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Original Paper

NAD$^+$-malic enzyme affects nitrogenase activity of *Mesorhizobium loti* bacteroids in *Lotus japonicus* nodules

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Abstract A gram-negative bacterium, *Mesorhizobium loti*, contains a NADP$^+$-malic enzyme (mlr5329) and a malate oxidoreductase (mlr0809) in the genome. We have screened transposon-induced mutants from the signature-tagged mutant library to survey their roles in nodule nitrogenase activity. The nodules induced by malate oxidoreductase mutants failed to fix N_2, although NADP$^+$-malic enzyme (NADP$^+$-ME) mutants induced nodules exhibiting no change in nodule nitrogenase activity. When malate-degrading enzyme activities were compared between malate oxidoreductase mutants and wild-type *M. loti*, NAD$^+$-malic enzyme (NAD$^+$-ME) activity was decreased significantly in malate oxidoreductase mutants, suggesting it is an NAD$^+$-ME mutant. We found that NADP$^+$-ME was not required for N_2 fixation. The fact that significant accumulations of sucrose, starch granules and malate were observed in the nodules induced by malate oxidoreductase mutant suggests that low nitrogenase activity of the nodules resulted in photosynthate accumulation. These data suggest that this malate oxidoreductase has a similar function to NAD$^+$-ME in both *Bradyrhizobium japonicum* and *Sinorhizobium meliloti.*

Key words: *Lotus japonicus, Mesorhizobium loti,* NAD$^+$-malic enzyme, NADP$^+$-malic enzyme, STM mutant.

The genus Rhizobium comprises a group of soil bacteria that form nodules on the roots and stems of legume host plants for symbiotic nitrogen fixation (Gottfert 1993). In legume nodules, rhizobia differentiate into bacteroids that are able to reduce atmospheric nitrogen to ammonia, which can be effectively used by host legume plants. Bacteroids developed in infected plant cells are surrounded by a peribacteroid membrane and form a stable metabolic unit. This structure is believed to function like an intracellular organelle, and is designated the symbiosome (Dao et al. 2008; Day and Copeland 1991; White et al. 2007). Under this symbiotic condition, bacteroids have been reported to use C_4 dicarboxylic acids as their sole respiratory substrate. For continuous supply of pyruvate to the TCA cycle, malic enzymes catalyze oxidative decarboxylation of malate to pyruvate in conjunction with the reduction of either NAD$^+$ (EC 1.1.1.38 and EC 1.1.1.39) or NADP$^+$ (EC 1.1.1.39 and EC 1.1.1.40) to NADH or NADPH, respectively (Driscoll and Finan 1996; Mitsch et al. 1998; Provorov and Tikhonovich 2003). Genetic evidence of such physiological roles of a malic enzyme in bacteroids has been reported (Chen et al. 1998; Dao et al. 2008; Day et al. 1994; Tajima et al. 1990). Driscoll and Finan (1993, 1996) showed that *Sinorhizobium meliloti*, which forms indeterminate nodules, contains both NAD$^+$-dependent malic enzyme (NAD$^+$-ME) and a NADP$^+$-dependent malic enzyme (NADP$^+$-ME) in alfalfa root nodules, and found that the NAD$^+$-dependent malic enzyme (*dme*) mutants of *S. meliloti* were Nod$^+$/Fix$^-$, whereas an NADP$^+$-dependent malic enzyme (TME)-defective mutant was Nod$^+$/Fix$^+$. Furthermore, Mitsch et al. (2007) investigated the biochemical background of *S. meliloti* bacteroids using genetic techniques to determine various types of *dme* mutants. These results showed that NADP$^+$-ME activity in nodules did not replace NAD$^+$-ME activity. In addition, *Escherichia coli* NAD$^+$-ME restored wild-type N_2-fixing activity by the *dme* (NAD$^+$-ME) mutant. In *Bradyrhizobium japonicum*, which forms determinate nodules, there was also clear genetic evidence of the significance of NAD$^+$-ME activity and the nitrogenase reaction of bacteroids, recently reported by Dao et al. (2008), who showed the role of *dme* mutant activity in bacteroids that have been identified in the soybean symbiont *B. japonicum* USDA110. Mutant analysis has shown that the *dme* mutant formed Nod$^+$/Fix$^-$. At present, it is clear that the DME in bacteroids affects nitrogenase activity in both *B.*

