PCR products from iron-depleted NTLirr samples were apparently lower than those from iron-depleted NTL4 samples (Fig. 2A). The results indicated that Irr_{At} also played a positive regulatory role in the induction of ferric siderophore receptor genes under iron limitation.

The mbfA *gene is negatively regulated by Irr under iron limitation and in the presence of manganese*

The *B. japonicum* blr7895 gene whose product has an N-terminal domain similar to ferritin-like rubrerythrin. The C-terminal domain of blr7895 is predicted to be an inner membrane protein. Therefore, blr7895 is called membrane bound ferritin (*mbfA*) (Rodionov *et al.*, 2006). The *mbfA* gene is negatively regulated by Irr under iron-depleted conditions. The interaction of *B. japonicum* Irr with the ICE motif of *mbfA* mediating the negative regulation was well established both *in vitro* and *in vivo* (Sangwan *et al.*, 2008). Significantly, the promoter region of *A. tumefaciens mbfA* gene (Atu0251) contains an ICE-like motif (TTTAGAATTATTCTAAA), which matches to the ICE motif of *B. japonicum mbfA* at 16 of 17 positions, suggesting a similar iron regulation of *mbfA* by Irr in *A. tumefaciens*.

Promoter-*lacZ* fusion assay was performed in order to determine the expression of *mbfA* in response to metals. The results showed that expression of *mbfA* in the wild-type NTL4 was regulated by iron concentrations. NTL4 expressing p*mbfA-lacZ* had lower β-

galactosidase activities in 50 μM 2,2'-dipyridyl (5.07 ± 0.62 U mg protein^{-1}) than in 50 μM $FeCl_3$ (13.64 ± 0.23 U mg protein^{-1}) (Fig. 3A). These results indicated that *mbfA* was repressed under iron limitation. The iron regulation of *mbfA* was lost in the NTLirr mutant. High constitutive expression of *mbfA-lacZ* was found in NTLirr both iron-replete and -depleted conditions, indicating that Irr was a repressor of *mbfA* (Fig. 3A). Importantly, it was found that expression of *mbfA-lacZ* in the wild-type NTL4 was further strongly repressed in the presence of 50 μM $MnCl_2$ (0.73 ± 0.02 U mg protein^{-1}). The manganese repression of *mbfA* was mediated by Irr since NTLirr showed derepression of *mbfA-lacZ* in the presence of manganese (Fig. 3A). Although the manganese-mediated gene repression by Irr *in vivo* has not been demonstrated before, it has been shown *in vitro* that binding of Irr to ICE motif was increased in the presence of manganese (Sangwan *et al.*, 2008; Small *et al.*, 2009). It is possible that manganese might be a metal cofactor for Irr_{At} to confer maximal repression of *mbfA*. Overproduction of Irr had a strong inhibitory effect on *mbfA-lacZ* expression. This was shown by lower levels of β-galactosidase activities were detected in NTL4/pIrr and NTLirr/pIrr under both high (Fe) and low (Dy) iron conditions, compared to a wild-type strain harboring the vector control NTL4/pBBR (Fig. 3A).

In order to further determine the metal-specific repression of *mbfA*, the *mbfA-lacZ* fusion in the wild-type strain (NTL4/pBBR) was tested for repression by various metal ions at a lower concentration (Fig. 3B). NTL4/pBBR expressing pP*mbfA-lacZ* was grown in the AB medium supplemented with 1 μM of $MnCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, or $CuSO_4$. The results showed that only manganese mediated repression of *mbfA-lacZ* (Fig. 3B). This indicated that *mbfA* expression was selectively repressed by manganese.

The NTLirr mutant is H_2O_2-hyperresistant but has reduced catalase activity

The role of Irr in the oxidative stress response has been reported previously. The *B. abortus irr* mutant strain showed an increased resistance to H_2O_2 killing as a result of elevated levels of catalase activity (Martinez *et al.*, 2006). To address the role of Irr in the response of *A. tumefaciens* to peroxide stress, H_2O_2 sensitivity test was performed. The mutant NTLirr/pBBR was approximately 10-fold more resistant than wild-type NTL4/pBBR to 400 μM H_2O_2 (Fig. 4A). Overproduction of Irr in strains NTL4/pIrr and NTLirr/pIrr, enhanced sensitivity to H_2O_2 approximately 10^2-fold compared with the parental strains NTL4/pBBR and NTLirr/pBBR (Fig. 4A). These data demonstrated that *A. tumefaciens* Irr has a negative regulatory role in the peroxide stress response. Moreover, high levels of Irr enhanced

sensitivity of cells to H_2O_2 killing, therefore, Irr levels must be kept at suitable levels to ensure survival under peroxide stress.

