Short Communication

Localization of the reb operon expression is inconsistent with that of the R-body production in the stem nodules formed by *Azorhizobium cauliformans* mutants having a deletion of praR

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*Azorhizobium cauliformans*, a kind of rhizobia, has a reb operon encoding pathogenic R-body components, whose expression is usually repressed by a transcription factor PraR. Mutation on praR induced a high expression of reb operon and the formation of aberrant nodules, in which both morphologically normal and shrunken host cells were observed. Histochemical GUS analyses of praR mutant expressing reb operon-uidA fusion revealed that the bacterial cells within the normal host cells highly expressed the reb operon, but rarely produced R-bodies. On the other hand, the bacterial cells within the shrunken host cells frequently produced R-bodies but rarely expressed the reb operon. This suggests that R-body production is not only regulated at the transcriptional level, but by other regulatory mechanisms as well.

Key Words: *Azorhizobium cauliformans*; R-body; reb genes; *Sesbania rostrata*; symbiosis
multiple transcription regulators. Among them, a transcription repressor PraR directly binds to the reb promoter region and represses the reb operon expression (Akiba et al., 2010; Matsuoka et al., 2017b). Besides PraR, Lon protease and a putative TetR-type transcription factor (AZC_3265 protein) indirectly repress the reb operon expression (Matsuoka et al., 2017a; Nakajima et al., 2012).

Thus, mutants with deletion in praR, lon, or AZC_3265 genes have a highly expressed reb operon (Akiba et al., 2010; Matsuoka et al., 2017a, b; Nakajima et al., 2012). A high expression of the reb operon results in R-body production. R-bodies were observed in the DpraR and DAZC_3265 cells both in free-living and symbiotic states (Matsuoka et al., 2017a, b). The stem nodules formed by these mutants highly expressing the reb operon are defective in nitrogen fixation (Akiba et al., 2010; Matsuoka et al., 2017a, b; Nakajima et al., 2012). The host cells infected with these mutants differentiates into two types (Akiba et al., 2010). In the first type, host cells maintain oval or elongated shapes (i.e., normal shapes) like the host cells infected with the WT bacteria, but the vacuoles in these cells gradually enlarge and the bacteria gradually disappear. In the other type, host cells gradually shrink with high-density bacteria remaining inside. These observations suggested that in the former type, plant host cells kill the bacteria, while the bacteria kill the plant host cells in the latter type. Interestingly, R-bodies are frequently observed in the bacteria in the shrunken host cells, but rarely in the bacteria in the normal shaped host cells (Matsuoka et al., 2017b). This phenomenon suggests that not all these bacteria in the stem nodules express the reb operon at roughly the same levels, and we expect that bacteria in the shrunken host cells have a higher expression of the reb operon than those in the normal shaped host cells. To prove these hypotheses, we analyzed the localization of the reb operon expression and R-body production within the stem nodules formed by a praR-deleted mutant in this study.

To elucidate the localization of the reb operon expression by β-glucuronidase (GUS) assay, we generated strains harboring a reb operon-uidA transcriptional fusion (reb-uidA fusion) on each chromosome from the wild-type and DpraR strains (namely, reb-uidA WT and reb-uidA DpraR strains, respectively), as follows. The plasmids and primers for strain construction are listed in Tables 1 and 2, respectively. Two fragments containing rebR and AZC_3265 were amplified from the WT genomic DNA by PCR using PrimeSTAR HS (Takara-Bio, Shiga, Japan) and primer pairs, Acp649-Acp741 and Acp711-Acp652, respectively. A uidA fragment was also amplified from the plasmid of pFAJ1819 (Xi et al., 1999) by PCR using PrimeSTAR HS and a primer pair, Tp80-Tp81. These fragments were then linearized of pK18mobsacB

| Primer | Sequence (5'-3') | Description for underlined sequence |
|--------|-----------------|-----------------------------------|
| Tp73   | GGGTACCCGAGCTCGAATTCGTAATC | Complement with Tp73 |
| Tp74   | GGGGATCCTCTAGAGTCGACCTGC | Complement with Tp80 |
| Acp649 | TCGAGGTCGGTACCCGCACCCCTCCCCCTCAAG | Complement with Tp73 |
| Acp741 | ACTCTCTCTTATCTACGTCACCAGTGGACCTG | Complement with Tp81 |
| Acp711 | AATCAACGGTACCCCGGGTGACGGAGGAAGGAA | Complement with Tp73 |
| Acp652 | CTTTAGAGGATCCTCCCATAATTCTGGACGGCCCGTTAT | Complement with Tp74 |
| Tp80   | TAGCTAAGGAGGAGGTCCCTTATGT | |
| Tp81   | CGGGTACCCGTGATTCATGT | |

Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| A. caulinodans    |             |                     |
| ORS571 (WT)       | WT          | Dreyfus et al. (1988) |
| Anx7 (ΔpraR)      | ΔpraR       | Akiba et al. (2010) |
| Anx242 (reb-uidA) | ORS571 derivative; reb-uidA | This study |
| Anx243 (reb-uidA ΔpraR) | Anx7 derivative; reb-uidA, ΔpraR | This study |
| E. coli          |             |                     |
| S17-1 Δpir        | F- thi pro hsdR [RP4-2 Tc::Mu Km::Tn7 (Tp Sm)] (Δpir) | Simon et al. (1983) |
| Plasmids         |             |                     |
| pK18mobsacB      | Suicide vector | Schafer et al. (1994) |
| pFAJ1819         | Mini transposon vector (used as a uidA donor in this study) | Xi et al. (1999) |

Table 2. Primer used in this study.
cloned into the linearized pK18mobsacB (Schäfer et al., 1994) in the direction of the rebR, uidA, and AZC_3789 fragments using the In-Fusion cloning kit (Clontech, Mountain View, CA, USA). The linearization of pK18mobsacB was performed by inverse PCR using the PrimeSTAR Max (Takara-Bio). The resultant plasmid (pTAC186) was conjugated into the WT and ΔpraR strains (Anx7) (Akiba et al., 2010), strains via E. coli S17-1 (λpir) (Simon et al., 1983), and strains with uidA at the position immediately downstream of the rebR open reading frame were obtained after allelic exchange. The resultant strains were inoculated on the stems of S. rostrata plants, which were grown at 30°C under a 24-h light regimen. To observe the stem nodules, optical and transmission electron microscopies were carried out as described previously (Matsuoka et al., 2017b). The histochemical GUS assay was carried out as follows. Stem nodules were longitudinally sectioned and stained with X-Gluc. D. Optical microscopic observations of the stem nodules formed with reb-uidA ΔpraR strain. i. Low magnification observation. ii. High magnification observation of the normal host cells around the meristematic and infection zone. iii. High magnification observation of the oval or elongated host cells (yellow arrowheads) with expanding vacuoles (v) and shrunken host cells (red arrowheads) around the mature zone.
ple were placed in 90% acetone for 15 min, and washed with 50 mM sodium phosphate buffer (pH 7.2). The washed samples were placed in GUS-staining buffer (50 mM sodium phosphate [pH 7.2], 0.5 mM K$_3$Fe(CN)$_6$, 0.5 mM K$_4$Fe(CN)$_6$, and 0.05% Triton X-100) containing 0.25 mM sodium phosphate [pH 7.2], 0.5 mM K$_4$Fe(CN)$_6$, and 0.5 with 50 mM sodium phosphate buffer (pH 7.2). The stained samples were washed with 50 mM sodium phosphate buffer, and embedded in Technovit 7100 (Heraeus Kulzer, Wehrheim, Germany). The embedded samples were sliced into 10-μm sections with a microtome (RM-2125RT; Leica, Germany), and observed using a bright-field microscope (DMLB; Leica, Germany).

Firstly, we observed the stem nodules formed with these strains to confirm that the stem nodule phenotypes are not affected with the integration of the reb-uidA fusion. The reb-uidA WT strain formed normal stem nodules with an inner region colored red. On the contrary, the inner region of the stem nodules formed with the reb-uidA ΔpraR strain were beige or pale pink at 7 days post inoculation (dpi), and white at 12 dpi (Fig. 1A).