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japonicum and *S. meliloti* even though these nodule forms are different. To date, there have been no reports of studies on the physiological role of NAD^+-ME and $NADP^+$-ME activities in supporting the nitrogenase activity of *Mesorhizobium loti*. *M. loti* is a microsymbiont of the model legume *Lotus japonicus*. In *M. loti,* there is one $NADP^+$-malic enzyme (mlr5329) in the Rhizobase (http://genome.kazusa.or.jp/rhizobase/) of *M. loti* (Kaneko et al. 2000). Although, we did not find NAD^+-malic enzyme, we found malate oxidoreductase (mlr0809), which has a similar amino acid sequence to NAD^+-malic enzyme. There is no other malic enzyme homologous gene in *M. loti*. Hence, it is of great interest to determine whether malate oxidoreductase plays a similar role to the *dme* of *B. japonicum* USDA110 or *S. meliloti* bacteroids in soybean or alfalfa nodules under symbiotic conditions, respectively.

Transposon insertion mutants, generated using signature-tagged mutagenesis (STM), are a powerful technique that can be used to identify the genes required for symbiotic nitrogen fixation function in root nodules (Shimoda et al. 2008). We have selected STM mutants that had a transposon inserted in the malic enzyme genes from the collection of transposon mutants and studied the function of these genes.

Materials and methods

Bacterial strains and media

Transposon insertion mutants (STM mutant) of *M. loti* MAFF303099 were obtained from Kazusa DNA Research Institute (Shimoda et al. 2008). *M. loti* strains were cultured in tryptone-yeast extract (TY) liquid medium (Beringer 1974) at 28ºC. Antibiotics were added to the media in the following concentrations: spectinomycin 100 μg mL^{-1}, streptomycin 100 μg mL^{-1} and phosphomycin 100 μg mL^{-1}.

Plant materials

Seeds of *L. japonicus* B-129 Gifu (Handberg and Stougaard 1992) were sterilized with a sodium hypochlorite solution, rinsed with sterile water, and allowed to germinate in sterile vermiculite with liquid B&D medium (Broughton and Dilwoth 1971) in double Magenda jars. Then 1×10^9 cells mL^{-1} was added in the jars. These plants were grown in an artificially growth cabinet controlled at 22ºC (16 hr/8hr, light/dark).

Acetylene reduction activity

For acetylene reduction activity, plants at 35 days after infection were used. The nodules were placed in a 20 ml vial and incubated at 25ºC by adding 2.6 ml of acetylene. After 30 min, the amount of ethylene formed was measured by gas chromatography (Shimazu GC-8A) to determine ethylene production as described previously (Kouchi et al. 1989)

Light microscopy

Nodules were fixed in 4% paraformaldehyde and 25% glutaraldehyde in 0.5 M Sodium phosphate buffer (pH 7.2) and then were dehydrated by passage through a graded ethanol series, and were infiltrated with a graded butanol series. The samples were embedded in Technovit 7100 (Kulzer Histo-Technik; Heraeus Kulzer, GmbH and Co., Wehrheim, Germany). Dried semi-thin microtome sections (2 to 3-μm) were stained with 0.1% toluidine blue for light microscopic observations (Hossain et al. 2006; Suganuma et al. 2003).

Metabolite determinations and enzyme assays

Proteins were extracted from excised nodules or cells. *M. loti* cells in the late-exponential phase were harvested by centrifugation at 6,000×g. The cells were suspended in 1 ml of sterile distilled water, and immediately disrupted by sonication. The suspension was then centrifuged at 10,000×g to remove cell debris. The resultant supernatants were subjected as soluble protein extracts. 1 g of freshly nodules was transferred and homogenized with an ice-cold mortar and pestle in a four-fold of grinding buffer; 100 mM Tris-HCl (pH 7.4), 1.5 M sucrose, 500 mM EDTA (pH 8.0), 0.4 M phenylmethylsulfonyl fluoride and 50 mM dithiothreitol with 30% Polyclar VT powder. After vigorous shaking with a vortex mixture, the nodule homogenate was centrifuged at 200×g. The collected supernatant was centrifuged at 5,000×g. The pellet was used as crude bacteroids.

