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LETTER TO THE EDITOR

Exploring the Intrinsic Limits of Nitrogenase Transfer from Bacteria to Eukaryotes

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Abstract Biological nitrogen fixation is widespread among the Eubacteria and Archae domains but completely absent in eukaryotes. The lack of lateral transfer of nitrogen fixation genes from prokaryotes to eukaryotes has been partially attributed to the physiological requirements necessary for the function of the nitrogenase complex. However, symbiotic bacterial nitrogenase activity is protected by the nodule, a plant structure whose organogenesis can be trigged in the absence of bacteria. To explore the intrinsic potentiality of this plant organ, we generated rhizobium-independent nodules in alfalfa by overexpressing the *MsDMI3* kinase lacking the autoinhibitory domain. These transgenic nodules showed similar levels of leghemoglobin, free oxygen, ATP and NADPH to those of efficient *Sinorhizobium meliloti* B399-infected nodules, suggesting that the rhizobium-independent nodules can provide an optimal microenvironment for nitrogenase activity. Finally, we discuss the intrinsic evolutionary constraints on transfer of nitrogen fixation genes between bacteria and eukaryotes.

Keywords Nitrogen fixation · Evolution · Lateral transfer · Bacteria · Eukaryotes · Oxygen

Introduction

Because nitrogen is a limiting nutrient in many agricultural crops, nitrogen supply is critical in attaining yield potential (Ladha and Reddy 1995). Agriculture is using more than 100 million metric tons of nitrogen-based fertilizer annually (www.fao.org) and unfortunately, development of fertilizer nitrogen is dependent on fast-depleting non-renewable energy resources (Triplett 1996). The economic relevance of this agronomic problem has prompted the investigation of biological nitrogen fixation.

Nitrogen fixation is distributed in evolutionarily diverse phyla from Eubacteria and also present in the phylum Euryarchaeota of Archaea (Dos Santos et al. 2012). All these microorganisms, collectively known as diazotrophs, contain an enzyme complex called nitrogenase, which is able to reduce gaseous nitrogen to ammonia (Peters and Szilagyi 2006). Several studies have shown bioinformatic evidence suggesting horizontal transfer of nitrogenase among microorganisms belonging to Bacteria Domain (Kechris et al. 2006). In addition, we recently demonstrated for the first time the transfer of a functional nitrogenase between two sequenced bacterial species (Setten et al. 2013). Recombinant nitrogen-fixing *Pseudomonas* strains expressing a heterologous nitrogenase displayed high nitrogenase activity and released significant quantities of ammonium to the medium (Setten et al. 2013). Importantly, inoculation of crops with recombinant bacteria

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increased the ammonium concentration in soil and plant productivity under nitrogen-deficient conditions, showing the potential applications of nitrogenase transfer (Setten et al. 2013).

Since plants are limited in their growth rate by the accessibility of fixed nitrogen, it would be expected that nitrogen fixation genes are acquired in the plant cell via lateral transfer. However, nitrogen fixation has been incorporated into eukaryotes only through symbiotic and endophytic bacteria (Dos Santos et al. 2012). One of the most solid arguments to explain the absence of nitrogen fixation within the Eukarya domain is the incompatibility between the physiological conditions required by the nitrogenase complex and the typical microenvironment of a eukaryotic cell (Dixon et al. 1997). This is presumably because nitrogenase is irreversibly inhibited by contact with gaseous oxygen, and on the other hand, the reduction of gaseous nitrogen requires a very large amount of energy and reducing power whereas eukaryotic cells have to produce energy and reducing power by aerobic respiration (Gallon 2001). However, during symbiotic nitrogen fixation, bacterial nitrogenase is protected from damage by oxygen and receives energy and reducing power from a plant organ: the nodule (Dixon and Kahn 2004). In legume root nodules, the parenchyma contains very few and small intercellular spaces, this arrangement is responsible for blocking the diffusion of oxygen (Witty and Minchin 1986). In addition, presence of the oxygen-binding protein leghemoglobin (Lb) within the cytoplasm of nodule cells also serves to buffer free oxygen in the nanomolar range while ensuring rapid transport of oxygen to the sites of respiration (Appleby and Bradbury 1983; Ott et al. 2005). Thus, the central region of nodule has both requirements of nitrogenase microenvironment, low effective concentration of oxygen and high rate of energy generation.

