ROLE OF THE EXTRACYTOPLASMIC FUNCTION SIGMA FACTOR *CARQ* IN OXIDATIVE RESPONSE OF *BRADYRHIZOBIUM JAPONICUM*

by

ANCHANA THAWEETHAWAKORN

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Abstract

ROLE OF THE EXTRACYTOPLASMIC FUNCTION SIGMA FACTOR CARQ

IN OXIDATIVE RESPONSE OF BRADYRHIZOBIUM JAPONICUM

ANCHANA THAWEETHAWAKORN, M.S.

The University of Texas at Arlington, 2013

Supervising Professor: Woo-Suk Chang

Bradyrhizobium japonicum is a nitrogen-fixing bacterium that can establish a symbiotic relationship with the soybean plant. To be a successful symbiont, *B. japonicum* must have an effective mechanism to deal with plant defense responses such as oxidative burst. Our previous transcriptomic study showed that *carQ* (bll1028) encoding extracytoplasmic function (ECF) sigma factor was highly expressed (108-fold induction) in response to H_2O_2-mediated oxidative stress. In this study, a *carQ* knock-out mutant was constructed to identify the role of *carQ* in the oxidative response of *B. japonicum*. The mutant exhibited a significant decrease in survival at 10 mM H_2O_2 for 10 min and distinguishable nodulation phenotypes compared to the wild type. Desiccation of mutant cells also resulted in lower percent cell survival in both early and late desiccation periods. Taken together, the findings will provide insights into how the ECF sigma factor regulates and contributes to the survival of the bacteria.

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Chapter 1

Background and Significance

1.1 Soybean Production in the United States

The soybean (*Glycine max*) is a hairy annual legume originating from East Asia (34). Soybeans are characterized as plant food that contains a high percentage of protein. Because of this high level of protein, soybeans are known as the "meat of the field" (42). The soybean's natural form is completely edible, and can be processed into many different forms, including the traditional non-fermented soybean as found in soy milk, edamame, and tofu, and fermented forms as found in soy sauce and miso-based products. This legume can also be processed into other forms that have numerous uses, such as soybean oil for use in cooking or other food products. Other important processed soybean products are protein-rich defatted soy flour that can be used for feeding livestock, or two forms of soy protein concentrates known as textured vegetable protein and soya protein isolate (24), normally used as ingredients in meat and dairy products. .

Soybeans were first introduced to the U.S. in the early 20th century, and the U.S. became the world's largest producer of soybean. It was estimated that approximately 77.5 million acres of soybean crops were planted in 2010 (11). About 90% of oilseed production in the U.S. is processed from soybeans (11). Due to the low cost of production, soybean crops are an affordable source of protein, and are mainly used for feeding livestock. The demand for soybeans grows every year, and most of this demand comes from livestock producers and other commercial enterprises; China is currently the world's largest consumer of soybeans (15).

1.2 Demand of Nitrogen Fertilizers and Their Environmental Impact

Agricultural practices of soybean rely tremendously on fertilizers to meet the steadily increasing demand. Fertilizers include any organic or inorganic substances extracted from natural or synthetic processes, which are added to the soil to supply the plant with additional nutrients necessary for increasing its fertility (44). In the U.S., up to 50% of crop production relies on the natural and synthetic fertilizers (46), and about 40% of the world population is dependent upon food production that requires an input of nitrogen fertilizer at some point in the growth cycle (43). In fact, the demand of synthetic nitrogen fertilizers has continually increased, and correlates with global population growth. Without nitrogen fertilizers, normal agricultural practice alone could never meet the global demand for crop production.

Even though the use of nitrogen fertilizers is a huge benefit to agricultural practices, their application can lead to environmentally negative impacts. Fertilizer pollution is classified as a non-point source pollutant, causing the contamination of bodies of water via diffusion (50). It is difficult to reduce or eliminate this pollutant from the environment. Compounds in nitrogen fertilizers, especially nitrate, have become a huge concern for their impact on the environment. Nitrate is highly soluble in water, and can easily run off from agricultural fields into watersheds, ending up in major rivers and eventually in the ocean. This leads to a process known as eutrophication, which consists of an acceleration of algal growth, often resulting in the formation of algal blooms (41). Excessive amounts of algae cover the surface of the water and block the sunlight penetration into the water, which is needed for photosynthesis to take place in aquatic plants. Additionally, dead algae are also a food source for marine microbes and thus microbe population also rapidly increases in the area. Ultimately, deficient photosynthesis and over population of marine microbes cause hypoxia (O_2 concentration in water less

than 2 mg/L), resulting in suffocation of aquatic life (48) and the formation of dead zones. The dead zones in the Gulf of Mexico are some of the largest dead zones in the world. The dead zones in this area are caused by the excessive run off of nitrogen and phosphorus from fertilizer usage in the Midwest into the Mississippi River. This runoff and eutrophication of the Gulf of Mexico coastline directly disrupts the marine ecosystem.The U.S. Environmental Protection Agency (EPA) has reported the loss of thousands of acres of wetland in up to half of the coastal states due to the increasing area of these aforementioned dead zones (49). Moreover, the Gulf is an important resource for petroleum and domestic natural gas in the country, providing an essential habitat for numerous fish and wildlife species (49). If the Gulf hypoxic zones continue to expand at their current rates, the commercial and recreational fishing industries, along with the coastal state economies in the U.S., will greatly suffer due to the decrease in productivity.

Another important environmental consequence from the use of nitrogen-based fertilizers is the increase in soil acidification. Nitrogen in the fertilizer is usually in form of nitrate (NO_3^-) or ammonium (NH_4^+) that is soluble in soil water and can be taken up via plant roots. After fertilizer application combines with water, excess ammonia in fertilizer will be converted to nitrate by bacteria in soil, and the resulting reaction will cause the release of protons, resulting in an increase in soil acidity (4). Acidification in soil has received attention in China, the United Kingdom, and the U.S. (23). Even though nitrogen fertilizers help to increase the crop yield, it lowers the pH in soil and affects the crop production over time. Therefore, the mismanagement of the fertilizer use needs to be considered to avoid further soil acidification..

1.3 Biological Nitrogen Fixation and *Bradyrhizobium japonicum*

In healthy growing conditions, soybeans actually take up nitrogen from Bacteria (rhizobia) that have adhered to the root areas, allowing for fixation of nitrogen without the need to rely on synthetic nitrogen fertilizers. This only occurs when the soil is enriched with nutrients, and already has an adequate rhizobia population run-on surface; however, this cannot apply to farmlands that do not have a history of soybean cultivation, or for farmlands that do not have crop rotations that leaves a long time interval for cultivate soybean. In this case, rhizobia bacteria in soil might not be present in an adequate amount to provide a stable nitrogen source for soybean growth. In practice, this problem can be solved by inoculation of soybean seeds with rhizobial bacteria prior to cultivation of soybeans in soil. Unfortunately, this strategy is not always successful, and most of the time, it results in nitrogen-deficiencies in soybeans (32). At this point, fertilizer application is a better alternative to introduce more nitrogen source in soil, but at the same time, this application will have environmental impacts as well as increase the cost of soybean production. In the environment, nitrogen is found abundantly in form of nitrogen gas (N_2), contributing to 78% of the total atmospheric gas concentration. However, this form of nitrogen cannot be utilized by most living organisms, due in part to the stable triple bond that exists between the two nitrogen atoms. Certain bacterial species are able to convert the stable form of nitrogen (N_2) into the more readily useable form as known as ammonia (NH_3)

B. japonicum is a slow growing, rod-shaped, motile, non-spore forming, Gram-negative soil bacterium that is capable of entering into a symbiosis with soybean and fixing nitrogen in specialized structures known as root nodules (22). The symbiotic relationship begins with the recognition of plant signal molecules, known as flavonoids, which serve as activators of nodulation (*nod)* genes. Expression of *nod* genes will induce

the production of Nod factors that are in turn recognized by plants. Nod factors will cause plant root hair modification and root hair curling, consequently trapping the bacteria within the root. The trapped bacteria then penetrate further into the root cortex via a tubular structure known as the infection thread, and begin to colonize within the developing root nodules. Within the nodule, bacteria will differentiate into endosymbiotic forms, called bacteroids, that are capable of converting atmospheric nitrogen into ammonia (45). In healthy growing conditions, soybeans take up nitrogen from bacteroids within nodules, allowing for fixation of nitrogen without the need to rely on synthetic nitrogen fertilizers.

In this symbiotic relationship, the plant can provide the bacteria a variety of essential nutrients, especially a carbon source, for energy and growth. Through a series of biochemical reactions within the nodule, ammonia will be converted into ureides, nitrogen-rich compounds that will be transported further from soybean nodules to other areas (1).

