Summary of Doctoral Thesis

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Title	Biochemical characteristics and plant tissue localization of plant growth-promoting bacteria isolated from sugar beet (*Beta vulgaris* L.)

Introduction and purpose

Sustainable agricultural production through environmentally friendly approaches is the most effective way to meet increasing food demands worldwide. Plant growth is affected immensely by the interactions of its associated microorganisms. Application of bacteria that positively effect on plant growth (PGPB) as alternative solution for chemical fertilizers can enable us to produce high yields with less use of chemicals. Better understanding about the role of PGPB to plant growth can contribute to develop microbial approach that can be successfully used for sustainable agriculture. Therefore, it is crucial to investigate growth promoting profiles of beneficial microbes from different sources of plant and soil for future application.

Sugar beet (*Beta vulgaris* L.) is one an essential sugar producing crop. Owing to its diverse bacterial community with excellent tolerance against local environmental stresses, this plant has gained attention as a study object. Previously, bacterial strains having a high affinity with the sugar beet (*Beta vulgaris* L. cv. Rycka) were examined for their sugar beet growth-promotive abilities (Kenkyuseika, vol. 539. 2015. Tsukuba Office, Agriculture, Forestry and Fisheries Research Council Secretariat, Japan). This study aimed to identify the most potential bacterial strain among six bacteria isolated from sugar beet with regard to its beneficial traits of PGPB and evaluate the selected strain's effectiveness on growth of vegetables seedlings in order to ascertain whether it can be employed as bioinoculant for agricultural practice.

Materials and methods

Bacterial stress tolerance:

Six bacterial strains (*Rhizobium* sp. HRRK005, *Polaromonas* sp. HRRK103, *Variovorax* sp. HRRK170, *Mesorhizobium* sp. HRRK190, *Streptomyces* sp. HRTK192, and *Novosphingobium* sp. HRRK193) were grown in R2A medium (BD, Sparks, MD, USA) under different range of

temperatures (10–40 °C) and pH (4.0–10.0). Growth was monitored at 660 nm by using a biophotorecorder.

Bacterial biochemical characteristics:

Production of indole-3-acetic acid (IAA) was quantified by high performance liquid chromatography (HPLC) analysis using ethyl acetate extraction method (Tien, Gaskins, & Hubbell, 1979). Siderophore production was evaluated using chrome azurol S shuttle assay (Schwyn & Neilands, 1987) and biofilm production was determined by microtiter plate assay (Yuttavanichakul *et al.*, 2012). Enzyme activities, including the 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, β-1,3-glucanase, chitinase, cellulase and lipase were assessed by methods reported by Penrose and Glick (2003), Singh *et al.* (1999), O'Brien and Colwell (1987), Crabbe *et al.* (1994) and Bhattacharya *et al.* (2009), respectively.

Preparation of inoculant and plant material and growth condition:

Wild type or GUS-labeled HRRK170 cells suspension was prepared as inoculant with cell amount adjusted to 8.5×10^7 CFU mL^{-1}. Plants, including sugar beet (*Beta vulgaris* L. cv. Rycka) and vegetables [cabbage (*Brassica oleracea* L. cv. Harunami), lettuce (*Lactuca sativa* L. cv. Cisco), tomato (*Solanum lycopersicum* L. cv. Momotaro), radish (*Raphanus raphanistrum* L. cv. Taibyo sobutori), eggplant (*Solanum melongena* L. cv. Senryo no.2), Chinese cabbage (*Brassica rapa* L. cv. Kigokoro 85, Kigokoro 65, Haregi 85, and Okiniiri), and green pepper (*Capsicum annuum* L. cv. Kyomidori, Kyonami, Ace, and Pitaro)] were grown under cycles of 14 h light at 23.5 °C and 10 h dark at 20.0 °C after inoculation.

Tissue localization and determination of infected cell density:

Sterilized seeds were sown on 1.5% agar and inoculated with 100 µL GUS-labeled HRRK170 cell suspension per seedling on 0.3% agar or 0.2 % gellan gum containing 0.05 % HYPONeX®, depending on purpose of experiment. Inoculated plants were grown for one week and GUS-stained by de-aerating for 90 min and incubated for 2 h at 30°C and observed by stereomicroscopy (SZX16, Olympus Co., Tokyo, Japan). Also, stained root sections were embedded in 5 % agar and sliced and the localization of cells inside plant tissues was observed under microscope. To observe HRRK170 cells which localized on plant root by scanning electron microscope (SEM), roots were cut from seedlings, fixed by 2% glutaraldehyde and 1% osmium tetroxide, and gradually dehydrated with 50 %, 75 %, and 99.5 % ethanol and 99.0 % tertiary

butyl alcohol. Then, samples were lyophilized and coated with gold using an MSP-mini magnetron sputter prior to imaging on a Scanning Electron Microscope (Miniscope TM3030).