Catalase is the enzyme that degrades H_2O_2. *A. tumefaciens* has two catalases named KatA and CatE, which were shown to play major protective roles against H_2O_2 toxicity in *A. tumefaciens* (Prapagdee *et al.*, 2004). KatA is a bifunctional catalase-peroxidase and is H_2O_2 inducible, whereas CatE is a growth phase regulated monofunctional catalase. Catalase activity gel staining assay was performed to determine the levels of catalases, KatA and CatE, using lysates prepared from NTL4 and NTLirr cells. In contrast to what observed in *B. abortus*, the catalase activity gel staining assay showed that the NTLirr mutant had lower levels of both KatA and CatE than wild-type NTL4 (Fig. 4B). The decreased basal levels of catalases in the NTLirr mutant might imply that, during normal growth, the NTLirr mutant may encounter a lower intracellular stress from H_2O_2 compared to the wild-type NTL4. The result indicated that the H_2O_2-hyperresistant phenotype of NTLirr was not due to the increased levels of H_2O_2-degrading enzyme catalases, and suggested that other Irr-mediated mechanism(s) was responsible for H_2O_2 resistance.

Role of MbfA in the peroxide protection of A. tumefaciens

Rubrerythrin (Rbr) proteins play a role in peroxide stress protection in an anaerobic sulphate-reducing bacterium *Desulfovibrio vulgaris* and an archaeon *Pyrococcus furiosus* (Lumppio *et al.*, 2001; Weinberg *et al.*, 2004). It has been shown that Rbr from *D.vulgaris* was able to partially rescue a catalases-deficient strain (*katG* and *katE* double mutation) of *E. coli* from H_2O_2 killing with 25% increase in survival (Lumppio *et al.*, 2001). The MbfA protein is a rubrerythrin-like membrane-bound ferritin. The high-level constitutive expression of *mbfA* in NTLirr raised the question of whether the overproduction of MbfA contributed to the H_2O_2-hyperresistant phenotype of NTLirr. The ability of MbfA to protect *A. tumefaciens* against H_2O_2 killing was assessed in wild-type NTL4 and the catalases-deficient strain (KC05, *katA* and *catE* double mutation) (Prapagdee *et al.*, 2004).

The catalases-deficient strain, KC05/pBBR, was highly sensitive to 200 μM H_2O_2 than wild-type, NTL4/pBBR (Fig. 4C). The KC05 strain complemented with functional KatA (KC05/pKatA) showed similar levels of H_2O_2 resistance to NTL4/pBBR (Prapagdee *et al.*, 2004). While complementation of KC05 by overproduction of MbfA, KC05/pMbfA, could partially reverse the H_2O_2-hypersensitive phenotype of KC05/pBBR (Fig. 4C). These data

suggested that MbfA plays a role in H_2O_2 resistance but to a lesser extent than catalase which directly degrades H_2O_2.

Discussion

Computational identification of the ICE motifs using *A. tumefaciens* genome revealed predicted genes belonging to the Irr regulon including genes involved in iron uptake (*fbpA*, *irgA*, *irpA*, *fhu*, *fat*, and *irp6*), genes encoding iron-containing proteins (*bfr*, *mbfA*, *fssA* and *fdx*), a gene involved in the heme biosynthesis (*hemA*), genes for the synthesis of Fe-S clusters (*suf*), and an iron-responsive transcriptional regulator (*rirA*) (Rodionov *et al.*, 2006). However, the direct experimental verification has not been done. The experimental data provided here show that physiological functions of *A. tumefaciens* Irr are to sense iron and manganese levels to control its target genes. Irr_{At} controls genes involved in the iron metabolism by a dual regulatory function as a positive regulator of genes responsible for siderophore synthesis and transport (Atu3675, *fhuA* and *irp6A*), and as a repressor of genes participating in iron storage (*mbfA*), and iron utilization (*hemA*). Furthermore, Irr_{At} plays a role in the peroxide stress response.

A. tumefaciens has an effective regulation of iron acquisition to ensure cellular iron demand. This is reflected by the fact that siderophore synthesis and transport genes are co-regulated by RirA and Irr. Siderophore synthesis and transport genes are turned on via derepression through RirA (Ngok-ngam *et al.*, 2009) and activation through Irr (Fig. 2) that help to increase efficiency for iron acquisition under iron deprivation.

Originally, the "iron response regulator" was named to a gene (*irr*) that mutated in the *B. japonicum* strain LODTM5, based on the observation that only iron could reverse a phenotype, PPIX accumulation under low iron conditions, of the mutant LODTM5 (Hamza *et al.*, 1998). The phenotype was primarily linked to iron availability. The accumulation of PPIX occurred when there was not enough iron for incorporating into overproduced PPIX to form heme, and supplementation with iron, not other metals, could prevent accumulation of PPIX. Therefore, *irr* seemed to response to iron only. It is shown here that *A. tumefaciens irr* responds to iron and also to manganese *in vivo*. Analysis of the β-galactosidase activities of promoter-*lacZ* fusion in wild-type NTL4 and the NTLirr mutant illustrated a novel role for Irr in the negative manganese-mediated regulation of a membrane-bound ferritin gene, *mbfA*. Repression of *mbfA* occurred under low iron conditions, moreover, maximal repression was observed in the presence of manganese, and this metal regulation was Irr-dependent (Fig. 3A). It has been shown *in vitro* that manganese enhanced DNA binding activity of *B. japonicum* Irr

(Sangwan *et al.*, 2008; Small *et al.*, 2009). Therefore, manganese may possibly be a metal cofactor for Irr_{At} activity *in vivo*. Binding to manganese might help to stabilize Irr protein which in turn directly increases repressor function of Irr. An indirect effect may also be possible if Irr regulates the expression of another protein that mediates *mbfA* repression in response to manganese. Experimental test of these possibilities will be an important subject for future study in order to gain more understanding of gene regulation by Irr_{At}.