1.4 The Plant Defense Response and Reactive Oxygen Species (ROS)

Plants can produce ROS as a defense mechanism against not only pathogenic but also symbiotic microorganisms. This rapid increase of ROS production by plants, called an oxidative burst; the primary form includes an increase in concentrations of superoxide radicals and hydrogen peroxide (3). Research done by Renata et al. revealed that superoxide radicals and hydrogen peroxide were found in the infection thread and nodules during plant early responses to an infection of soil bacteria (40). Hydrogen peroxide can be generated via the reduction of oxygen by photosystem I (PS I) and from the Rubisco oxyganase reaction together with the photorespiratory pathway (3).Typically, hydrogen peroxide is one of the primarily ROS production during the soybean nodule senescence. This ROS is severely cytotoxic in that it can cause oxidative damage to

membrane-bound proteins, and promote lipid peroxidation and hydroxyl radical generation (12).

To avoid the host defense mechanism and become a successful symbiont with soybeans, *B. japonicum* must have an effective regulation method to control the plant defense response. Several mechanisms have been suggested to deal with plant defense responses, including the suppression of ROS production by Nod factors, the synthesis of surface polysaccharide, the antioxidant system, and ethylene inhibitors.

1.5 Extracytoplasmic Function (ECF) Sigma Factor Subfamily and the Oxidative Stress Response

Bacterial sigma factors are part of a complex multi-subunit RNA polymerase that plays an important role in the recognition of primers and the initiation of the transcription process. After receiving the stimulus from the environment, the sigma factors will bind with the core RNA polymerase enzyme to become a more complex structure known as the RNA polymerase holoenzyme (29). Different sigma factors can replace one another, which results in different transcription regulation patterns in response to a particular metabolic pathway or environmental stress. The vast majority of sigma factors belong to the sigma 70 family. However, based on sequence similarity and protein domain architectures, sigma factors within sigma 70 family are separated into four subfamilies (18). The first subfamily is usually called primary or housekeeping sigma factor. This group is mainly involved in the transcription of genes in rapidly growing bacterial cells. The second sigma subfamily mainly regulates genes unnecessary for growth. Both first and second sigma subfamilies contain four distinct conserved domains (Figure 1-1). The third subfamily sigma factors, also called secondary sigma factors, are usually known as alternative sigma factors. In most case, the conserved amino acid sequence of region 1

and 3 were usually absent. This subfamily frequently increases under the general stress condition or during the developmental process (18, 20). ECF sigma factor is the forth subfamily of sigma 70 group and their function is associated with aspects of cell surface and transport, such as secretion and extracytoplasmic stress. This subfamily represents the most diverse amino acid sequences. The size of this fourth sigma subfamily is also smaller than other subfamily (typically 25- 35 kDa), and only contains conserved domain 2 and 4. Moreover, ECF sigma factors can autoregulate their own expression and their genes are usually co-transcribed with genes encoding cognate anti-sigma factors (18).

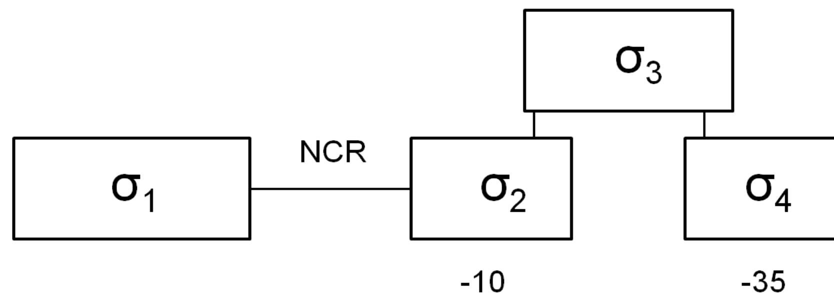

Figure 1-1 Diagram shows 4 conserved domains of sigma factors based on sigma 70 (18).

By reviewing several studies, the combating oxidative stress is most likely one of the main roles of ECF sigma factors. For instance, *Rhizobium etli* rpoE4 particularly plays an important role in saline, osmotic and oxidative stresses (hydrogen peroxide) (30). *Rhodobacter sphaeroides* RpoE (an ECF sigma factor) controls the activation of $RpoH_{II}$-dependent genes, which encode the oxidative-stress defense system involved in the singlet oxygen dependent response (36). *Caulobacter crescentus* SigT is responsible for the survivability of bacteria during osmotic and oxidative stresses (2). The other example

is ECF sigma factor CarQ in *Myxococcus Xanthus*, a sigma factor involved in light-induced carotenogenesis. The end product of the carotenogenesis can degrade the triplet-state porphyrin molecule and singlet oxygen (6).

The genome of *B. japonicum* USDA 110 contains a total of 23 sigma factors. Seventeen out of the twenty-three sigma factors are putative ECF-type sigma factors (16). Three of them (rpoH1, rpoH2, rpoH3) belong to RNA polymerase factor sigma-32, and two of them belong to RNA polymerase sigma-54 factor (35).

An analysis of *B. japonicum* gene expression by whole-genome expression profiling under the H_2O_2 shock treatment revealed that *carQ* (bll1028), encoding an ECF sigma factor, had the highest expression among the differentially expressed genes at up to 108-fold induction (21). The result from this study suggests that this ECF sigma factor might be involved in the detoxification of ROS. Due to its high expression, the response of *carQ* gene to H_2O_2 stress will be the main focus of this study.

Chapter 2

Materials and Methods

2.1 Experimental Overview

In order to investigate the role of *carQ* gene involved in combating oxidative stress of H_2O_2, a *carQ* knock-out mutant was constructed using site-specific mutagenesis by means of deletion mutation, as well as construction of a *carQ* complementary strain to confirm the rescue of the mutant phenotypes. Growth rate stress sensitivity analysis of the wild type strain *B. japonicum* USDA110, along with *carQ* mutant and *carQ* complementary strains, was tested under H_2O_2 oxidative stress, separated into two experiments; 1) the filter disk assay to measure the zone of inhibition of bacteria toward H_2O_2, and 2) stress test of H_2O_2 on liquid culture to monitor the bacterial survivability of all three strains over time. Determination of symbiotic phenotype was performed by pouch experiment to differentiate the significant differences among the three bacterial strains in term of an average distance of nodules from the root tip, nodule numbers, nodule sizes, nodule dry weight, and whole plant dry weight. In addition, nitrogenase activities for wild type, *carQ* mutant, and *carQ* complementary strains were measured by acetylene reduction assay (ARA) via monitoring the enzyme activity in nodules of soybeans inoculated with bacterial cultures. Moreover, all three strains were tested under the severe desiccation (i.e., 27% relative humidity [RH]) condition in order to observe the impact of desiccation induced oxidative stress responses.

2.2 Bacterial Strains, Plasmids, and Growth Conditions

The wild type strain of *B. japonicum* , *carQ* mutant, and *carQ* complementary strains were cultured in arabinose-gluconate (AG) medium at pH 6.8 which contains 125 mg of Na2HPO4, 250 mg of Na2SO4, 320 mg of NH4Cl, 180 mg of MgSO4·7H2O, 10

mg of CaCl2, 4 mg of FeCl3, 1.3 g of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1.1 g of 2-(*N*-morpholino) ethanesulfonic acid (MES), 1 g of yeast extract, 1 g of L-arabinose, and 1 g of D-gluconic acid sodium sulfate per liter (39). All *B. japonicum* strains were incubated aerobically at 30°C with vigorously shaking at 200 rpm for 2 days. Appropriate antibiotics were supplied for each strain ($\mu g \cdot ml^{-1}$): chloramphenicol, 30 (all *B. japonicum* strains); kanamycin, 150 (*carQ* mutant strain); tetracycline, 50 (*carQ* complementary strain). *Escherichia coli* strains and plasmids were grown aerobically in Lubia-Bertani (LB) medium at pH 7.0 which contains 10 g of tryptone, 5 g of yeast extract, and 10 g of NaCl per liter of nanopure water (5). All *E. coli* cultures were grow at 37°C (except DH5α pRK78 incubated at 30°C), vigorously shaking at 200 rpm overnight where appropriate antibiotics were applied in $\mu g \cdot ml^{-1}$: gentamicin, 15 (pjQ200SK and pRK78); tetracycline, 15 (pBBR1MCS-3 and pRK290); ampicillin, 50 (pNM480); kanamycin, 50 (pRK2013 and pNM480); streptomycin, 50 (pRK2073). Additional 15 g per liter of agar was added in case of making agar plates for every types of medium. In oxidative stress experiments, all *B. japonicum* strains were exposed to 10 mM of H_2O_2 for 10 min, the condition that developed intermediate effects on cell survival of the wild type strain of *B. japonicum* under H_2O_2 stress (21). All bacterial strains and plasmids in this study are listed in Table 2-1.