At an early stage of nodule development, such as 7 dpi, the process of the symbiosis breakdown caused by praR deletion is well observed in single nodules (Akiba et al., 2010; Matsuoka et al., 2017b). In the present study, we have also observed the dynamics of host-bacterium interaction in the stem nodules formed with reb-uidA ΔpraR strain at 7 dpi (Fig. 1B). Undifferentiated host cells (host cells prior to differentiation into the two types described above) were abundantly observed around a meristematic and infection zone (Fig. 1B (i)), and R-bodies were rarely observed in the bacterium inside such host cells (Fig. 1B (ii)). The oval or elongated host cells with expanding vacuoles were abundantly observed around a mature zone, and bacteria remained inside such host cells (Fig. 1B (i)). R-bodies were also rarely observed in these bacteria (Fig. 1B (iii)). Shrunken host cells were abundant around the mature zone (Fig. 1B (i)), and R-bodies were observed in many bacteria inside these host cells (Fig. 1B (iv)).

These observations of the strains harboring the reb-uidA fusion are consistent with those of the WT (ORS571) and ΔpraR strains (Akiba et al., 2010; Matsuoka et al., 2017b), respectively (Figs. 1A and B), which strongly suggest that the uidA fusion did not affect the stem nodule phenotypes.

Next, we investigated the localization of GUS activity reflecting the reb expression in the stem nodules at 7 dpi. As expected, the inner regions of the stem nodules formed with the reb-uidA ΔpraR strain were deeply stained while those with the reb-uidA WT strain were not (Fig. 1C).

The stem nodules formed with the reb-uidA ΔpraR strain were sectioned after staining, and microscopically observed (Fig. 1D). The bacteria inside the undifferentiated host cells, which were abundantly observed around the meristematic and infection zone, showed a high expression of the reb operon (Fig. 1D (i and ii)). Likewise, the bacteria inside the oval or elongated host cells with expanding vacuoles, which were abundantly observed around the mature zone, also showed a high expression of the reb operon (Fig. 1D (i and iii)). Based on the localization of the R-body production as shown in Fig. 1B, these bacteria highly expressing the reb operon are supposed to rarely produce R-bodies. On the other hand, a high expression of the reb operon was rarely detected in the bacteria inside the shrunken host cells, which were abundantly observed around the mature zone (Fig. 1D (i and iii)). The bacteria in these shrunken host cells are supposed to produce R-bodies based on the result in Fig. 1B.

Despite our initial expectation, ΔpraR bacterial cells found in the shrunken host cells, in which R-bodies accumulated, did not show a high expression of the reb operon. One of the reasons for the low expression of the reb operon might be because these bacterial cells are weakened or killed, and, thus, are unable to express the reb operon. This could explain why, as shown in our previous result, many of the bacterial cells accumulating R-bodies in the shrunken host cells have collapsed appearances (Matsuoka et al., 2017b). Why do these bacterial cells collapse? The answer is still unclear. However, it is unlikely that R-body formation within the bacterial cells leads to the breakdown and death of the bacterial cells. In fact, R-body producing ΔpraR bacterial cells in free-living states are intact (Matsuoka et al., 2017b) and the growth rates of the ΔpraR strain are not significantly different from those of the WT strain (Akiba et al., 2010).

The next question to be answered is why R-bodies were rarely detected in the ΔpraR bacterial cells in the normal shaped host cells, although they highly expressed the reb operon. Previously, we found that the ΔpraR cells under free-living conditions highly expressed the reb operon but R-bodies were observed in only about 10% of these bacterial cells (Matsuoka et al., 2017b). Thus, it could be said that even though the bacteria highly express the reb operon, they do not necessarily produce visible R-bodies. This suggests that production of R-bodies is regulated not only at a transcriptional level, but also by some other regulatory mechanisms. For example, translation or polymerization of the small proteins encoded by the reb genes might be usually repressed in most of the bacterial cells that express the reb operon. As another assumption, R-bodies might be usually scavenged by an unknown degradation pathway.

It is still unclear how bacteria kill the host cells using R-bodies and why the bacteria themselves also collapsed after producing R-bodies. Clarification of these questions will advance our knowledge of symbiosis between plants and bacteria, which have the ability to produce R-bodies.

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