The finding that Irr_{At} was a manganese-responsive repressor of *mbfA* raised the question of whether heme biosynthesis gene, *hemA*, was also negatively regulated by Irr in response to manganese. Thus, *hemA* promoter-*lacZ* fusion assay in NTL4 and NTLirr was performed. β-galactosidase activities obtained from wild-type NTL4 containing p*hemA-lacZ* showed that *hemA* expression was approximately 2-fold repressed in the presence of manganese (2.70 ± 0.11 U mg protein^{-1}) relative to maximal expression under iron-replete conditions (5.13 ± 0.18 U mg protein^{-1}), although the magnitude of repression was not as strong as observed for *mbfA* (approximately >18-fold, Fig. 3A). Furthermore, derepression of *hemA* under high manganese conditions was observed in NTLirr (5.16 ± 0.16 U mg protein^{-1}).

Irr_{At} plays a role in the peroxide stress response (Fig. 4A). The Irr-mediated peroxide resistance of *A. tumefaciens* resembles the *Bacillus subtilis* peroxide resistance controlled by PerR (peroxide-responsive repressor) in several respects. *B. subtilis* PerR is a manganese-dependent repressor that senses H_2O_2 and regulates peroxide stress genes (*katA*, *ahpCF*, *mrgA*) and heme biosynthesis genes *hemAXCDBL* (Chen *et al.*, 1995; Bsat *et al.*, 1996). Inactivation of *perR* results in loss of metal repression of peroxide stress genes and confers increased resistance to peroxide killing (Bsat *et al.*, 1998). KatA and AhpCF are H_2O_2-degrading enzymes whereas MrgA is a ferritin-like Dps protein that binds DNA and sequesters iron to protect DNA from oxidative damage. Derepression of heme biosynthesis genes in the *perR* mutant strain seems reasonable to be required for a heme protein like KatA. Similar to the *perR* mutant strain, the NTLirr mutant had increased resistance to H_2O_2 killing, at least in part, due to the derepression of a peroxide protective gene *mbfA* coding for a membrane bound ferritin. Like PerR, Irr controls a gene (*hemA*) involved in heme biosynthesis. Importantly, both PerR and Irr function as a transcriptional repressor in response to manganese. Irr mediates maximal repression of *mbfA* and *hemA* in the presence of manganese.

Experimental data show that there are at least two regulatory mechanisms, OxyR and Irr dependent, for sensing and responding to the peroxide stress in *A. tumefaciens*. OxyR is a metal-independent activator of an inducible peroxide-detoxification gene *katA* (Nakjarung *et al.*, 2003). Another mechanism as demonstrated here, a peroxide protective gene *mbfA* is

under the negative control of Irr and this regulation has a close link with the levels of iron and manganese concentrations (Fig. 3A). Thus far, no other peroxide defense gene controlled by Irr has been experimental verified yet. Further study will have to be carried out to identify other Irr-regulated peroxide stress response genes and to further clarify the molecular mechanism of metal-dependent Irr mediating cellular response to peroxide stress.

Iron regulation is crucial for bacterial survival and infection. Loss of the iron-responsive transcriptional regulator could be lethal to bacteria (Touati *et al.*, 1995; Hassett *et al.*, 1996). The iron regulators from plant pathogens have been shown to play a critical role for their virulence during the plant-pathogen interaction (Franza *et al.*, 1999; Subramoni and Ramesh, 2005; Cha *et al.*, 2008). RirA and Irr are iron regulators in *A. tumefaciens*. Mutation in the *A. tumefaciens rirA* gene reduced the bacterial ability to cause tumor on tobacco leaves (Ngok-ngam *et al.*, 2009). The attenuated virulence in the *rirA* mutant most likely resulted from the increased sensitivity to oxidants and the decreased ability to induce virulence genes (*virB* and *virE*) (Ngok-ngam *et al.*, 2009). Nonetheless, the disruption of *irr* in the NTLirr strain had no apparent defects in tumorigenesis on tobacco leaves (R. Sukchawalit, unpublished). Generation of H_2O_2 (Wojtaszek, 1997) and iron withholding (Scalbert, 1991) are important mechanisms that plant use to inhibit invading bacteria during infection. In fact, the NTLirr mutant was more resistant to H_2O_2 killing (Fig. 4A) and was able to grow better under iron-limiting conditions (in the presence of an iron chelator, 2,2'-dipyridyl) (R. Sukchawalit, unpublished) than wild-type NTL4. These abilities of the NTLirr mutant may, at least in part, contribute to the fully virulent phenotype.