Table 2-1 List of bacterial strains and plasmids

Strain or Plasmid	Relevant genotype or phenotype	Source or Reference
E.coli strain		
DH5α	supE44 ΔlacU169 (Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1 F' (traD36, proAB+ lacIq, Δ(lacZ)M15) endA1 recA1 hsdR17 mcrA supE44λ gyrA96 relA1	Bethesda Research Laboratories
B. japonicum strains		
USDA110	Cm^R Wild-type	USDA, Beltsville, MD
carQ mutant	Cm^R Km^R *carQ*::Km	This work
carQ complement	Cm^R Km^R Tc^R *carQ*::Km with pBBR1MCS3 vector with *carQ* insert	This work
Plasmids		
pKD4	Km^R expression vector,	(8)
pRK2013	Km^R cloning vector	(13)
pNM480	Amp^R promoterless *lacZ* fusion vector	(33)
pCarQ-lacZ	Amp^R promoterless *lacZ* fusion vector with upstream region of *carQ* plus *carQ* 172 bp	This work
pRK2073	Sm^R cloning vector	(28)
pRK290	Tc^R cloning vector	(9)
pRK290- pCarQ-lacZ	Amp^R Tc^R pRK290 vector containing pCarQ-lacZ	This work
pjQ200SK	Gm^R cloning vector	(38)
pJQ200SK-*carQ*	Gm^R cloning vector containing *carQ* gene	This work
pRK78-pJQ200SK-*carQ*-Km	Gm^R cloning vector with suicide vector and internal *carQ* gene substitute with kanamycin cassette	This work
pBBR1MCS-3	Tc^R cloning vector	(26)
pBBR1MCS-3-*carQ*	Tc^R cloning vector contain *carQ* gene	This work

Amp, Cm, Km, Gm, Sm, and Tc refer to ampicillin, chloramphenicol, kanamycin, gentamicin, streptomycin, and tetracycline, respectively.

2.3 Construction of the *B. japonicum carQ* Mutant Strain

A *carQ* knock-out mutant was constructed using site-specific mutagenesis by means of deletion mutation. The *B. japonicum carQ* gene (bll10128) was amplified by PCR using the following primers: 5' AATTGGGCCCTGTCCGACGAAACAATTTGGCG 3' and 5' TAATGGGCCCTCCGCCTGATGTTGTGGTTTGT 3', forward and reverse respectively. *ApaI* restriction sites were added to both forward and reverse primers as indicated by the underlined sequence. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) as follows: initial denaturation at 95°C for 3 min, 34 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The purification of PCR results were performed by using the QIAquick PCR Purification Kit (Qiagen Sciences, Germantown, MD), and gel electrophoresis, a PowerPac™ Basic (Biorad, Hercules, CA), was used to confirm the correct size of amplified PCR products. Then, the amplified 0.85 kb *carQ* gene was inserted into a pJQ200SK suicide vector (38) using the heat shock method to construct a 6.0 kb recombinant plasmid. Insertion was confirmed by restriction enzyme digestion and gel electrophoresis. The cloning plasmid was transformed into the Lambda Red recombinase expression plasmid, DH5α pKD78, using electroporation method (8) to create electrocompetent cells, pRK78-pJQ200SK-*carQ-λ,* using Micro Pulser™ (Biorad, Hercules, CA), at 1.8 kV. A 1.47kb kanamycin antibiotic cassette from pKD4 plasmid was amplified by PCR using the following primers: 5' GTGTAGGCTGGAGCTGCTTC 3' and 5' CATATGAATATCCTCCTTAG 3', forward and reverse respectively. PCR amplification was performed on C1000 Thermo Cycler as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. Kanamycin antibiotic cassette fragments then were amplified again with

60 bp primers which combined between a kanamycin antibiotic cassette primer with an additional 40 bp *carQ* homology sequence: 5’ ATTGCCGACGGCAACCGGACGTC GATGCACATCCTCTATTGGTAGGCTGGAGCTGCTTC 3’ and 5’ CTAAGGAGGA TATTCATATGGGCGGATTTGCTTAAGGGCGCGGCGTCGACCGCTTCGCT 3’, forward and reverse respectively. PCR amplification was performed on the C1000 Thermo Cycler as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 72°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min. The PCR fragment and the electrocompetent cells were ligated to construct a recombinant plasmid (pRK78-pJQ200SK-*carQ*-Km) by electroporation using Micro Pulser^{TM}, at 2.2 kV. The resulting construct was transferred from *E. coli* to the wild type strain USDA110 by tri-parental mating with the helper strain pRK2013. Transconjugants were selected for sucrose-induced lethality (SecB^{S}), and chloramphenicol and kanamycin resistances (Cm^{R} and Km^{R}). The mutant strain DNA was extracted and confirmed by PCR using the 2 following primer sets: the 1st set had a forward sequence of 5' CGAACGTCATCGCCATCAAC 3' and reverse of 5’ CCAGTCATAG CCGAATAGCC 3’. The 2nd set of primers had a forward sequence of 5’ AGGATCTCGTCGTGACCCAT 3' and a reverse sequence of 5’ CCAGTCATAG CCGAATAGCC 3’. PCR amplification was performed on C1000 Thermo Cycler as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 72°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min.

In this study, a mutant of *carQ* gene was constructed in order to assign the physiological roles of a given sigma factor gene. To confirm the result of gene knockout construction, colonies of 8 transconjugants were randomly selected from an agar plate after performing tri-parental mating, and were cultured with AG medium and selective

antibiotic resistances for 4 days. All samples were sub-cultured and extracted the DNA after OD reached 0.8. PCR was performed in order to indentify the desired mutant. Two PCR fragments were amplified via 2 specific primer sets as described previously. A former 756 bp target fragment included a 133 bp downstream region of the *carQ* gene, 123 bp internal region from the 5' end of *carQ* gene, and 500 bp from the 5' end of kanamycin cassette, whereas a latter 652 bp target fragment included a 106 bp upstream region of *carQ*, 46 bp from the 3' end of *carQ* gene, and 500 bp from the 3' end of kanamycin antibiotic cassette (Figure 2-1 A). A recombinant plasmid strain and wild type strain, USDA110, were amplified with the same primer sets to serve as positive and negative controls, respectively. To evaluate the mutant, if the kanamycin antibiotic cassette was correctly replaced with an 433 bp internal region of *carQ* gene, 2 PCR products should clearly appear on the agarose gel at 756 bp and 652 bp, respectively, and should appeared at the same level with amplicons amplified from a recombinant plasmid which served as a positive control. For a negative control, no amplified PCR products were detected, and no band was found on agarose gel (Figure 2-1 B).

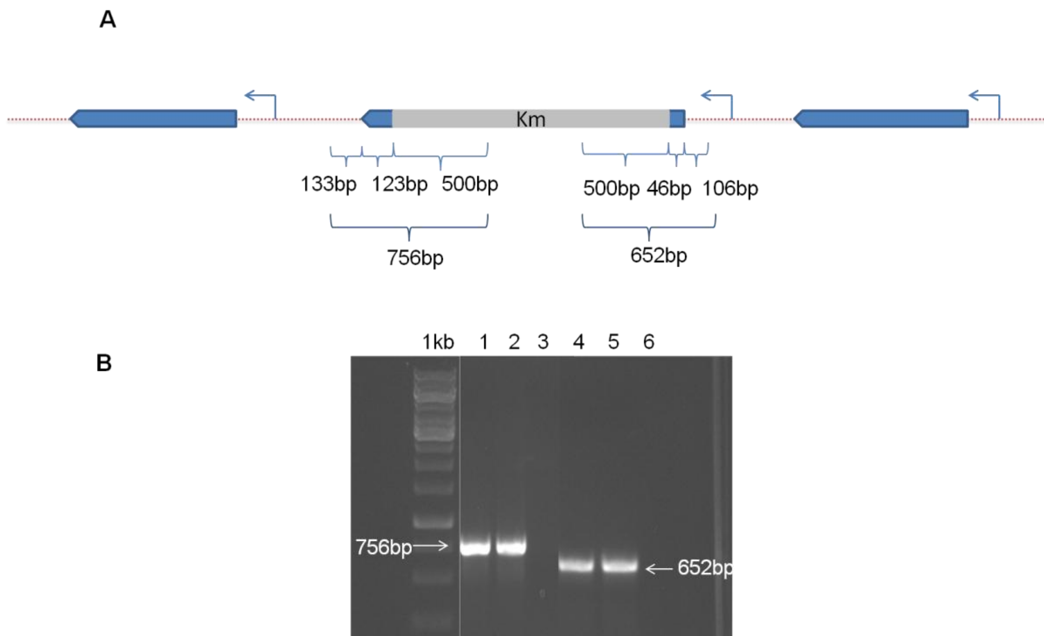

Figure 2-1 Confirmation of the mutant strain construction. (A) diagram indicated two regions on bacterial chromosome which were amplified by PCR to confirm a desired mutant; the region of carQ gene (blue) remained after replaced by kanamycin antibiotic cassette, upstream and downstream region of carQ gene, and inserted kanamycin antibiotic cassette (gray) within a carQ gene. (B) the bands of PCR products amplified from a mutant strain correspond to the size indicated on picture A: lane 1, 1 kb DNA marker; lane 1 and 4, a mutant colony; lane 2 and lane 5, positive control; lane 3 and lane 6, negative control.