It is well known that a small subpopulation of alfalfa (*Medicago sativa* L.) plants grown without nitrogen compounds can develop root nodules in the absence of bacteria, i.e. spontaneous nodules (Caetano-Anollés et al. 1991). This result suggests that leguminous plants have the capacity to produce nodules in absence of bacteria. Supporting this idea, recent works have demonstrated that the natural and/or induced mutations into autoinhibition domain from a Ca^{2+}/calmodulin-dependent protein kinase (CCaMK), named "does not make infection 3" (DMI3), is sufficient to activate nodule morphogenesis and the appropriate induction of early nodulation gene expression in the absence of bacterial elicitation in *Medicago truncatula* and *Lotus japonicas* (Gleason et al. 2006; Mitra et al. 2004; Tirichine et al. 2006). These nodules are genuine nodules with an ontogeny and morphology similar to rhizobial-induced root nodules, suggesting that symbiotic bacteria are not required for nodule organogenesis (Gleason et al. 2006; Mitra et al. 2004; Tirichine et al. 2006). Unfortunately, the characterization of DMI3-induced nodules was restricted to morphological analysis using model plants. In this article, we report the identification and characterization of alfalfa *DMI3* gene required for nodule organogenesis. Alfalfa is one of the most important forage legumes in temperate areas, and thus has a great economic relevance (Guiñazú et al. 2010). So, studying putative nitrogen fixation-related genes is important for better understanding legume-rhizobium symbiosis and would have a substantial biotechnological relevance. In this context, we explored the biochemical characteristics of nodules produced by transgenic alfalfa plants overexpressing protein kinase DMI3 without its autoinhibitory domain. Our results indicate that bacterial-independent alfalfa nodules may be a suitable microenvironment for nitrogenase activity.

Methods

For RNA isolation, total RNA was extracted by using an RNeasy Plant Mini Kit (QIAGEN Cat#74903, Germany) according to Soto et al. (2008) with very slight modifications (Online Resource 1). Full-length cDNA of the *MsDMI3* gene was amplified by PCR and this fragment was cloned into a pGEM-t easy vector (Online Resource 1). The nucleotide sequences of *MsDMI3* gene obtained here have been deposited in the EMBL Nucleotide Sequence Database Accession No.: GQ890699. *MsDMI3* with a truncated C-t autoinhibitory domain, named *MsDMI3*/1-340, was amplified by PCR (Online Resource 1) and this amplification fragment was cloned into pBI121 (AF485783). The resulting plasmid, named 35S-*MsDMI3*/1-340 (Online Resource 2), was sequenced by primer walking (Soto et al. 2011). *Agrobacterium rhizogenes*-mediated plant transformation was conducted essentially as described in Soto et al. (2011) with very slight changes (Online Resource 1). Concentrations of NADPH and $NADP^+$ were determined using the method described by Dalton et al. (1993) with slight modifications (Online Resource 1). The Lb levels in nodule soluble extracts were quantified by the pyridine-hemochrome method (Appleby and Bergersen 1980). Free-oxygen was quantified using a needle-type fiber-optic oxygen microsensor with a tip diameter of 50 μM (Ott et al. 2005).

Results

To explore the potential functions of alfalfa DMI3, a 1572-bp cDNA fragment was isolated from roots by RT-PCR using primers designed for the *Medicago truncatula* DMI3 gene (MTR_8g043970). The PCR product was cloned into a pGEM-t easy vector and sequenced. This

cDNA fragment (GQ890699) shares 98 % identity with the DMI3 kinase from *Medicago truncatula* (XP_003628124), hence it was named *MsDMI3* (*Medicago sativa* does not make infection 3). The DMI3 tree showed a complete congruence with the organismal tree, suggesting that the plant DMI3 was acquired by vertical transfer (Fig. 1). The extended distribution and high level of sequence conservation at amino acid and domain levels of DMI3 (Online Resource 3) indicate an ancient origin of this kinase, in concordance with its proposed ancient function in arbuscular mycorrhiza and rhizobium symbiosis (Gleason et al. 2006).