Experimental procedures

Bacterial strains, plasmids and growth conditions

A. tumefaciens strains (wild-type strain NTL4 and its mutant derivatives) and plasmids used in this study are described in Table 1. *A. tumefaciens* strains were grown aerobically in Luria-Bertani (LB) medium at 28^{o}C with shaking at 150 rpm or on LB plates containing 1.5% agar. The medium was supplemented with 60 μg ml^{-1}gentamicin (Gm), 30 μg ml^{-1} kanamycin (Km), 100 μg ml^{-1} carbenicillin (Cb), or 5 μg ml^{-1} tetracycline (Tc) as required. *E. coli* strains (DH5α and BW20767) were used for DNA cloning and grown aerobically at 37^{o}C in LB medium supplemented with 100 μg ml^{-1} ampicillin (Ap), 30 μg ml^{-1} Gm, 15 μg ml^{-1} Km or 15 μg ml^{-1} Tc as required.

Molecular techniques

General molecular techniques were performed using standard procedures (Sambrook *et al.*, 1989). Plasmid DNA was isolated using the QIAprep kit (Qiagen). DNA restriction and modifying enzymes were purchased from Promega, Fermentas, or New England Biolabs and used according to the suppliers' recommendations. The primers used are listed in Table 2. PCR products and restriction fragments were purified using PCR clean-up and gel extraction kits (Qiagen). DNA sequence analysis was performed using a BigDye terminator cycle sequencing kit (PE Biosystems) on an ABI 310 automated DNA sequencer (Applied Biosystems). Plasmids (50-100 ng) were transferred into *A. tumefaciens* strains by electroporation (Cangelosi *et al.*, 1991).

Construction of an irr *mutant*

The *A. tumefaciens irr* gene was inactivated by a single homologous recombination of the suicide plasmid into the chromosomal *irr* gene. The 223 bp DNA fragment of *irr* internal region was amplified by PCR using primers BT696 and BT697 designed from the sequence of a putative *irr* gene (Atu0153) (Wood *et al.*, 2001), Pfu DNA polymerase (Promega) and genomic DNA isolated from wild-type NTL4 as the template. The 223 bp PCR product was cloned into the unique *Sma*I site of the pKNOCK-Gm suicide plasmid (Alexeyev, 1999), which is unable to replicate in *A. tumefaciens*, generating the recombinant plasmid, pKNOCKirr. The cloned DNA region was confirmed by automated DNA sequencing. The pKNOCKirr was transferred into wild-type NTL4 by conjugation (Cangelosi *et al.*, 1991). The single homologous recombinants were selected on LB agar plates containing 25 μg ml^{-1} chloramphenicol and 60 μg ml^{-1} gentamicin. Correct integration of the pKNOCKirr into the chromosomal *irr* gene was confirmed by southern blot analysis.

Reverse transcriptase PCR (RT-PCR) analysis

Bacteria grown overnight in LB medium were sub-cultured into 20 ml of fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were harvested by centrifugation at 6,000 rpm for 5 min. In some experiments, exponential phase cells were treated with 50 μM $FeCl_3$, 50 μM $MnCl_2$ or 250 μM 2,2'-dipyridyl for 15 min before harvesting. Total RNA was extracted from untreated and treated cells using the modified hot phenol method as described previously (Ngok-ngam *et al.*, 2009).

The RNA samples were treated with DNase I using the DNA-*free*™ Kit (Ambion), according to the manufacturers' protocols. Reverse transcription (converting mRNA to cDNA before PCR) was accomplished using SuperScript™ II Reverse Transcriptase (Invitrogen) with random hexamer primers (BioDesign, Thailand). Reverse transcribed RNA samples (0.1 μg) from each condition were used in the PCR reaction. Control reactions, where reverse transcriptase was omitted, were run in parallel to ensure that there was no DNA contamination. Positive controls were performed using genomic DNA isolated from wild-type NTL4. Gene-specific primers for *hemA*, *Atu3675*, *fhuA*, *irp6A*, and 16S rRNA (see Table 2) were used for PCR reactions using the Taq PCR Master Mix Kit (Qiagen). PCR reactions were carried out with an initial denaturation step at 95°C for 5 min, followed by 25 cycles of denaturation at 95°C for 30 sec, annealing at 55°C (*hemA*, *Atu3675*) or 58°C (*fhuA*, *irp6A*, *sitA* and 16S rRNA) for 30 sec and extension at 72°C for 30 sec, with a final extension step at 72°C for 5 min. RT-PCR products were visualized through gel electrophoresis on a 2% agarose gel and ethidium bromide staining. The 16S rRNA, a housekeeping gene, was used as a loading control and to quantitate the amount of RNA in RT-PCR reactions.

Overexpression of Irr and MbfA

The full-length of wild-type *irr* and *mbfA* (Atu0251) genes were amplified from *A. tumefaciens* NTL4 genomic DNA by PCR with specific primers for *irr* gene (BT694 and BT695) and for *mbfA* gene (BT1625 and BT1677), using Pfu DNA polymerase. The PCR products were cloned into the unique *Sma*I site of an expression vector, pBBR1MCS-4 (Kovach *et al.*, 1995), creating the recombinant plasmids, pIrr and pMbfA. The cloned DNA region was confirmed by automated DNA sequencing. Plasmids were used for complementation.