The activities of nodule enzymes were measured as previously described for malic enzyme, MDH (Chen et al. 1998; Dao et al. 2008) and sucrose synthase (Morell and Copeland 1985). The organic acid content of nodules was analyzed using a L7100 HPLC system. Separation of the organic acids was achieved by using an ion exclusion column (RSpsk KC-811, Shodex). Organic acids were quantified using an UV-VIS spectrum detector. The sucrose content of the nodules was analyzed using a HPLC system comprising a Model Nanospace SI-2 (Shiseido, Tokyo, JAPAN). Separation of the sucrose was achieved by using an anion exchange column (Sucrebead I, Shiseido).

Results

Screening of STM mutants of malate oxidoreductase and $NADP^+$-malic enzyme from STM library

We identified two malate oxidoreductase mutants in M. loti by screening the signature-tagged mutant library (STM) (Shimoda et al. 2008). The STM clone of STM17 or STM38 had a Tn insertion in the malate oxidoreductase gene (mlr0809) (Figure 1A). The STM clone of STM4 or STM14 had a Tn insertion in the $NADP^+$-ME gene (mlr5329) (Figure 1B).

Symbiotic and growth phenotypes of STM mutants

The symbiotic phenotypes of STM mutants were examined by inoculating *Lotus japonicus* seedlings growing under nitrogen-deficient conditions. The dry weight, nodule number, shoot and root lengths, and acetylene reduction activities of plants inoculated with the wild type and two mutants (STM4, STM14) were

similar, showing that NADP$^+$-ME mutation had no effects on N_2 fixation (Figures 2, 3). On the other hand, the dry weight of plants (Ljstm17, Ljstm38) inoculated with malate oxidoreductase mutants (STM17, STM38) showed the Fix$^-$ phenotype. Nodules in Ljstm17 or Ljstm38 were smaller (Figure 2F), and shoot and root length were shorter in comparison with plants inoculated with the wild type (Figure 2C, D). Acetylene reduction activity was significantly reduced when plants were inoculated with STM17 or STM 38 mutants (Figure 2E).

After inoculation of *L. japonicus* with the mutants and wild type for four weeks, we examined the color of the nodules. Nodules inoculated with STM17 mutants were pale pink (ca. 70%) and white (ca. 30%) nodules (Figure 3B). In contrast, all nodules formed with wild-type or Ljstm14 were pink (Figure 3C, D). Nodule sections were examined by light microscopy (Figure 3E–G). The infection appeared normal in all nodules; however, in the nodule infected with STM17 mutants, there were many more starch granules in uninfected cells than in those infected with *M. loti* or STM14 mutant, probably because nodules infected with malate oxidoreductase mutants cannot utilize photosynthesis as an energy source for nitrogen fixation. These data suggest that inoculation with the malate oxidoreductase mutant caused a significant reduction in N_2-fixing activity.

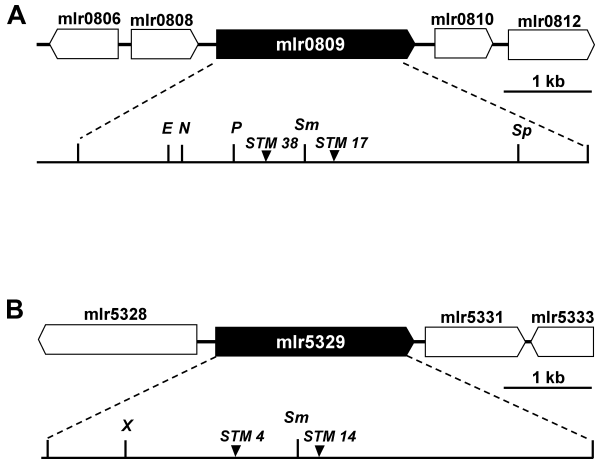

Figure 1. Delineation of the malic enzyme gene region with direction of transcription on *M. loti* MAFF303099 chromosome. Transposon (Tn5) insertions in the malate oxidoreductase genes (mlr0809) (A) and the NADP$^+$-dependent malic enzymes (mlr5329) (B) are indicated by closed arrowheads, respectively. Restriction enzyme sites are shown in (A) and (B) are: *E*, *Eco*RI; *N*, *Not*I; *P*, *Pst*I; *Sm*, *Sma*I; *Sp*, *Sph*I; *X*, *Xho*I.