2.4 Construction of the *B. japonicum carQ* Complementary Strain

In order to restore the wild type phenotype, a *carQ* complementary strain was constructed. A 0.823 kb of the *B. japonicum carQ* gene (bll10128), including upstream and downstream regions, was amplified by PCR using the following primers: 5' AATTGGGCCCTGTCCGACGA CGAAACAATTTGG 3' and 5' ATTACTCGAG

TGTTTCGTCCGCGGTAACAC 3’, forward and reverse respectively. *ApaI* and *XhoI* restriction sites were added to forward and reverse primers, respectively, as indicated by the underlined sequence. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The purified PCR products then were ligated with *ApaI-XhoI* digested pBBR1MCS-3 plasmid to yield a recombinant plasmid pBBR1MCS-3-*carQ*. The plasmid pBBR1MCS-3-*carQ* was confirmed by using double restriction enzyme digestion (New England Biolabs) with *ApaI* and *XhoI* and single digestion with *ApaI*. The correct sizes of cut recombinant plasmids were observed through gel electrophoresis, a PowerPac^{TM} Basic (Biorad, Hercules, CA), at 110 V for 45 min. The result recombinant plasmid pBBR1MCS-3-*carQ* was transferred back into the *B. japonicum carQ* mutant strain by tri-parental mating using pRK2073-Sm^{R} plasmid as a helper strain. After 2-day incubation, transconjugants were grown on the selective antibiotic plates which contained chloramphenicol, kanamycin, and tetracycline to select for antibiotic resistance. Transconjugants DNA were extracted and confirmed by PCR using the 2 following primer sets: the 1st set had a forward sequence of 5' TGATTCTTCTCGCTTCCGGC 3' and reverse of 5’ CACGTCTTCCTGGTCGATGT 3’. The 2nd set of primers had a forward sequence of 5’ ACATCGACCAGGAAGACGTG 3' and a reverse of 5’ GTGCGCTGTTCCAGACTATC 3’. PCR amplification was performed on C1000 Thermo Cycler as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 10 min.

To confirm the complementary strain, colonies of 7 transconjugants were randomly selected from an agar plate after tri-parental mating was performed, and were

subsequently cultured with AG medium and selective antibiotic resistances for 4 days. PCR was performed in order to identify a correct complementary strain. 990 bp and 1.1 kb fragments were amplified by 2 specific primer sets as described on the previous section. A former 990 bp target fragment included a 642 bp upstream region of *carQ* gene and 348 bp from the 5' end of *car*Q gene, whereas the latter 1.1 kb fragment included an 825 bp downstream region of *carQ* and a 284 bp from the 3' end of the *carQ* gene (Figure 2-2 A). A recombinant plasmid and a *car*Q mutant strain were both amplified with the same primer sets to serve as positive and negative controls, respectively. To evaluate the desired complementary strain, if a recombinant plasmid was successfully inserted into a *carQ* mutant strain, 2 PCR products should have clearly appeared on the agarose gel at 990 bp and 1.1 kb respectively. Importantly, these two PCR products should appear at the same level on the agarose gel with amplicons amplified from a recombinant plasmid, which served as a positive control. For a negative control, none of amplified PCR products were detected and no band appeared on the agarose gel (Figure 2-2 B).

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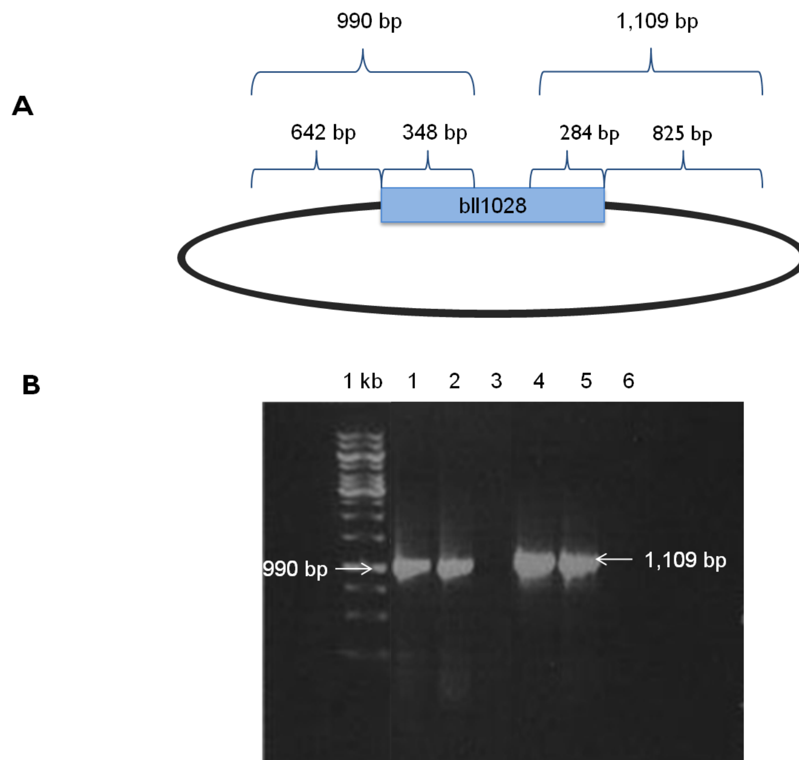

Figure 2-2 Confirmation of a complementary strain (A) diagram indicated two regions on the bacterial plasmid which were amplified by PCR to confirm the desired complementary strain: the region of *carQ* gene and upstream and downstream region of *carQ* gene (blue); plasmid region (black). (B) The bands of the PCR products amplified from the complementary strain correspond to the size indicated on picture A: lane 1, 1 kb DNA marker; lane 1 and lane 4, complementary strain colony; lane 2 and lane 5, positive control; lane 3 and lane 6, negative control.

2.5 Growth Curve Measurements

Initial cultures of wild type, *carQ* mutant, and *carQ* complementary strains were grown in AG medium with appropriated antibiotic until they reached the mid exponential phase at OD_{600} 0.8, and then subcultured into 125 ml flasks, 3 replicates of 30 ml for each strain, at OD_{600} 0.005. All samples were incubated at 30 °C in a shaking incubator. OD_{600} of each culture was monitored every 12 h until each culture reached the stationary phase, and at each time point, each culture was grown on selected antibiotic plates at different serial dilution factors from 1:1000 to 1:1,000,000 for a total of 3 replicates for each sample. All plates were incubated at 30°C for 4 days and the resulting colony forming units (CFUs) were then counted. This experiment was repeated 3 times.

2.6 Filter Disk Assay on Agar Plates

All three *B. japonicum* strains, including wild type, *carQ* mutant, and *carQ* complementary strains, were compared in regards with their susceptibility toward the H_2O_2 at 10 mM concentration by performing a filter disk assay. Each strain was grown in AG medium with appropriated antibiotics and harvested when the optical density at 600 nm (OD_{600}) reached the exponential phase at 0.8 OD_{600}. Each culture was sub-cultured to 0.05 OD_{600} in 0.9% soft AG agar with appropriated antibiotics. After all agar plates were solidified, 2 of 6 mm diameter filter disks (Becton Dickinson and Co., Franklin Lakes, NJ) were placed on each soft agar plate. The first filter disk was soaked with 10 µL of 10 mM H_2O_2 and placed on the top half of the plate. The second filter disk which served as a control was soaked with 10 µL of ddH_2O and placed on the bottom half of the plate. The zone of inhibition, a clearing area surround the filter disk, was measured in millimeters after 4-day incubation at 30°C. In this zone of inhibition test, each *B. japonicum* strain was comprised of 6 replicates.

2.7 Fulminant Shock in Liquid Culture

A total of 200 ml culture of each *B. japonicum* strain, including wild type, *carQ* mutant, and *carQ* complementary strains in AG medium and selective antibiotics, were incubated aerobically at 30°C with vigorously shaking at 200 rpm until the OD_{600} reached 0.8. Each 200 ml culture was divided into a total of six 30 ml cultures. Three cultures were treated with 10 mM H_2O_2 and another three were treated with sterilized ddH_2O to serve as a control. All cultures were incubated aerobically at 30°C with vigorously shaking at 200 rpm, and survival was monitored in each individual culture, measured at 0, 10, 20, 30, 60, 120 min after treatment. Each culture was washed with fresh AG medium and spread on AG agar plates containing chloramphenicol at 1:1000 to 1:1,000,000 dilution factor. After a 4-day incubation at 30°C, colony forming units were counted in order to determine the percent survival of each strain over time. The experiment was repeated three times for a total of 9 replicates for each strain.