Siderophore detection

The yeast extract mannitol (YEM) agar plate containing chrome azural S (CAS) was used for detection of siderophore production and was prepared as previously described (Kitphati *et al.*, 2007). Overnight cultures grown in LB medium (5 μl at an OD_{600} of 0.1) were spotted onto a YEM+CAS plate containing 200 μM 2,2'-dipyridyl, and incubated at 28°C for 2 days. Siderophore production is indicated by the presence of a halo zone around the bacteria.

Construction of promoter-lacZ *fusions and β-galactosidase activity assay*

DNA fragments containing the promoter region of *hemA* (Atu2613) and *mbfA* (Atu0251) genes were amplified by PCR using Pfu DNA polymerase (Promega), genomic DNA isolated from wild-type NTL4 as the template, and specific primers for *hemA* gene (BT3039 and BT3040) and *mbfA* gene (BT1707 and BT1665. The PCR products were cloned into the unique *Sma*I site of pKNOCK-*lacZ* (the promoterless *lacZ* vector), a derivative of pKNOCK-Ap plasmid (Alexeyev, 1999). The recombinant plasmid was then digested with *Hin*dIII to yield DNA fragments containing the promoter of interested gene-*lacZ* fusion which were then subcloned into the *Hin*dIII of the pUFR027 vector (DeFeyter *et al.*, 1990). The resultant recombinant plasmids were named p*hemA-lacZ* and p*mbfA-lacZ*, respectively. The cloned DNA region was confirmed by DNA sequencing and was transferred into wild-type NTL4 and mutant strains.

Bacteria grown overnight in LB medium were sub-cultured into fresh LB medium. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were washed and resuspended in the minimal AB medium (Cangelosi *et al.*, 1991) to give an OD_{600} of 0.1. Cells were treated with 50 μM $FeCl_3$, 50 μM $MnCl_2$ or 50 μM 2,2'-dipyridyl. In some experiments, cells were treated with 1 μM $MnCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, or $CuSO_4$. Cells were further incubated at 28°C with shaking for 18 h. Cells were harvested and β-galactosidase activity was measured as described previously (Kitphati *et al.*, 2007). β-galactosidase specific activity is presented in units per milligram of protein. The data are means of triplicate samples ± SD.

Sensitivity to H_2O_2

Bacteria grown overnight in LB medium were sub-cultured into 10 ml of fresh LB medium to give an OD_{600} of 0.1. Exponential phase cells (OD_{600} of 0.5 after incubation for 4 h) were washed once and adjusted. Ten-fold serial dilutions were made. An aliquot (10 μl) of each dilution was spotted onto LB agar plates containing 200 and 400 μM H_2O_2. Cells spotted on an LB agar plate were used as a control. Plates were incubated at 28°C for 2 days. Each strain was tested in duplicate and the experiment was repeated at least twice to ensure the reproducibility of the results.

Catalase activity gel staining assay

Bacteria grown overnight in LB medium were sub-cultured into fresh LB medium to give an OD_{600} of 0.1 and grown at 28°C for 24 h. Cells were harvested and resuspended in 50 mM

sodium phosphate buffer (PB), pH 7.0, containing 1 mM phenylmethylsulphonyl fluoride (a protease inhibitor). Cells were disrupted by brief sonication followed by centrifugation at 12,000 *g* for 10 min. Clear lysates were collected and protein concentrations were determined using the Bradford BIO-RAD protein assay. Total proteins (25 μg) from lysate samples were separated on 7.5% non-denaturing gel and catalase isozymes were visualized by activity staining as described previously (Gregory and Fridovich, 1974). The gel was soaked in PB containing 50 μg ml^{-1} of horseradish peroxidase (Sigma) for 45 min at room temperature, and was then soaked in PB containing 5 mM H_2O_2 for 10 min. After, briefly washing twice with distilled water, the gel was stained with freshly prepared 0.5 mg ml^{-1} diamino benzidine in PB until the background became dark. Catalase isozymes appeared as colorless bands against a dark background.

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Figure legends

Figure1. Primary sequence alignment generated using CLUSTALW (http://clustalw.genome.jp) for Irr proteins from selected members of α-Proteobacteria and PerR. Protein sequences were obtained from the GenBank database including *Brucella abortus* Irr (AAO89498), *Bartonella quintana* Irr (CAF25629), *Rhizobium leguminosarum* Irr (CAD37806), *Agrobacterium tumefaciens* Irr (AAK85974), *Sinorhizobium meliloti* Irr (CAC41686), and *Bradyrhizobium japonicum* Irr (AAC32183. In Irr, the heme regulatory motif (HRM) consisting amino acid GCPWHD residues which binds ferric heme, and three consecutive histidine residues (HHH) which bind ferrous heme, are highlighted on a gray background.

Figure 2. *A. tumefaciens* Irr is a negative and positive regulator of iron-responsive genes.

A. RT-PCR analysis of 16S rRNA, *hemA*, Atu3675, *fhuA*, and *irp6A* transcripts. RNA samples were isolated from wild-type NTL4 and mutant NTLirr cultures treated with 50 μM $FeCl_3$ (Fe) or 250 μM 2,2'-dipyridyl (Dy) for 15 min.