Enzyme activities in M. loti and STM mutants

When three malate-metabolizing enzymes (NAD$^+$-ME, NADP$^+$-ME and malate dehydrogenase) were analyzed in culture cells and bacteroids, the NAD$^+$-ME activity of

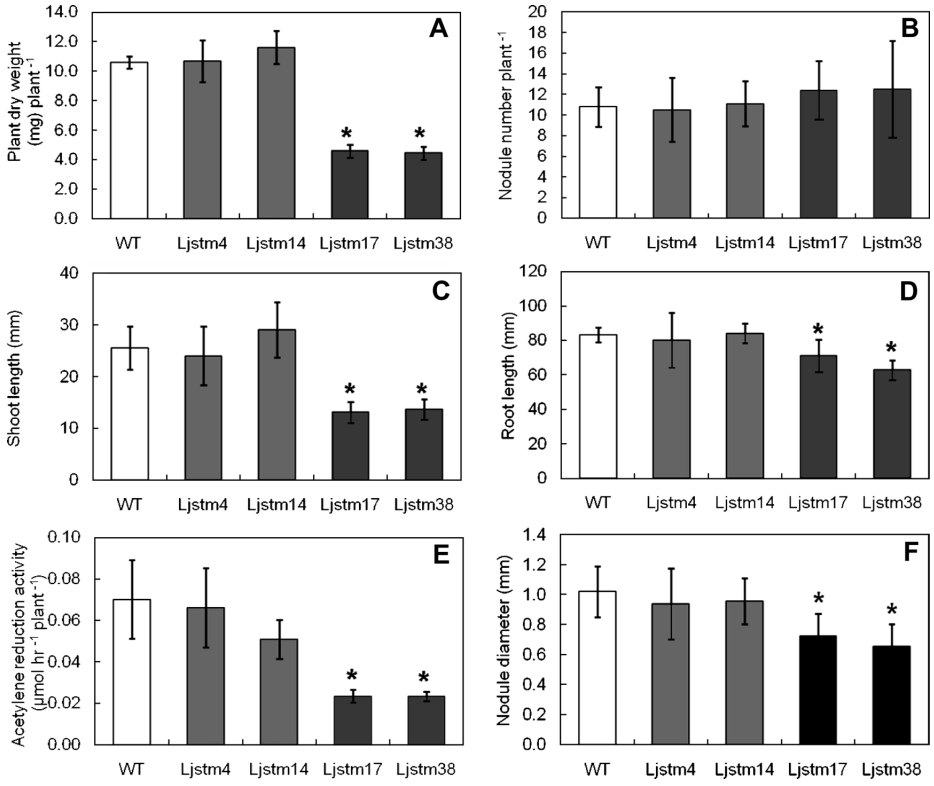

Figure 2. Acetylene reduction activity and symbiotic phenotypes of plants inoculated with various mutants and wild-type. Plant dry weight (A), nodule numbers (B), Shoot (C) and root (D) lengths, acetylene reduction activity (E), and the sizes of nodule (F) were determined 28 days after inoculation. The data represent the means±SDs of ten individual plants. Asterisks indicate significance differences from wild-type based on the LSD test ($P<0.05$).