2.8 Desiccation Experiment

Each *B. japonicum* strain, including wild type, *carQ* mutant, and *carQ* complementary strain, was grown in 5 ml AG medium with selective antibiotics and incubated aerobically at 30°C with vigorously shaking at 200 rpm for 2 days. All strains were subcultured in 200 ml AG media with chloramphenicol and incubated aerobically at 30°C with vigorously shaking at 200 rpm until the strains reached mid exponential phase at OD_{600} of 0.8. For each strain, subcultures were divided into 8 of 20 ml cultures in 50 ml Eppendorf tubes. Each 20 ml culture was transferred to a 0.4 µm sterilized MF^{TM} polycarbonate membrane filter (0.45 mm HA, 47 mm diameter; Millipore) and placed into a 60 x 15 mm sterilized Petri dish. Four of membrane filters were incubated in the desiccator containing sterilized water to generate the 100% relative humidity (RH) to

induce a hydrating condition, whereas another four membrane filters were incubated in the desiccator filled with a saturated potassium acetate solution to give a 27% relative humidity in order to induce a severe desiccating condition (7). The desiccators were incubated in the dark at 30°C and survival was monitored at 0, 4, 24, 72 h by resuspending cells from membrane filters with Bergersen's minimal salts medium (MM) and spread onto AG agar plates at dilution factors ranging from 1:10,000 to 1:1,000,000. Percent survivals were determined after cells were incubated at 30°C for 4 days. This experiment was repeated three times.

2.9 Symbiotic Phenotype Observation

Soybean seeds were sterilized by the following steps: seeds were placed into a 250 ml beaker and covered with aluminum foil to protect seeds from all light sources, while approximately 200 of the soybean seeds were washed with 30% Clorox for 10 min with moderate aggravation and immediately rinsed with sterilized ddH_2O three times. The soybean seeds were washed with 10% HCl for 10 min with the moderate aggravation and immediately rinsed with sterilized ddH_2O three times.

Seed germination was performed immediately after soybean seeds were sterilized. Approximately 15 soybean seeds were placed on sterilized 3M paper and placed in a sterile Petri dish. Soybean seeds were selected with the following criteria: light brown color, about 8 x 5 x 5 mm (length, width, and height), have spontaneously wrinkled seed coat around the seed, and no black spot or shape disproportion. To maintain moisture within the Petri dish, a sterilized 3M paper soaked with sterilized ddH_2O was placed on the top of the soybean seeds before being covered by a Petri dish lid. All Petri dishes were cover with aluminum foil and incubated in the dark at 30°C for 3 days.

Germinated seeds that have their roots immersed approximately 1.5 cm were selected and placed into sterilized plastic pouches (Mega International). A total of three seeds were placed in one pouch, and the position of each root tip was marked on the pouch. A straw was placed into either end of a pouch where it left the space between its bottom end and the bottom of a pouch approximately 5 cm. Two pouches (with the straw side facing out) were placed into a hanging paper folder using paper clips to attach them together, then this folder was placed into a folder rack.

To prepare the inoculum for the pouch experiment, the wild type, *carQ* mutant, and complimentary *B. japonicum* strains were grown in 20 ml of AG medium with the appropriate antibiotics at pH 6.8, and incubated aerobically at 30°C with vigorously shaking at 200 rpm until all cultures OD_{600} reached the mid-log phase (1.0 OD_{600}). Cells were harvested by centrifugation at 4,000 rpm for 10 min and washed with half-strength nitrogen-free Broughton and Dilworth (B&D) medium (pH 6.8) containing 500 µM $CaCl_2$, 250 µM KH_2PO_4, 250 µM K_2HPO_4, 5 µM Fe-citrate, 125 µM $MgSO_4 \cdot 7H_2O$, 125 µM K_2SO_4, 0.5 µM $MnSO_4 \cdot H_2O$, 1 µM H_3BO_3, 0.25 µM $ZnSO_4 \cdot 7H_2O$, 1 µM $CuSO_4 \cdot 5H_2O$, 0.05 µM $CoSO_4 \cdot 7H_2O$, and 0.05 µM $Na_2MoO_4 \cdot 2H_2O$ (27). Strain suspensions in the half-strength B&D medium were all adjusted to have an OD_{600} of 0.1.

After pouches and all cultures were prepared, each pouch was nourished with 20 ml of half-strength B&D medium. For each bacterial strain, 1.0 ml (ca. $1x10^8$ cells/ml) of 0.1 OD600 of culture was inoculated on each seed. All pouches were incubated in a growth chamber with 15 h of day and 9 h of night at 27°C. All pouches were watered with 20 ml of half-strength B&D medium for every 2 days. After 30 days incubation, the physical properties of soybean seeds inoculated with different bacterial strains were observed, including average nodule size, nodule number, nodule dry weight, plant dry weight, and the distance of nodules from the root tip. In one pouch experiment, 4

pouches were used for each strain (12 seeds total), and this experiment was repeated three times.

2.10 Acetylene Reduction Assay

An acetylene reduction assay was used to quantify the nitrogen-fixing activity of *B. japonicum* wild type, *carQ* mutant, and *carQ* complementary strains by detecting the ethylene production via gas chromatography (GC). A total of 6 soybean seeds were sterilized, germinated, and inoculated with bacterial culture as described in the pouch experiment section. After 30 days, each soybean-intact root nodule was transferred into a 27 ml sterilized glass jar, and sealed with a serum cap. 10% of the air inside the glass jar was replaced with an equal amount of pure acetylene gas, the air in the glass jar mixed via shaking. 250 µl of gas from the sample was drawn by using a gas-tight syringe (Hamilton Co., Reno, NV) and injected into the GC (GC-2014; Shimadzu) equipped with a hydrogen flame detector. The area of ethylene peak (3.6 min) and acetylene peak (4.75 min) were recorded at the end of the run (8 min). After acetylene reduction assay measurement, the nodules of each sample were dried in the oven (Chicago Surgical & Electrical Co.) at 65°C for 3 days. The nodule dry weight was measured by using an analytical balance (Denver Instruments APX-60). This experiment was repeated three times for each *B. japonicum* strain.

Chapter 3

Results

3.1 The *carQ* Mutant Has a Longer Generation Time

Growth of *B. japonicum* wild type, *carQ* mutant, and complementary strains was monitored via OD_{600} until all cultures reached the stationary phase. The generation times of the wild type, mutant, and complementary strains were 8.5 ± 0.2, 9.6 ± 0.1, and 8.7 ± 0.1 h, respectively, indicating a longer generation time in the mutant strain. The mutant strain also reached stationary phase faster with a lower maximum OD when compared to the other two strains (Figure 3-1).

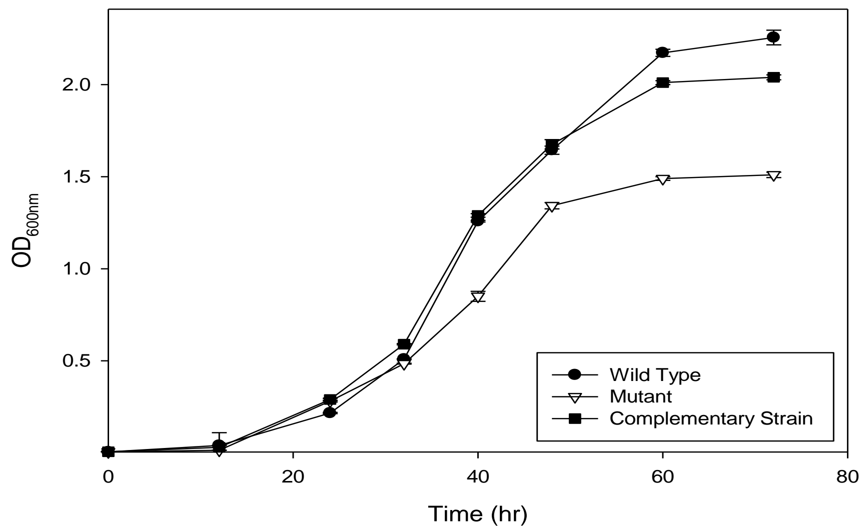

Figure 3-1 Growth of *B. japonicum* USDA110 (wild type), *carQ* mutant, and complementary strain in AG media. Each time point represents an average OD of three replicates with standard error.

3.2 The *carQ* Mutant Is More Susceptible to H_2O_2-Mediated Oxidative Stress

In order to demonstrate how the *carQ* gene is involved in the bacterial oxidative stress response, two different experiments were conducted: filter disk assay on solid agar plates and fulminant shock treatment in liquid culture. In the former, the susceptibility of bacterial response to the H_2O_2 exposure was indicated by the zone of inhibition, a clear zone around the disk. Under this treatment, a *carQ* mutant showed a significant increase in susceptibility to H_2O_2 compared with the wild type USDA110 and complementary strains ($P < 0.05$). The average diameter of the zone of inhibition for the *carQ* mutant was 25.6 ± 0.3 mm, whereas that of the wild type and complementary strains were 17.7 ± 0.2 and 16.7 ± 0.2 mm, respectively (Figure 3-2, Table 3-1).