Calculation of HRRK170 cell density using color development by GUS staining:

GUS-labeled HRRK170 cells were and serially diluted, centrifuged and suspended in 2 mL GUS-staining solution and GUS-stained. After removing cells by centrifugation, the absorbance (OD_{615}) of the supernatant was measured using a spectrophotometer. In parallel, the cell number which was determined by plate dilution method in each diluted cell suspensions was applied for constructing correlogram for calculation of infected cell density in plant. Ten seedlings inoculated with GUS-labeled cells were GUS-stained in 20 mL staining solution, and the absorbance (OD_{615}) was measured to identify infected cell number per g weight of plant.

Evaluating plant growth promoting effect of HRRK170:

Sterilized sugar beet and vegetables seeds were sown on plug trays containing approximately 100 g soil and inoculated with 1 mL wild type HRRK170 cells suspension per 4 seeds. Seeds were covered with aluminum foil for 7 days, and then grown for three weeks. When salt (NaCl) and drought (Polyethylene glycol or PEG) stresses mitigating effect of HRRK170 was investigated using two cultivars of Chinese cabbage (Kigokoro 85 and Haregi 85), seeds were sown on 1.5 % agar plate for three days in dark condition and transferred to sterilized soil that was treated with intended concentrations of NaCI and PEG stressors, inoculated with 1 mL wild type HRRK170 cells suspension per four seedlings and grown for three weeks. Fresh and dry weight of plants were evaluated as a growth parameter.

Statistical analyses

The statistical analysis was performed using IBM SPSS Statistics for Windows v.23.0 (IBM, Armonk, NY, USA). Data were subjected to the Student's t-test. A Tukey's honestly significant difference test with post-hoc comparison at the 5% confidence level was used to compare mean values among treatments.

Results

In chapter 2, we screened six bacterial strains as bioinoculant by biochemical characteristics and stress tolerance. Among them, HRRK170 had the highest potential for plant growth promotion, given its ability to produce plant growth substances and enzymes such as siderophores and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, concomitantly with

active growth in a wider range of temperatures (10–30 °C) and pH (5.0–10.0). Previously, some strains of *Variovorax* sp. was noted as a plant growth promoting bacteria mostly due to its ACC-deaminase content. The effectiveness of plant growth-promoting bioinoculants not only depends on advantageous biochemical profile such as production of PGPP compounds, but also bacterial stress tolerance and ability to colonize roots which is an advantage to compete with other soil microorganisms and sustain beneficial interactions with the host plant in natural condition. HRRK170 strain efficiently localized on the root of sugar beet during the early interaction as visualized by microscope using GUS-labeled HRRK170 cells. Considering these beneficial traits as a bioinoculant, this bacterial strain was used for further study.

In chapter 3, HRRK170 was inoculated to vegetables and sugar beet seedlings for observation of plant tissue localization and evaluation of growth promotion. It colonized either as spots or widely on the root surface of all vegetable seedlings tested, but significant growth promotion occurred only in two vegetables (Chinese cabbage and green pepper). Inoculation of HRRK170 was inefficient or detrimental effect on plant growth (tomato, radish and lettuce). To elucidate this contradictory phenomenon, the number of HRRK170 cells infecting each plant was estimated by the absorbance value (OD_{615}) GUS-staining solution. The color development of the reaction solution is based on the production of this indigo-blue chromophore caused by enzymatic hydrolysis of X-Gluc, and its absorbance value (OD_{615}) correlated with the cell number, indicating that it could be used as a determining infected cell density to plant surface area. When HRRK170 infected to plant with low or higher density, beneficial interaction was not occurred, indicating that HRRK170 has an optimum cell density for its full function. This results showed that *Variovorax* sp. HRRK170 promoted plant growth within a certain range of cell densities.

In chapter 4, amelioration of salt and drought stress by HRRK170 was evidenced by inoculating two cultivars of Chinese cabbage seedlings. Since important PGP compounds IAA and biofilm production of HRRK170 increased under PEG and NaCl effected conditions. Efficient localization of HRRK170 on the seedlings roots grown under stressed was confirmed by GUS-staining microscopic observations. When infected cell density to seedlings in each given condition investigated using the similar technique as mentioned in above, it was indifferent other than 1% and 1.5% NaCl effected conditions, which caused higher infected cell density. Additionally, cv. Kigokoro 85 which was comparatively susceptible to stresses, gained more

benefit from inoculation of the HRRK170 as reflected by significantly improved growth under PEG effected condition, compared to the un-inoculated plants.

Conclusion and consideration

Study results suggested that HRRK170 was the most potential bioinoculant among six strains for its advantageous biochemical characteristics and higher stress tolerance. HRRK170 could function as a plant growth promoter to sugar beet vegetables. However, it has an optimum cell density for successful function as bioinoculant. The results represented in this study could be considered for efficient application of this strain. In addition, HRRK170 displayed stress mitigating effect when plant was effected by salt and drought stresses. Plants stress tolerance improved by the presence of HRRK170 cells, presumably due to efficient localization of HRRK170 on plant root and increased production of PGP compounds. Consequently, HRRK170 could be applied as bioinoculant or biofertilizer in order to facilitate plant growth and protect from damage causes by salt and drought stresses.