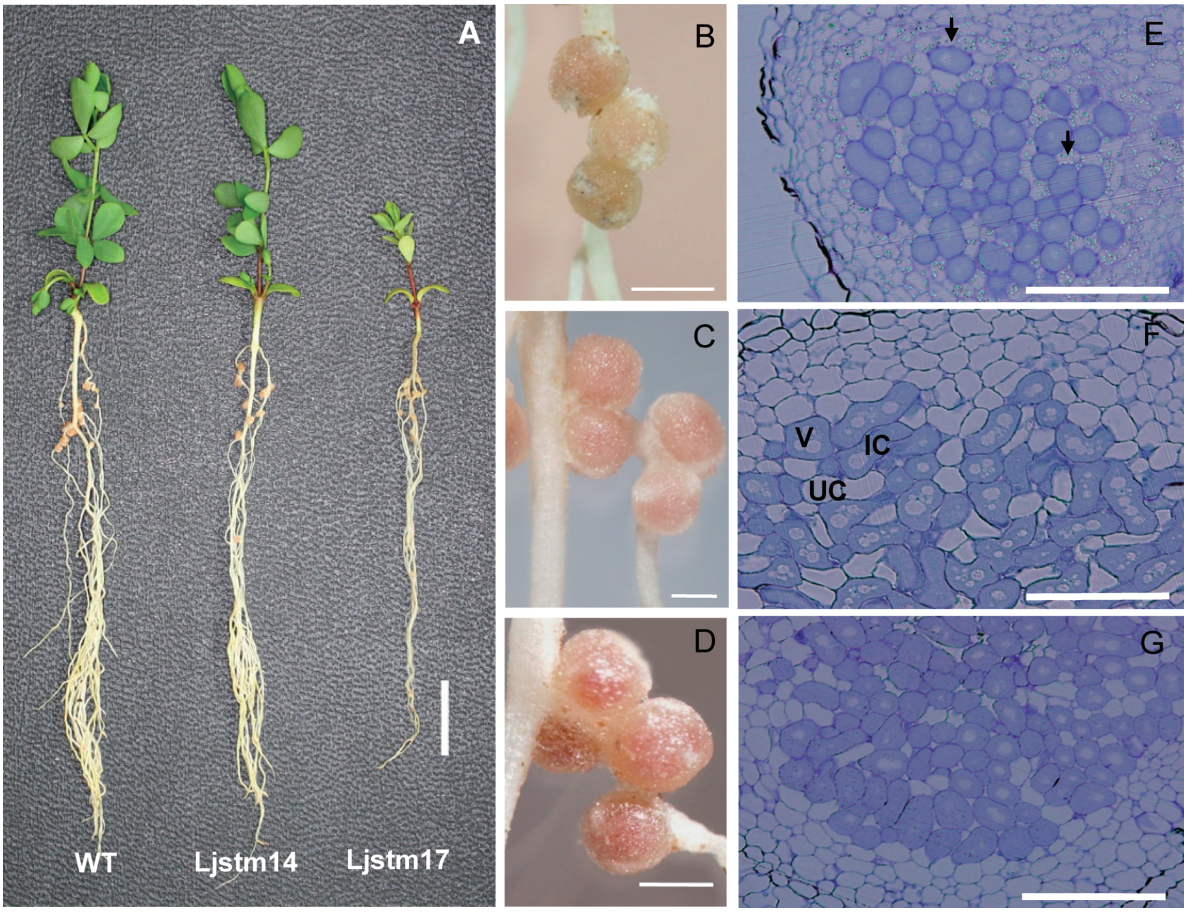

Figure 3. Growth and nodulation phenotypes of plants inoculated with *M. loti* wild-type, STM14 and STM17 mutants, respectively (From left to right). The plants were photographed 28 days after inoculation (A). Nodules on the roots induced by STM 17 (B), STM14 (C) and Wild-type (D). Light micrographs of nodules stained with 0.1% toluidine blue. A section of nodule induced by STM17(E), STM14 (F) and wild-type (G). Large numbers of starch granules were accumulated in uninfected cells in the central zone as well as in the nodule cortex (indicated by arrows). V, vacuoles; IC, infected cell; UC, uninfected cell. Bars indicate 10 mm (A), 1 mm (B–D), and 0.2 mm (E–G).

