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Enhancement of the nitrogen fixation efficiency of genetically-engineered *Rhizobium* with high catalase activity

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The *vktA* catalase gene, which had been cloned from *Vibrio rumoiensis* S-1^T having extraordinarily high catalase activity, was introduced into the root nodule bacterium, *Rhizobium leguminosarum* bv. *phaseoli* USDA 2676. The catalase activity of the *vktA*-transformed *R. leguminosarum* cells (free-living) was three orders in magnitude higher than that of the parent cells and this transformant could grow in a higher concentration of exogenous hydrogen peroxide (H_2O_2). The *vktA*-transformant was inoculated to the host plant (*Phaseolus vulgaris* L.) and the nodulation efficiency was evaluated. The results showed that the nitrogen-fixing activity of nodules was increased 1.7 to 2.3 times as compared to the parent. The levels of H_2O_2 in nodules formed by the *vktA*-transformant were decreased by around 73%, while those of leghemoglobins (Lba and Lbb) were increased by 1.2 (Lba) and 2.1 (Lbb) times compared with the parent. These results indicated that the increase of catalase activity in rhizobia could be useful to improve the nitrogen-fixing efficiency of nodules by the reduction of H_2O_2 content concomitantly with the enhancement of leghemoglobins contents.

[**Key words:** Catalase; *Rhizobium*; Root nodule bacteria; Nitrogen fixation; Hydrogen peroxide]

Root nodule bacteria form nodules in the root of leguminous plant and fix nitrogen in bacteroids like microsymbiotic organelles. Nitrogen fixation is a process that needs large amounts of ATP, which is supplied by oxidative phosphorylation. Whereas, the process of nitrogen fixation is extremely sensitive to molecular oxygen and the partial pressure of oxygen inside the nodules is maintained at lower levels, resulting in the strongly reduced condition with the production of active oxygen such as hydrogen peroxide (H_2O_2) (1). Leghemoglobins present in nodules play a role in the effective diffusion of oxygen and their autoxidation results in the production of O_2^- and H_2O_2 (2,3). On the other hand, it is also reported that the oxygen species such as H_2O_2 are released in the early stages of the formation of infection thread so that the host plant could control the nodulation by oxidative burst (4). Mehdy reported a striking release of reactive oxygen species under plant defense conditions against pathogens (5). Since active oxygens such as H_2O_2 are known to damage the proteins, lipids and DNA components (6), these reports suggest that the response of root nodule bacteria to active oxygens such as H_2O_2 would exert a strong influence on both the nodulation process and nitrogen-fixing activities.

It is reported that catalase plays an important role in the defense of cells against toxic forms of oxygen such as H_2O_2 and affects both the nodulation and nitrogen- fixing activities. *Sinorhizobium meliloti* has three kinds of catalases, KatA, KatB and KatC, and the phenotypes of both nodulation and nitrogen fixation were not impaired in the *katA*:: Tn*5* mutant (7). However, the double mutants of Δ*katA* Δ*katC* or Δ*katB* Δ*katC* showed poor nodulation and nitrogen-fixing capabilities (8,9). Besides, there were also reports that the activities of both catalase and nitrogen fixation were decreased during the nodule senescence (10), suggesting that these catalases have an important role in the nodulation and nitrogen-fixing activities. For *Bradyrhizobium* cells, it was reported that the *katG* minus mutant resulted in the loss of both catalase activity and exogenous H_2O_2 consumption, suggesting that KatG plays a role in the breakdown of H_2O_2 (11,12). However, in another study, it was reported that the root nodule bacteria had lower catalase activity than the other genera of aerobic or facultative anaerobic bacteria tested, resulting in higher susceptibility to H_2O_2 (13). These results prompted the construction of the root nodule bacteria with higher catalase activity to improve the nitrogen-fixing activity of nodules.

Vibrio rumoiensis S-1^T which showed extraordinarily high catalase activity was isolated and the catalase gene (*vktA*) was cloned (14,15). In this study, the *vktA* was introduced into *Rhizobium leguminosarum* cells and the strain with a remarkably high catalase activity was constructed. Results here show that the increase of catalase activity in rhizobial cells could be a valuable way to improve the nodulation and nitrogen-fixing ability of nodules.

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MATERIALS AND METHODS

Bacterial strains and growth conditions *Rhizobium leguminosarum* bv. *Phaseoli* USDA2676 was kindly supplied by Dr. Van Berkum (Agricultural Research Service, USDA, Beltsville, MD). *Escherichia coli* JM109 and MM294 harboring helper plasmid (pRK2013) (16) were grown at 37 °C in Luria–Bertani (LB) medium and *Rhizobium* strain was grown at 30 °C in tryptone-yeast extract (TY) medium (17). Kanamycin was added to the media at the concentration of 50 μg/ml unless otherwise noted.