In further support of the vertical origin of high plant DMI3, this gene is present in the primitive vascular plant *Selaginella moellendorffii* (SM00005G06300) and the ancestral moss *Physcomitrella patens* (PP00167G00470) (Fig. 1). Since phylogenetic analysis of DMI3 was consistent with rRNA data, orthologous DMI3 assignment is possible in any land plant. Thus, the congruent pattern demonstrated that DMI3 from alfalfa (GQ890699) is indeed the ortholog of DMI3 genes from *Medicago truncatula* (XP_003628124), *Lotus japonicas* (CAJ76700) and *Oryza sativa* ssp. Japonica (NP_001055895) previously described (Chen et al. 2007; Gleason et al. 2006; Tirichine et al. 2006) (Fig. 1).

In order to obtain alfalfa nodules induced by MsDMI3 kinase, the *MsDMI3* gene without the autoinhibitory domain (*MsDMI3*/1-340) was cloned within the binary vector pBI121 and the resulting recombinant vector (35S-*MsDMI3*/1-340) was used to stably transform alfalfa roots. The release of autoinhibition from DMI3 protein was sufficient to activate nodule morphogenesis in alfalfa in the absence of bacterial elicitation, as previously reported to other species of legumes (*Medicago truncatula* and *Lotus japonicus*) (Online Resources 4 and 5). Additionally, spontaneous and nitrogen-fixing nodules were produced by nitrogen deficit condition (MS medium without nitrogen) and *Sinorhizobium meliloti* B399 (a high-efficient nitrogen-fixing bacterium) infection, respectively.

The biochemical characterization showed that nodules induced by truncated MsDMI3 had a low amount of free oxygen and high levels of Lb, ATP and NADPH, like the nodules produced by the efficient nitrogen-fixing *Sinorhizobium meliloti* B399 strain (Table 1). Contrary, the spontaneous nodules induced by nitrogen starvation showed altered levels of oxygen, Lb, ATP and NADPH (Table 1). In spite of the increase in free oxygen for oxidative phosporylation, the nodule ATP/ADP ratio was more than 6-fold lower in spontaneous nodules than in nitrogen-fixing or DMI3-induced nodules (Table 1). Probably, high concentrations of Lbs improve energy metabolism in nitrogen-fixing and DMI3-induced nodule cells, by increasing the total amount of oxygen (i.e. free plus protein bound) and thus the flux of oxygen to sites of respiration. Interestingly, the phenotype of spontaneous nodules is similar to that observed in *Medicago truncatula* when Lb expression was inhibited by RNAi. In that case, it was proposed that the low Lb level leads to increased free oxygen concentration and a decrease in both ATP/ADP and NADPH/NADP rates (Ott et al. 2005). These data show strong biochemical and metabolic plasticity of legume nodules. Importantly, our results suggest that the rhizobium-independent nodules from alfalfa could have, at least under certain conditions, a lower effective concentration of oxygen and high availability of reducing power and energy needed for the functional expression of a heterologous nitrogenase complex.

Discussion

It has been proposed that the absence of nitrogen-fixing organelles in eukaryotes results from the relative timing of