STM17 mutants decreased significantly in both free-living cells and bacteroids in comparison to *M. loti* or STM14 mutants (Table 1). We suggested that malate oxidoreductase (mlr0809) is a NAD^+-ME in *M. loti*; however, not only STM14 mutants but also STM17 mutants showed decreased $NADP^+$-ME activity in aerobically grown cells and bacteroids (Table 1). These data suggest that malate oxidoreductase (mlr0809) exhibited activity with NAD^+ as a cofactor, but this protein showed some activity with $NADP^+$.

Accumulation of metabolites in nodules inoculated with STM mutants

Chen et al. (1998) reported that most metabolic intermediates involved in the TCA cycle of *B. japonicum* A1017, except succinate, amino-n-butyrate and hydroxyl-n-butyrate, do not inhibit NAD^+-ME activity. In addition, the metabolic intermediates in glycolysis stimulated NAD^+-ME activity. To our knowledge, the activations of mechanisms by which these metabolites accumulate in either acetyl-CoA or glycolysis pathways are still unclear in *L. japonicus* nodules. We examined the effect of the STM17 mutation on the accumulation of metabolites in nodules (Figure 4). The malate, citrate and sucrose contents of the nodules were increased in Ljstm17 nodules. When we measured sucrose synthase activity in the nodules, the activity of Ljstm17 nodules was lower (156.5 ± 25.8 nmol min^{-1} mg $protein^{-1}$) than in

Table 1. Various malate metabolizing enzyme activity in free-living and bacteriods of *M. loti* MAFF303099

Strain	Specific enzyme activity (nmol min^{-1} mg $protein^{-1}$)		
	NAD-ME	NADP-ME	Malate dehydrogenase
Aerobic			
Wild type	68.8 ± 3.6	43.1 ± 5.2	925.7 ± 158.9
STM14	71.0 ± 5.5	14.2 ± 2.4	837.7 ± 30.3
STM17	12.3 ± 1.9	31.9 ± 1.8	826.5 ± 22.5
Bacteroid			
Wildtype	64.7 ± 3.0	32.1 ± 1.6	1282.3 ± 95.5
STM14	74.3 ± 6.6	23.3 ± 2.8	1273.8 ± 110.5
STM17	2.5 ± 1.1	18.1 ± 1.1	1234.6 ± 15.1

Values are means ± SDs of triplicate assays of each sample.

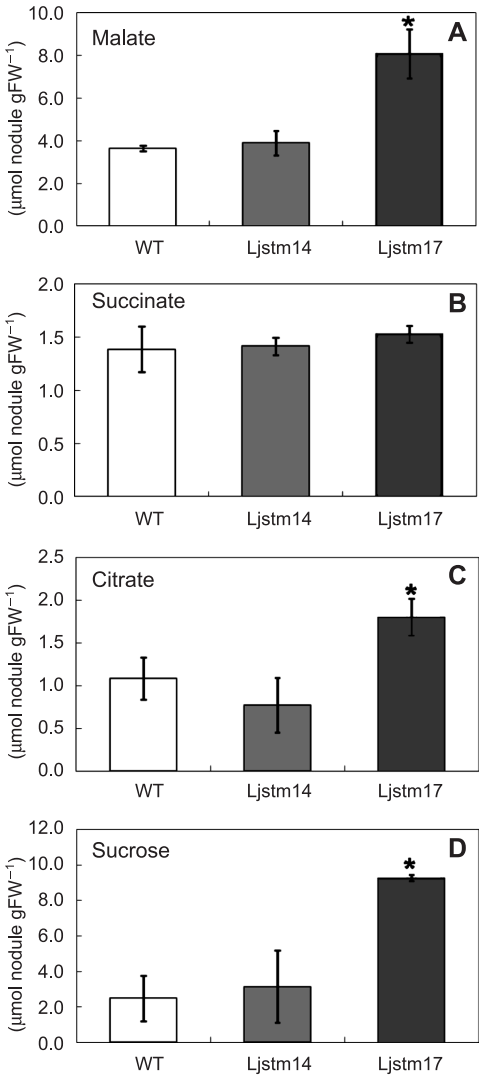

Figure 4. Organic acid and sucrose contents in *L. japonicus* nodules induced by STM14, STM17 mutants and wild-type. Amounts of malate (A), succinate (B), citrate (C) and sucrose (D) in the nodule were measured. All values are indicated as means±SDs of triplicate assays of each sample. Asterisks denote significance differences from wild-type based on the LSD test ($P<0.05$). FW, Fresh weight.

wild-type or Ljstm14 nodules (479.8 ± 5.9, 487.6 ± 39.5 nmol min^{-1} mg protein^{-1}, respectively). The decrease of sucrose synthase activity might be feedback inhibition by the accumulation of organic acids or sucrose.