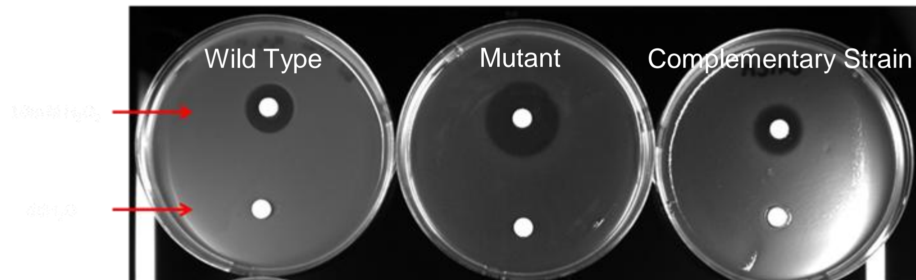

Figure 3-2 Inhibition zones of hydrogen peroxide on three *B. japonicum* strains. Left, wild type; Middle, *carQ* mutant; Right, complementary strain.

Table 3-1 Average diameter of the zone of inhibition compared among the three *B. japonicum* strains.

	Wild type	*carQ* mutant	Complementary strain
Diameter (mm)	17.67 ± 0.21	25.58 ± 0.30	16.67 ± 0.21

For the latter experiment, cell survival of the wild type, *carQ* mutant, and complementary strains was monitored under 10 mM H_2O_2 at various time points up to 120 min. As shown in Figure 3-3, more than 73% of the wild type and complementary strains survived throughout the treatment periods. However, the average percent cell survival of the *carQ* mutant was approximately 30% lower compared to that of the wild type ($P < 0.05$), suggesting the importance of the *carQ* gene and its involvement in response to oxidative stress.

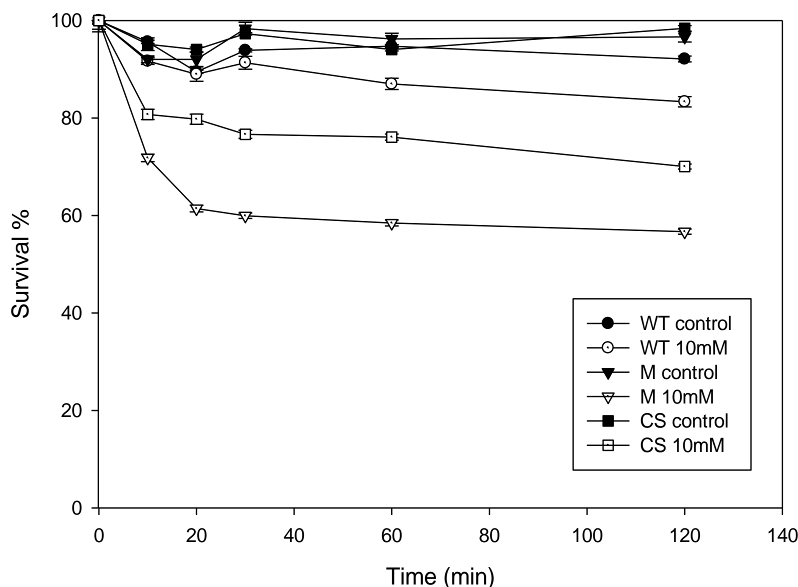

Figure 3-3 Survival of *B. japonicum* USDA110 (WT), *carQ* mutant (M), and *carQ* complementary strain (CS) after challenge with 10mM of H_2O_2.

3.3 Cell Viability of the *carQ* Mutant under Desiccation Conditions

Rhizobia have been known for their sensitivity to drought (i.e., desiccation), one of the major environmental stresses that reduce efficiency in development of symbiotic relationships between rhizobia and their host plants. Previous studies of genome-wide transcriptional analyses of *B. japonicum* under desiccation conditions revealed that genes involved in oxidative stress and sigma factors were highly expressed during desiccation (7). This result suggests i) the possible involvement of drought and oxidative stress and ii) co-regulation of those genes by sigma factors. Therefore, it is likely that ECF sigma factor CarQ is also involved in response to desiccation. To observe the role of the *carQ* gene in a desiccation stress response, the cell survival of the wild type, *car*Q mutant, and complementary strains was compared under hydrated (100% RH) and desiccating conditions (27% RH). Under the desiccating condition, survivability of the mutant strain was significantly decreased compared to that of the wild type in all 3 incubation periods ($P < 0.05$), and showed the most significant decrease of percent cell survival after 24 h of incubation (Figure 3-4).

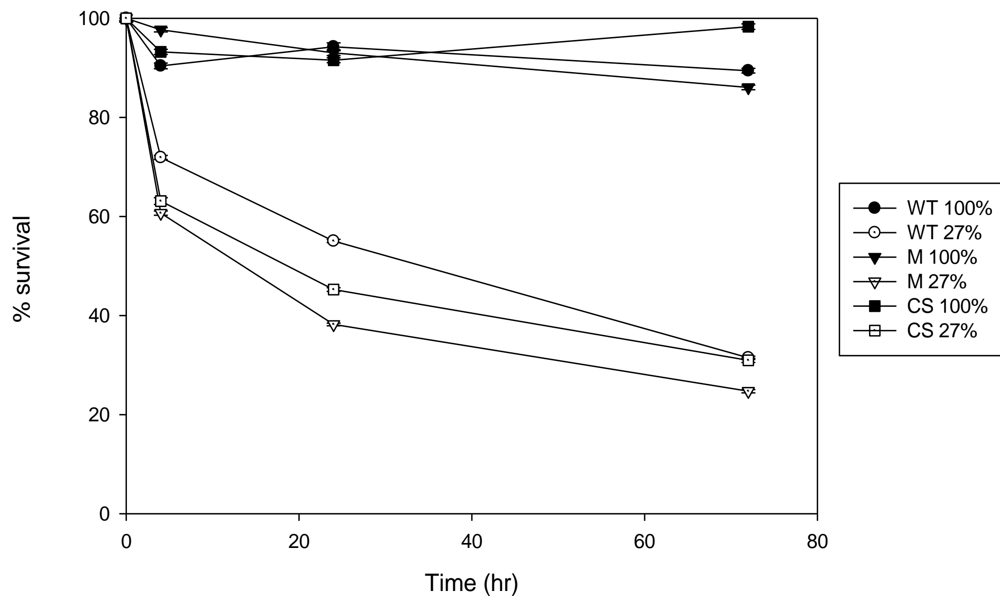

Figure 3-4 Survival of *B. japonicum* cells, following incubation under desiccating (27% RH) and hydrated (100% RH) conditions. WT, wild type; M, *carQ* mutant; CS, complementary strain.

3.4 The *carQ* Mutant Displays Different Symbiotic Phenotypes

Nodulation assay and plant tests were conducted to identify the role of *carQ* gene on bacterium-plant symbiotic development, especially in nodule development and its phenotypes. Soybean seeds were inoculated with the wild type, *carQ* mutant, or complementary strains at OD_{600} 0.1 (ca. 1 x 10^8 cells/ml) and the plants were grown for 4 weeks with 15 h of day and 9 h of night. Each individual soybean plant was collected and the nodule development and phenotypes were observed, including the average distance of a nodule from the initial root tip (mm), dry weight of nodules (mg), nodule numbers, nodule sizes (mm), and dry weight of plant (mg). The results showed that phenotypic

parameters were significantly different ($P < 0.05$) between the wild type and the mutant strains (Figure 3-5). The average distance of nodules from the root tip per plant formed by three different strains were as follows: the wild type, +1.6 ± 2.4 mm; the *carQ* mutant, **-**18.9 ± 2.7 mm; the complementary strain, -8.8 ± 2.2 mm. The positive and negative values indicate the position of nodules formed from to the root tip mark line: positive (+) nodules formed above the root tip, while negative (**-**) nodules below the root tip. The average of nodule dry weight per plant inoculated with the wild type, *carQ* mutant, and complementary strain was 4.7 ± 0.5, 2.1 ± 0.2, and 3.9 ± 0.4 mg, respectively. Soybeans inoculated with the mutant strain showed an approximately 50% lower dry weight of nodules per plant compared to the wild type and complementary strains. Moreover, in samples inoculated with the wild type and complementary strains, there were about 36% (5.3 ± 0.4 nodules) and 23% (4.7 ± 0.3 nodules) more nodules per plant, respectively, compared to samples inoculated with the mutant strain, which had an average 3.6 ± 0.3 nodules per plant. The average of nodule sizes formed by the mutant strain was also smaller compared to the wild type and complementary strains. Interestingly, mutation on the *carQ* gene also affected plant growth as indicated by the significantly lower plant dry weight compared to the wild type and complementary strains after 4 weeks. Average plant weights were 223.2 ± 9.6, 147.8 ± 6.0, and 185.6 ± 9.9 mg for those inoculated with wild type, *carQ* mutant, or complementary strains, respectively.