B. Detection of siderophore production using a siderophore indicator CAS agar plate. Cells were spotted onto YEM+CAS agar plate containing 200 μM 2,2'-dipyridyl. The halo zone surrounding bacteria indicates the production of siderophores. NTL4/pBBR and NTLirr/pBBR strains are the wild-type and the *irr* mutant containing the plasmid vector pBBR1MCS-4, respectively. The complemented strain, NTLirr/pIrr, is the *irr* mutant expressing functional Irr from the plasmid pIrr.

Figure 3. The *mbfA* gene is negatively regulated by Irr under iron limitation and in the presence of manganese.

A. β-galactosidase activities were obtained from wild-type NTL4 and mutant NTLirr expressing plasmid vector (pBBR), functional Irr (pIrr), Mur (pMur) or RirA (pRirA). Cells containing plasmid p*mbfA-lacZ* were grown in the AB medium supplemented with 50 μM $FeCl_3$ (Fe), 50 μM 2,2'-dipyridyl (Dy) or 50 μM $MnCl_2$ (Mn).

B. Selectivity of metal repression of *mbfA*. β-galactosidase activities were obtained from wild-type (NTL4/pBBR) containing plasmid p*mbfA-lacZ* grown in the AB medium without supplemented (-) or supplemented with 1 μM of $MnCl_2$, $ZnCl_2$, $NiCl_2$, $CoCl_2$, or $CuSO_4$. β-galactosidase specific activity was presented in units per mg protein (U mg $protein^{-1}$). The data are means of triplicate samples $\pm$ SD.

Figure 4. The NTLirr mutant is H_2O_2-hyperresistant but has reduced catalase activity and role of MbfA in the peroxide resistance.

A. Sensitivity to H_2O_2. Cells were adjusted, diluted and spotted on an LB agar (LA) plate containing 400 μM H_2O_2 and incubated at 28^{o}C for 48 h. 10-fold serial dilutions are indicated above each column. Cells spotted on an LA plate were used as a control. NTL4/pBBR and NTLirr/pBBR strains are the wild-type and the *irr* mutant, respectively, containing the plasmid vector. NTL4/pIrr and NTLirr/pIrr strains are the wild-type and the *irr* mutant, respectively, expressing functional Irr.

B. Catalase activity gel staining assay. Cell lysates prepared from wild-type NTL4 and the NTLirr mutant strains were subjected to electrophoresis on a 7.5% non-denaturing gel. Catalase isozymes (CatE and KatA) were visualized by activity staining as described in the experimental procedures.

C. Role of MbfA in the peroxide protection of *A. tumefaciens*. Wild-type (NTL4) and the catalase-deficient, *katA* and *catE* double mutant (KC05) contain a plasmid vector (pBBR) or express functional MbfA (pMbfA). Cells were spotted on LA plates containing 200 μM H_2O_2.

TABLE 1. Bacterial strains, plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
A. tumefaciens		
NTL4	Wild-type stain, a Ti plasmid-cured derivative of strain C58	S. K. Farrand
NTLirr	*irr* mutant, derivative of NTL4 in which *irr* was disrupted by pKNOCKirr, Gm^r	This study
KC05	*katA catE* double mutant, Km^r and Tc^r	Prapagdee *et al.* (2004)
E. coli		
DH5α	host for general DNA cloning	Grant *et al.* (1990)
BW20767	host for pKNOCK plasmid	Metcalf *et al.*(1996)
Plasmids		
pGEM-T-easy	a cloning vector, Ap^r	Promega
pKNOCK-Gm	a suicide vector, Gm^r	Alexeyev (1999)
pKNOCKirr	pKNOCK-Gm containing a 223 bp fragment of *irr* coding region, Gm^r	This study
pBBR1MCS-4	an expression vector, Ap^r	Kovach *et al.* (1995)
pIrr	full-length *irr* coding region cloned into pBBR1MCS-4, Ap^r	This study
pMbfA	full-length *mbfA* coding region cloned into pBBR1MCS-4, Ap^r	This study
pUFR027	a cloning vector, Tc^r	DeFeyter *et al.* (1990)
p*hemA-lacZ*	*hemA* promoter-*lacZ* fusion fragment cloned into pUFR027, Tc^r	This study
p*mbfA-lacZ*	*mbfA* promoter-*lacZ* fusion fragment cloned into pUFR027, Tc^r	This study

[a] Gm^r, gentamicin resistance; Km^r, kanamycin resistance; Tc^r, tetracycline resistance; Ap^r, ampicillin resistance