Introduction of *vktA* gene into *R. leguminosarum* *Vibrio rumoiensis* S-1^T showing considerably high catalase activity was isolated from a drain pool of a fish product processing plant and the DNA fragment (4.9 kbp) including catalase gene (*vktA*), which is controlled by its own promoter activity, was ligated into pBluescriptII SK + to construct pBSsa1 (14). The *Bam*HI–*Xho*I fragment including a coding region of *vktA* (4.9 kbp) was isolated from the pBSsa1 and ligated into a broad host range vector, pBBR1MCS-2 at the site of *Bam*HI-*Xho*I (18). The ligation was confirmed by electrophoresis after the *Sal*I digestion of the recombinant plasmid with the bands of 2.3 and 2.6 kbp including *vktA* gene, in addition to a 5.1 kbp band of the vector (Fig. 1). Then, the recombinant plasmid or vector was introduced into *R. leguminosarum* cells to construct the *vktA*-transformant and vector control by triparental mating according to Simon (19). Briefly, *E. coli* JM109 (donor), MM294 (helper) and *R. leguminosarum* (recipient) cells in the exponential phase were mixed together and centrifuged for 30 s. After the cell pellets were suspended in 50 μl TY medium, mating was done on Millipore filter (pore size 0.45 μm) placed on TY agar plate at 30 °C. Cell suspensions were spread on minimal agar plate (8.3 mM sodium succinate, 5.9 mM sodium glutamate, 1.3 mM K_2HPO_4, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.8 mM $FeCl_3 \cdot 6H_2O$, 3.0 mM $CaCl_2 \cdot 2H_2O$, 0.9 μM biotin, pH 7.0) containing kanamycin.

Assay and staining of catalase activity Catalase activity was measured by the rate of H_2O_2 breakdown at 240 nm (3,20). Protein was measured by the method of Bradford using bovine serum albumin (BSA) as a standard (21). Activity staining of catalase on 7.5% polyacrylamide gels was carried out according to Clare et al. (22). Briefly, the gels were soaked in horseradish peroxidase (50 μg/ml) in 50 mM phosphate buffer, pH 7.0 for 45 min, and then in 5.0 mM H_2O_2 for 10 min. The gels were then rapidly rinsed with distilled water and placed in diaminobenzidine (0.5 mg/ml) in 50 mM phosphate buffer until the staining was completed.

Plant growth, inoculation and acetylene reduction activity Seeds of *Phaseolus vulgaris* (L.) cv. Yukitebou were kindly provided by the Tokachi Federation of Agricultural Cooperatives, Obihiro, Japan. The seeds were surface-sterilized with 70% (v/v) ethanol for 1 min, 10% (v/v) sodium hypochlorite for 30 s in this order, and then sufficiently washed with sterilized distilled water. Seeds were put in a seed bag with Norris & Date medium (23) or in pots stuffed with vermiculite, and then inoculated with approximately 10^6 cells per seed. The plants were incubated at light (22 °C for 14 h) and dark (16 °C for 10 h) conditions, respectively. The nitrogen-fixing ability of the nodules formed in the plant roots was measured by the acetylene reduction activity, i.e., the whole plants were sealed in a glass container containing 10% acetylene and incubated at 30 °C for 60 min. A portion of the gas from the sealed container was withdrawn and subjected to a gas chromatography (Hitachi K53, Tokyo, Japan) equipped with a column of Porapak N (50/80 mesh) (Waters, Milford, U.S.A).

Preparation of bacteroids The bacteroids were separated by the methods of Percoll gradient immediately after the nodules were detached from the plant roots (24). Briefly, the nodules (around 10 g) were ground in 30 ml of buffer [50 mM 3-(*N*-morpholino) propanesulfonic acid (MOPS)-KOH, pH 7.0, 0.3 M mannitol, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM dithiothreitol (DTT), 1% (w/v) BSA (Fraction V), and 1.0% (w/v) soluble polyvinylpyrrolidone]. The homogenates were then filtered through 4 layers of cloth before centrifugation at 13,000× *g* for 5 min. The precipitates were suspended in 10 ml of buffer (50 mM MOPS-KOH, pH 7.4, 0.3 M mannitol, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT), and 1 ml of the suspension was loaded on the top of Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden) gradient (82%, 72%, 60%, 45%, 30%), followed by centrifugation in SW41-Ti swing rotor (XL-80, Beckman, U.S.A.) at 18,400× *g* for 20 min. The bacteroids fractions (boundary layer between 60 and 72%) were collected, diluted with 5 volumes of buffer (50 mM MOPS-KOH, pH 7.4, 0.3 M mannitol, 5 mM $MgCl_2$, 1 mM EDTA), and centrifuged at 13,000× *g* for 5 min.

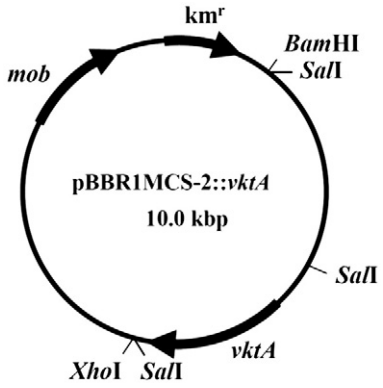

FIG. 1. Plasmid map of pBBR1MCS-2 harboring *vktA* gene. The *Bam*HI–*Xho*I fragment (4.9 kbp) including the *vktA* region (2.3 kbp) excised from pBSsa1 was ligated into pBBR1MCS-2 at the site of *Bam*HI–*Xho*I (see Materials and methods).