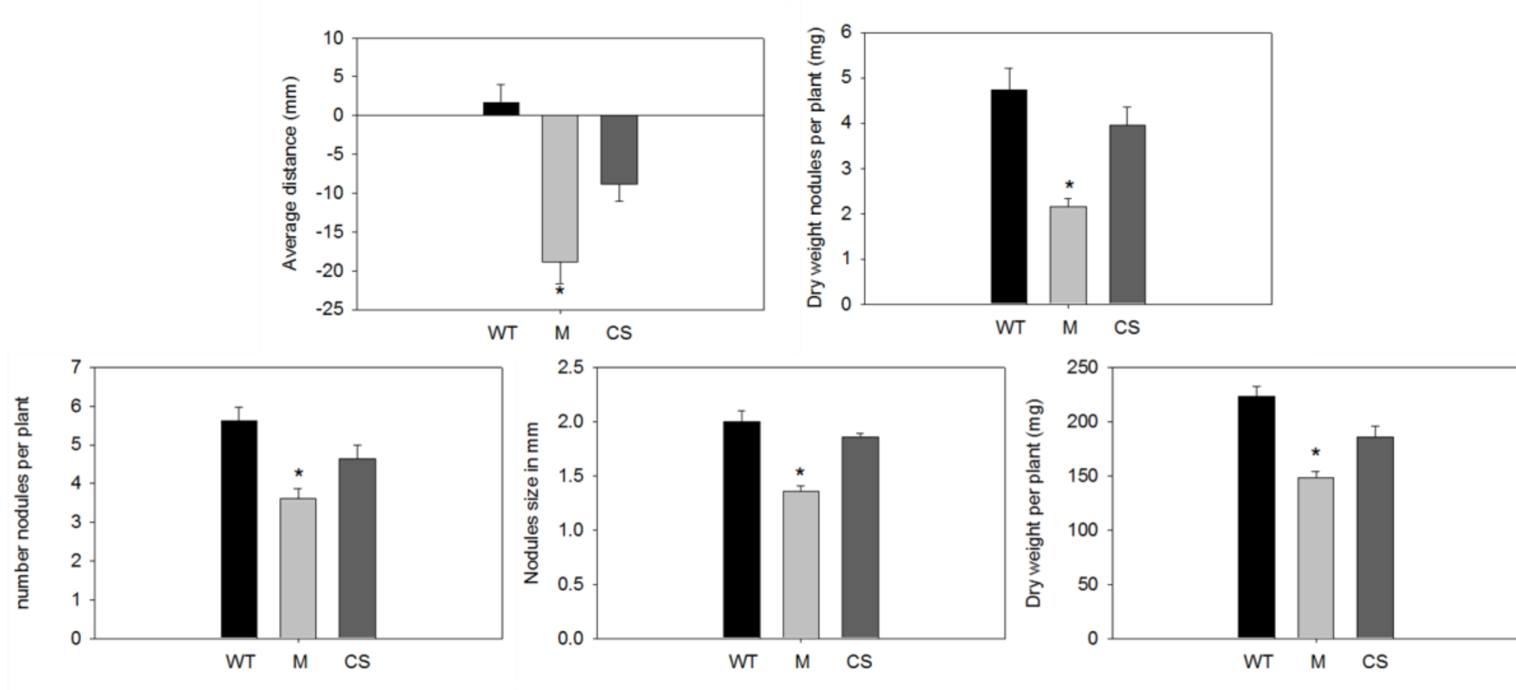

Figure 3-5 Comparison of symbiotic phenotypes among USDA110 (WT), *carQ* mutant (M), and complementary strain (CS) after inoculation for 4 weeks. (A) Average distance (mm) of nodules from the root tip, (B) dry weight of nodules per plant (mg), (C) nodule numbers per plant, (D) nodule sizes, (E) nodule dry weight (mg) per plant. Error bars represent the standard error of the mean and the asterisk indicates statistical significance ($P < 0.05$).

3.5 The *carQ* Mutant Shows Reduced Nitrogen-Fixing Capabilities

Due to a lower number of nodules and smaller nodule size in plants inoculated with the *carQ* mutant strain, an acetylene reduction assay was conducted to measure the nitrogenase activity by means of indirect measurement of ethylene production. The result from this experiment showed that mutation on the *carQ* gene affected the nitrogenase activity inside the nodule compared to the wild type and complementary strains (Figure 3-6). The average amount of ethylene production of the soybean samples inoculated with the *carQ* mutant was significantly decreased, with 0.3 ± 0.1 μmole per min per g of dry weight nodules ($P < 0.05$) of ethylene being produced, whereas the ethylene production of soybean samples inoculated with the wild type and complementary strain was 0.4 ± 0.1 and 0.4 ± 0.1 μmole per min per g of dry weight nodules, respectively.

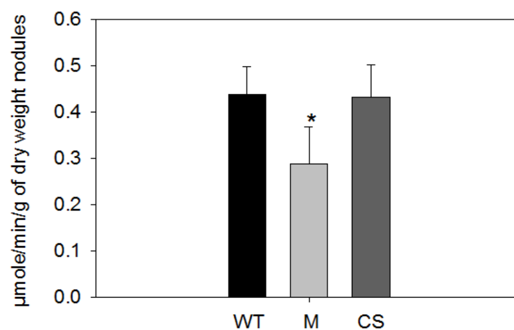

Figure 3-6 Comparison of nitrogenase activity measured by ethylene (C_2H_2) production among USDA110 (WT), carQ mutant (M), and complementary strain (CS) after inoculation for 4 weeks. Error bars represent the standard error of the mean and the asterisk indicates statistical significance ($P < 0.05$)

Chapter 4

Discussion

Soybean plants are able to produce an oxidative burst as a defense mechanism against foreign bacteria including both pathogenic and symbiotic partners. During the oxidative burst state, H_2O_2 is the primary ROS source released by the plant at the invasion site (25). Therefore, symbiotic bacteria need to develop an effective mechanism, not only to protect them from H_2O_2 present in the infection thread, but also to successfully establish a symbiotic relationship with their host plant. Rhizobial ECF sigma factors are a notable group of genes well known for their roles in response to environmental stresses (18). The results in this study reveal that CarQ plays an important role in the oxidative response of *B. japonicum*.

Sigma factors function in promoter recognition to initiate the transcription process. *B. japonicum* USDA110 contains a total of 17 putative ECF-type sigma factors. To date, four sigma factors, including CarQ, have been characterized. The first ECF sigma factor characterized is PhyR-σ^{ECFG} (bll7795), which is involved in heat and desiccation resistance under the carbon starvation condition (16). A study on EcfS (blr4928) showed that a mutation on the gene caused defects in the nodule phenotypes and nitrogen fixation activity (47). EcfG (blr7797) plays a key role in combating H_2O_2-mediated oxidative stress under micro-oxic conditions (31). Characterization of a *carQ* (bll1028) gene in this study has revealed the complex role of this gene as it is involved in the oxidative response, desiccation, symbiotic phenotypes, and nitrogen fixation activity. This suggests that *carQ* is probably an important gene involved in many types of stress responses for bacterial survival.

Previous studies on *B. japonicum* genome-wide transcriptional analysis in response to H_2O_2-mediated oxidative stress revealed that the highest level of expression

of the *carQ* sigma factor gene can be found in both oxic (108 fold) and micro-oxic conditions (35 fold) (21, 31). However, under micro-oxic conditions, there were no significant findings of properties of a *carQ* mutant in physiological and symbiotic analyses compared with the wild type (31). This may imply that the *carQ* gene may play a crucial role in the early state (i.e., aerobic conditions) of symbiotic development. Furthermore, it could be possible that a concentration of oxygen is a key factor that triggers expression of the *carQ* gene in response to H_2O_2-mediated oxidative stress.

Disruption of the *carQ* gene causes a change in growth behavior in *B. japonicum*. The mutant strain had a longer generation time and a lower maximum OD compared to the wild type and complementary strains. The difference in growth patterns may be caused by a lack of the gene itself, resulting in the deferment to a different metabolic pathway or more likely by regulation of genes involved in central metabolisms controlled by the ECF sigma factor. In addition, the filter disk assay and fulminant shock experiments on liquid culture revealed that lack of the *carQ* gene activity under non-stressful conditions did not affect cell viability and growth, but it showed a significant change in cell susceptibility and survival under the 10 mM H_2O_2 shock treatment. These results indicate that this gene primarily responds to H_2O_2-mediated oxidative stress. Even though the mutant gene caused a significant decrease in cell survival after 20 min incubation, percent survival was only slightly decreased throughout the treatment, suggesting that *B. japonicum* is still able to maintain an effective detoxification process. The result also suggests that the expression of *carQ* is a part of the regulatory network of stress response genes, which in the absence of *carQ*, the oxidative stress response is suspected to be compensated by other regulatory genes within the stress response gene family. For instance, the result from previous genome-wide transcriptional analysis indicated a relatively high amount of expression in a group of oxidative stress response

genes, particularly bll4012 and bll0753 (*ohr*) genes, as well as other *B. japonicum* sigma factor genes, such as blr7337 (*rpoH2*) and blr3038 (*SigD*) (21).