Table 2. Primers used in this study

Gene-Primer name	Sequence 5'→3'	Expected product size (bp)
Gene inactivation		
irr-BT696	GCCAGCGCGTTGCTTTGGGT	223
irr-BT697	AAGAAGTGATGGTGATCCGA	
Complementation		
irr-BT694	GATGTGAATCAATTTTGCAC	441
irr-BT695	AACGTCAACCGCGCTTGCGA	
mbfA-BT1625	CGCCTTGATCTCGACACGAG	1044
mbfA-BT1677	TTCACGCGTTGCCGATGATA	
RT-PCR		
16S rRNA-BT1421	GAATCTACCCATCTCTGCGG	280
16S rRNA-BT1422	GATGTGAATCAATTTTGCAC	
Atu0152-BT2852	CTGGTGTTGAACGACTTCAC	435
irr-BT1100	TCGTCATGCAGCTCCTCGAC	
hemA-BT1345	AAGCCATGAAAGCCGCCATC	216
hemA-BT1090	GGTCGGAGAAGATAATGAGG	
Atu3675-BT3080	GCGGCTTATGAATTACATCG	292
Atu3675-BT3081	ATAGATCTGGTCGATCAGCC	
fhuA-BT1346	GGTGACGAAGGGTATTGGCG	202
fhuA-BT1096	CGCGACATAACCTTTCACCG	
irp6A-BT2734	CACCGTCAAGGATGTGACCG	232
irp6A-BT2735	CTTCATGCCGCCGAAAGTCG	
Promoter-*lacZ* fusion		
mbfA-BT1707	CCAGCAGATCGATGAAAGCG	378
mbfA-BT1665	AAGGTGATCGGCATAGGAGC	
hemA-BT3039	GATGTGAATCAATTTTGCAC	441
hemA-BT3040	AATGGTTGGTGCCAGAAATG	

FIG. 1

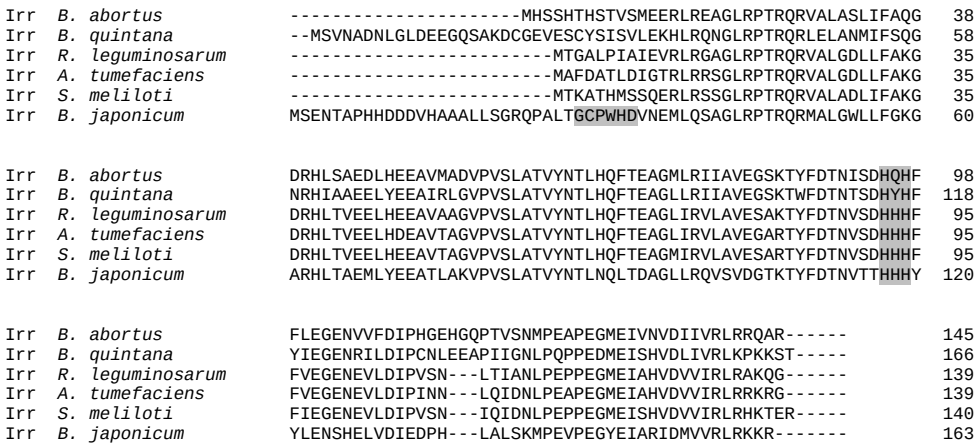

Irr B. abortus ----------MHSSHTHSTVSMEERLREAGLRPTRQRVALASLIFAQG 38
Irr B. quintana --MSVNADNLGLDEEGQSAKDCGEVESCYSISVLEKHLRQNGLRPTRQRLELANMIFSQG 58
Irr R. leguminosarum ----------MTGALPIAIEVRLRGAGLRPTRQRVALGDLLFAKG 35
Irr A. tumefaciens ----------MAFDATLDIGTRLRRSGLRPTRQRVALGDLLFAKG 35
Irr S. meliloti ----------MTKATHMSSQERLRSSGLRPTRQRVALADLIFAKG 35
Irr B. japonicum MSENTAPHHDDDVHAAALLSGRQPALTGCPWHDVNEMLQSAGLRPTRQRMALGWLLFGKG 60
Irr B. abortus DRHLSAEDLHEEAVMADVPVSLATVYNTLHQFTEAGMLRIIAVEGSKTYFDTNISDHQHF 98
Irr B. quintana NRHIAAEELYEEAIRLGVPVSLATVYNTLHQFTEAGLLRIIAVEGSKTWFDTNTSDHYHF 118
Irr R. leguminosarum DRHLTVEELHEEAVAAGVPVSLATVYNTLHQFTEAGLIRVLAVESAKTYFDTNVSDHHHF 95
Irr A. tumefaciens DRHLTVEELHDEAVTAGVPVSLATVYNTLHQFTEAGLIRVLAVEGARTYFDTNVSDHHHF 95
Irr S. meliloti DRHLTVEELHEEAVTAGVPVSLATVYNTLHQFTEAGMIRVLAVESARTYFDTNVSDHHHF 95
Irr B. japonicum ARHLTAEMLYEEATLAKVPVSLATVYNTLNQLTDAGLLRQVSVDGTKTYFDTNVTTHHHY 120
Irr B. abortus FLEGENVVFDIPHGEHGQPTVSNMPEAPEGMEIVNVDIIVRLRRQAR------ 145
Irr B. quintana YIEGENRILDIPCNLEEAPIIGNLPQPPEDMEISHVDLIVRLKPKKST----- 166
Irr R. leguminosarum FVEGENEVLDIPVSN---LTIANLPEPPEGMEIAHVDVVIRLRAKQG------ 139
Irr A. tumefaciens FVEGENEVLDIPINN---LQIDNLPEAPEGMEIAHVDVVIRLRRKRG------ 139
Irr S. meliloti FIEGENEVLDIPVSN---IQIDNLPEPPEGMEISHVDVVIRLRHKTER----- 140
Irr B. japonicum YLENSHELVDIEDPH---LALSKMPEVPEGYEIARIDMVVRLRKKR------- 163