Western blot analysis One-dimensional SDS-PAGE was performed under the condition of 12.0% (w/v) acrylamide gel (running gel) with 375 mM Tris–HCl, pH 8.9, 0.1% SDS, 0.075% ammonium persulfate (APS) and 0.1% TEMED, and 4.5% (w/v) acrylamide gel (stacking gel) with 31 mM Tris–HCl, pH 6.8, 0.025% SDS, 0.02% APS and 0.025% TEMED, constant current of 15 mA. The proteins which separated on the gel were blotted onto a polyvinylidene difluoride membrane (PVDF: Fluorotrans, Biocraft) at room temperature. Polyclonal anti-VktA and a goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (American Qualex, San Clemente U.S.A.) were used as primary and secondary antibodies, respectively. The antigen-antibody complex was detected using diaminobenzidine.

Immunoelectron microscopy Cells were fixed with 3.0% paraformaldehyde and 0.1% glutaraldehyde in phosphate-buffered saline (PBS: 5.1 mM KH_2PO_4, 6.7 mM $Na_2HPO_4 \cdot 12H_2O$, 116.4 mM NaCl, pH 7.0) overnight. After washing with PBS containing 50 mM NH_4Cl, samples were dehydrated with ethanol and embedded in L.R. white resin (The Resin Company, London, U.K.) by polymerization at 4 °C for 48 h. Ultrathin cuts of the resin were treated with the polyclonal antiserum against VktA and then with a secondary anti-rabbit antibody, which was coupled with gold particles (15 nm in diameter) (Amersham). These specimens were observed by a transmission electron microscope (Hitachi H-800). Each grid with a section was transferred to 10% BSA-TBS (2 mM Tris–HCl, 0.15 M NaCl, 2 mM EDTA·Na_2, pH 7.0) for 1 h, immunostained with anti-catalase (VktA) antibody in TBS containing 10% BSA for 2 h, followed by washing with TBS for 25 min, then, stained with goat anti-rabbit IgG conjugated to 5 nm colloidal gold overnight in the same buffer. The grids were washed with distilled water for 25 min, stained for 15 min with uranyl acetate, washed with distilled water again, stained for 5 min with lead acetate, then finally washed briefly with distilled water. Grids were retrieved by forceps and air-dried prior to observation by an electron microscope (Hitachi H-800).

Assay of H_2O_2 content The nodules formed in the plant root were detached 35 days after planting, ground in 1 M $HClO_4$ and then neutralized with 0.5 M KOH as described previously (25). The extracts were centrifuged at 13,000× *g* for 1 min at 4 °C and the H_2O_2 present in the supernatant was measured by the method of Quantitative Hydrogen Peroxide Assay (OXIS International, Portland, U.S.A.).

Assay of leghemoglobin components The extraction and quantification of leghemoglobin components by capillary electrophoresis (Quanta 4000E, Waters) were carried out (26). The fresh nodules were extracted with 100 mM potassium phosphate buffer (pH 6.8) at 4 °C and passed through a hydroxyapatite mini-column. Twenty-five μl of the eluate was mixed with 5 μl of 100 mM potassium phosphate buffer (pH 6.8) containing 50 μg potassium hexacyanoferrate (III), 50 μg nicotinate, and 20 μl of sodium azide solution, and injected for 90 s by the hydrostatic method. Electrophoresis was conducted under 25 kV for 45 min using 75 mM sodium phosphate buffer (pH 2.0) as electrolyte buffer. The leghemoglobin components were detected by OD_{185}.

RESULTS

Catalase production in *vktA*-transformed *R. leguminosarum* As shown in Fig. 2A, the *vktA*-transformed *R. leguminosarum* exhibited a remarkably high catalase activity up to around 10,000 unit/mg protein. This activity was 3 orders of magnitude greater than that of the parent strain. To confirm the production of VktA catalase in *R. leguminosarum* cells, the activity staining of catalase in the *vktA*-transformed *R. leguminosarum* was performed (Fig. 2B). Results of the electrophoresis showed the obvious band of VktA in both logarithmic and stationary phases, and the intensity of VktA in stationary phase was stronger than that in logarithmic phase, indicating that the activity of catalase increased from logarithmic to stationary phase (Lane of transformant in Fig. 2B). Although the parent strain of *R. leguminosarum* had originally two bands of different mobility from VktA, their intensities were considerably weak compared to that of VktA and was not obvious in extracts from *vktA*-transformed *R. leguminosarum* (Lane of parent in Fig. 2B). In addition, the production of VktA catalase in *R. leguminosarum* cells was ascertained by immunoblot analysis using the anti-VktA antiserum. The result showed that a weak but positive antigen-antibody reaction occurred as a single band between the anti-VktA antiserum and the VktA, indicating the antibody specificity of anti-VktA antiserum against VktA (Lane of transformant in Fig. 2C II). These results clearly showed that the efficient production of VktA catalase was responsible for the high activity of catalase in the cells.