Desiccation is an inevitable problem in the practice of agriculture, mainly causing degradation of soil quality. This severe environmental cue can endanger plants and influence the viability of rhizobia in soil, affecting the symbiotic relationship between plants and their symbionts (51). Although several soybean cultivars have been engineered to become more resistant to drought, a corresponding bacterial symbiont *B. japonicum* has yet to show improvement in this stress condition (7). Previous research also indicated that desiccation can interfere with the interaction between plant and their symbiotic microorganisms by limiting nitrogen fixation (52). In addition, desiccating conditions could induce ROS generation in rhizobial species (51). Interestingly, our study also showed that cell viability of the *carQ* mutant significantly decreased when the mutant was exposed to a severe desiccating condition (i.e., 27% RH) compared to the wild type. The previous work on genome-wide transcriptional analysis of *B. japonicum* under severe desiccation conditions corroborates our results by illustrating the upregulation of several oxidative stress-related genes and sigma factor genes, including *carQ* (7).

All results in the phenotypic determination of the mutant showed a significant difference compared to the wild type strain. The *carQ* mutant showed lower numbers of nodules and the decrease in nodule size, nodule dry weight, and plant dry weight. The inoculation of root with the mutant strain caused delayed nodulation, as indicated by a longer distance of nodule formation from the root tip when compared with the wild type. Interestingly, soybeans inoculated with the complementary strain did not show the exact same results as the wild type, as compensation of this gene was supposed to restore the complete wild type phenotypes. This incomplete restoration might be explained by i) weak activity of the promoter, and/or ii) the natural loss of the recombinant plasmid

containing *carQ* gene during the treatment periods due to the absence of selection force (e.g., antibiotic absence). The results from the acetylene reduction assay showed that the *carQ* gene may play a significant role in nitrogen fixation, since the *carQ* mutant showed a significant decrease in ethylene production.

In order to gain insights into the regulatory mechanisms of this gene and how it affects its target genes during H_2O_2 stress responses, genome-wide transcriptomic analysis will be conducted. We will apply RNA-seq technology as one of the most powerful tools to reveal differential gene expression between the wild type and the *carQ* mutant under oxidative stress. By using this sequencing method, we hope to find relevant gene groups that are regulated by *carQ* ECF sigma factor in response to oxidative stress.

Chapter 5

Conclusion

The findings of this study indicate that *carQ* is one of the key genes in *B. japonicum*, playing a crucial role not only in protection against oxidative stress derived from the soybean plant, but also in developing the symbiotic relationship. The mutant exhibited a significant increase in susceptibility to H_2O_2-mediated oxidative stress compared to the wild type. Mutant cells also showed a significantly lower percent cell survival in both early (after 4 h) and late (after 24 h) desiccating periods. Nodulation phenotypes of the *carQ* mutant were distinguishable compared to those of the wild type, including lower numbers of nodules, decreased nodule size, decreased nodule dry weight, and decreased plant dry weight. In addition, nodules containing *carQ* mutant bacteroids showed decreased nitrogenase activity. These findings will bring a huge benefit and serve as an important piece of information to improve bacterial-plant symbiosis in hopes of increasing plant productivity, as well as reducing the world's dependence on nitrogen fertilizers. Future studies on the functionality of the ECF sigma factor CarQ are necessary to better understand how the organism adapts and responds to a variety of stresses from the environment, and to gain insight into how this gene regulates and contributes to the survival of the bacteria.

Appendix A

Reporter Gene Construction

Materials and methods for *carQ* reporter gene construction

A *carQ*-lacZ fusion was constructed as followed (Figure A1-1): the predicted promoter region 538 bp of *B. japonicum carQ* gene (bll10128) was amplified by PCR using the following primers: 5' ATATGAATTCG AAGACTCGGAGTTACCA 3' and 5' TTAAAAGCTTAAGCTTGCACG ATACGCAGGATG 3', forward and reverse respectively. respectively. *EcoRI* and *HindIII* restriction sites were added to forward and reverse primers, respectively, as indicated by the underlined sequence. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) as follows: initial denaturation at 95°C for 3 min, 34 cycles of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, followed by final extension at 72°C for 5 min. The purification of PCR results were performed by using the QIAquick PCR Purification Kit (Qiagen Sciences, Germantown, MD), and gel electrophoresis, a PowerPacTM Basic (Biorad, Hercules, CA), was used to confirm the correct size of amplified PCR products. The PCR fragment was cloned into the EcoRI and HindIII sites of pNM480, a β-galactosidase fusion vector, to give pCarQ-lacZ. To conjugate this cloned plasmid into *B. japonicum*, a 9.82 kb of *EcoRI*- digested pCarQ-lacZ and the EcoRI-digested pRK290 conjugative plasmid were ligated to construct a 29.8 kb recombinant plasmid. The resulting construct was mobilized into the wild type *B. japonicum* strain USDA110 by tri-parental mating with the helper strain pRK2013. Transconjugants were selected for chloramphenicol and tetracycline resistances (CmR and TetR). The in-frame fusion site was confirmed by PCR using the following primers: 5' ATATGAATTCGAAGA CTCGGAGTTACCA 3' and 5' AGTGTGATCATCTGGTCGCT 3', forward and reverse respectively. PCR amplification was performed on C1000 Thermo Cycler (Biorad, Hercules, CA) as follows: initial denaturation at 95°C for 3 min, 34 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 1.45 min, followed

by final extension at 72°C for 5 min. The purification of PCR results were performed by using the QIAquick PCR Purification Kit (Qiagen Sciences, Germantown, MD), and gel electrophoresis, a PowerPacTM Basic (Biorad, Hercules, CA), was used to confirm the correct size of amplified PCR products (Figure A1-2).

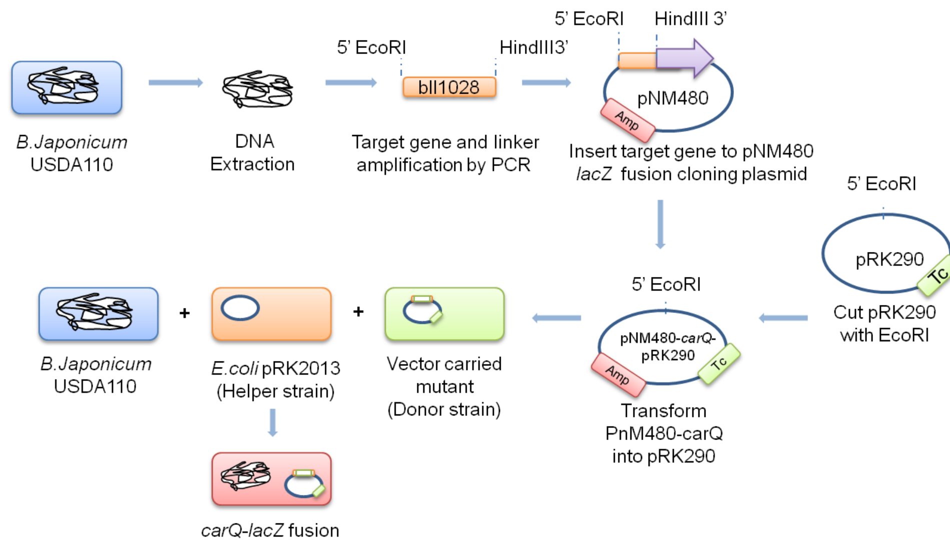

Figure A1-1 strategy for the construction of a *carQ* reporter gene

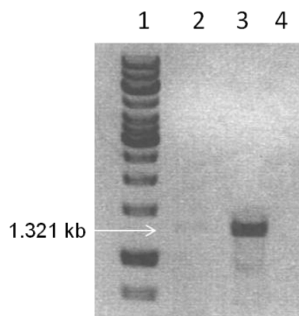

Figure A1-2 Confirmation of a *carQ* reporter gene. The bands of the PCR products amplified from the *carQ-lacZ* fusion strain correspond to the size indicated on picture: lane 1, 1 kb DNA marker; lane 2, *carQ-lacZ* fusion strain; lane 3, *carQ- lacZ* fusion plasmid (positive control) ; lane 4, USDA110 (negative control)

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Biographical Information

Anchana Thaweethawakorn is from Thailand. She graduated with a Bachelor of Science degree in Radiologic Technology in 2004 from Chaing-Mai University at Chaing Mai, Thailand. In 2005, she participated in the Industrial Projects for Undergraduate Students (IPUS) competition in Thailand. Her project involved the design and creation of a humanoid abdominal-pelvic phantom for qualification control of the x-ray machine, winning second prize of the IPUS science project awards. After this, she came to the United States in 2006 to study English at the English Language Institute (ELI) at UTA. She pursued a second Bachelor of Science degree in Biology at UTA in 2011 and continued her studies, obtaining a Masters of Science degree in Biology at the same school. After graduation, she plans to attend the Doctoral program for Microbiology at UTA in spring 2014.