Discussion

Although the physiological roles and biochemical mechanisms of malic enzymes have been reported to support nitrogenase activity in various microorganisms, such as *E. coli*, *S. meliloti* and *B. japonicum* (Dao et al. 2008; Voegele et al. 1999; Mitsch et al. 2007), in this paper, we reported whether *M. loti* STM mutants, named NADP$^+$-dependent malic enzyme mutant (STM14, STM4) and malate oxidoreductase mutant (STM17, STM38), respectively, could infect *L. japonicus*. Both STM 4 and STM 14 mutants induced nodules like the wild type, indicating that NADP$^+$-ME was not required for symbiotic N_2 fixation (Figure 3). Nitrogenase activity in Ljsym4 and Ljstm14 nodules was slightly reduced but retained more than 90% of that of nodules induced by the wild type (Figure 2E). The function of the *tme* (NADP$^+$-ME) mutant has been studied in *S. meliloti* (Driscoll and Finan 1996, 1997). The *tme* mutant in *S. meliloti* induced Fix$^+$ nodules that had a similar phenotype to *M. loti* STM4 and STM14 mutant. There is one NADP$^+$-ME gene in both *M. loti* and *S. meliloti,* but two in *B. japonicum* (Kaneko et al. 2000, 2002), suggesting that the regulation of NADP$^+$-ME expression and/or NADP$^+$-ME activity in *M. loti* may differ from that in *B. japonicum.*

The growth of malate oxidoreductase mutant showed the Fix$^-$ phenotype and the level of nitrogenase activity appeared to be significant decreased (Figure 2E). This is because of nitrogen deficiency in the plants, showing that the nodule size of the infected STM17 mutant was smaller than that of wild-type nodules (Figure 2F). Malic enzyme activity in STM17 mutants exhibited specific activity with NAD$^+$ as a cofactor, but also showed some activity with NADP$^+$ (Table 1). These results suggest that malate oxidoreductase has a fundamental role of NAD$^+$-ME that is similar to *S. meliloti*, and that NAD$^+$-ME has a crucial role in maintaining nitrogenase activity in a broad range of rhizobia in symbiotic nitrogen fixation systems. In particular, malate to pyruvate flux is required in bacteroids to maintain efficient N_2 fixation. A significant change in both sucrose and organic acid contents of the nodules was observed in Ljstm17 nodules (Figure 4). In our previous reports on the omega-cassette insertion mutant of NAD$^+$-ME in *B. japonicum*, the nitrogenase activities of nodules were reduced and the amount of sucrose and malate in nodules changed (Dao et al. 2008). NAD$^+$-ME protein in *S. meliloti* is subjected to substrate inhibition, and shows allosteric regulation by acetyl-CoA, and its activity is positively regulated by succinate and fumarate (Voegele et al. 1999). Accumulation of malate in Ljstm17 nodules might lead to the accumulation of citrate, which induces the reduction of respiratory activity. The Ljstm17 nodule contained more starch granules and sucrose than the wild type. Our data suggest that the STM17 mutant cannot utilize photosynthate as an energy source. In conclusion, it is currently clear that NAD$^+$-ME in bacteroids affects nitrogenase activity in not only *B. japonicum* and *S. meliloti*, but also *in M. loti*; however, NADP$^+$-ME did not affect nitrogenase activity. The function of NADP$^+$-ME is unclear because NAD$^+$-ME protein (mlr0809) also showed some activity with NADP$^+$ as a cofactor. In the future, we will perform physiological studies on the double mutant generated by these mutants to more precisely establish their role in symbiotic N_2 fixation.

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