FIG. 2

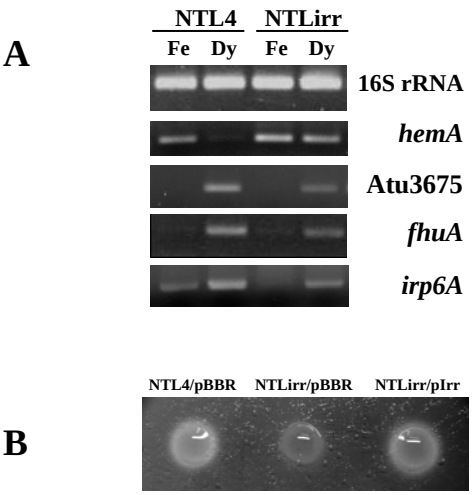
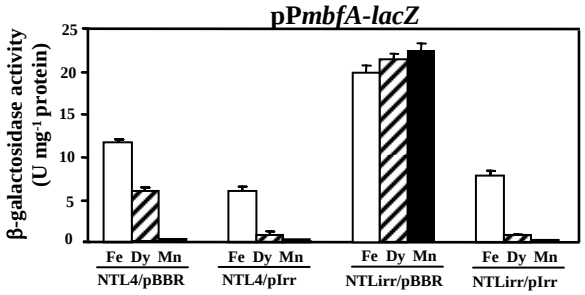

A
NTL4
NTLirr
Fe
Dy
Fe
Dy
16S rRNA
hemA
Atu3675
fhuA
irp6A
B
NTL4/pBBR
NTLirr/pBBR
NTLirr/pIrr

FIG. 3

A


pP*mbfA-lacZ*
β-galactosidase activity
(U mg-1 protein)
25
20
15
10
5
0
Fe Dy Mn
NTL4/pBBR
Fe Dy Mn
NTL4/pIrr
Fe Dy Mn
NTLirr/pBBR
Fe Dy Mn
NTLirr/pIrr

B

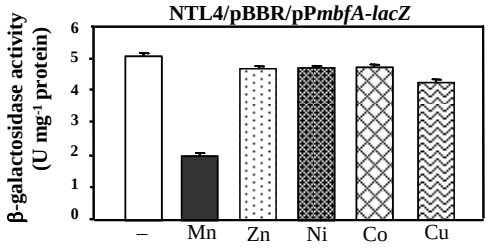
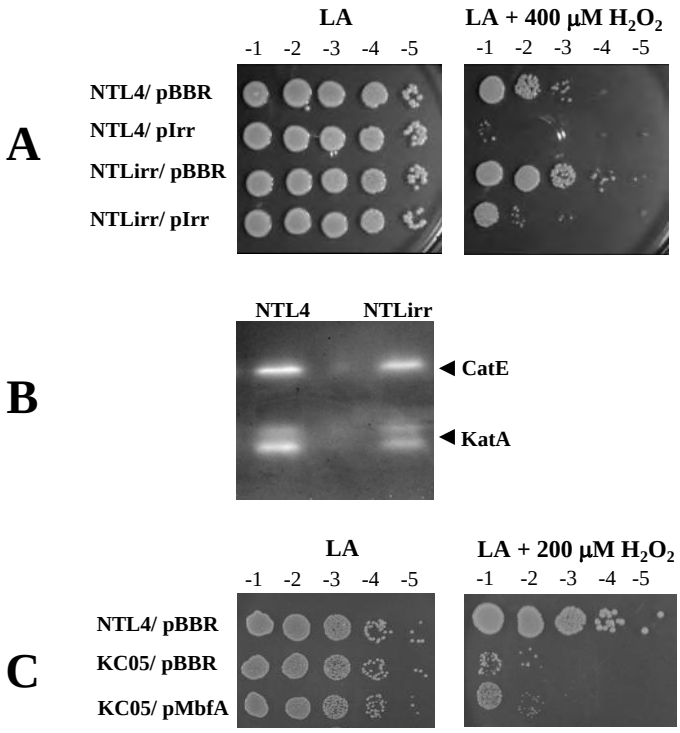

NTL4/pBBR/pP*mbfA-lacZ*
β-galactosidase activity
(U mg-1 protein)
6
5
4
3
2
1
0
–
Mn
Zn
Ni
Co
Cu

FIG. 4


A
LA
LA + 400 μM H2O2
-1 -2 -3 -4 -5
-1 -2 -3 -4 -5
NTL4/ pBBR
NTL4/ pIrr
NTLirr/ pBBR
NTLirr/ pIrr
B
NTL4
NTLirr
CatE
KatA
C
LA
LA + 200 μM H2O2
-1 -2 -3 -4 -5
-1 -2 -3 -4 -5
NTL4/ pBBR
KC05/ pBBR
KC05/ pMbfA