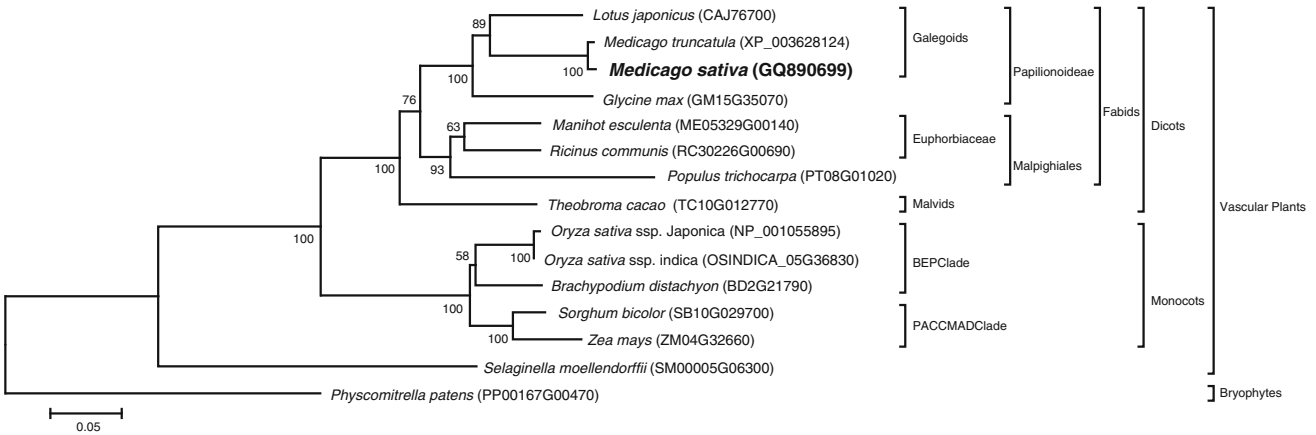

Fig. 1 Phylogenetic tree of DMI3 protein sequences. Bootstrap percentages are indicated at the branch points. In all cases, tree topologies were obtained using the NJ method and minimum evolution methods were identical

Table 1 Pigment concentration, oxygen levels and nucleotide pools in alfalfa nodules induced by nitrogen deficient conditions (spontaneous nodules), *Sinorhizobium meliloti* B399 (nitrogen-fixing nodules) or overexpression of MsDMI3/1-340 (DMI3-induced nodules)

Type of nodules	Leghemoglobin (mM)	Oxygen levels (% of air)	Nucleotide pools (nmol/g fresh weight)			
			ATP	ADP	NADPH	$NADP^+$
Spontaneous nodules	0.2 ± 0.1	17.2 ± 1.1	47 ± 11	89 ± 5	15 ± 2	23 ± 3
Nitrogen-fixing nodules	1.3 ± 0.1	3.6 ± 0.7	97 ± 7	26 ± 1	24 ± 5	11 ± 3
DMI3-induced nodules	1.4 ± 0.2	4.1 ± 0.3	93 ± 2	26 ± 3	27 ± 3	9 ± 4

All values are the mean of 4 replicates ± SD

the origin of the eukaryotic cell and the origin of biological nitrogen fixation (McKay and Navarro-González 2004). This attractive idea suggests that nitrogen-fixing microorganisms were not present at the time of eukaryogenesis. This hypothesis is difficult to test, but even if it remains true, it is not sufficient to explain the absence of nitrogen fixation in eukaryotes. This is because one of the most important mechanisms of evolution is gene transfer (Syvanen 2012), and there has not been a *nif* transfer event from bacteria to eukaryotes (Dos Santos et al. 2012). The results presented here indicate that bacterial-independent nodules could be a suitable microenvironment for nitrogenase activity. This could eliminate the restriction of an appropriate physiological eukaryotic frame for the functional nitrogenase complex. However, even disregarding this constrain, the nitrogenase enzyme is not a protein but an enzyme complex consisting of a large number of genes (Dixon et al. 1997). Although the number of genes required to form a functional nitrogenase is not known, it is estimated that at least 16 essential genes (structural and biosynthesis genes) are required for efficient nitrogen fixation. Since the probability of transfer of a function is inversely proportional to the number of genes involved in this transfer, the absence of nitrogen fixation in eukaryotes could be because this fact is extremely unlikely. In addition, bacterial *nif* genes have to adapt to eukaryotic expression (for example, the incorporation of eukaryotic promoters and terminators within *nif* genes), which makes transfer of a functional nitrogenase between domains even less likely. In this context, and given the high efficiency in plant energy production through the collection of light energy, symbiosis may have been the most-parsimonious adaptation of eukaryotes in response to selection pressure on growth under nitrogen deficiency conditions.

Conclusion

The absence of gene transfer of *nif* genes from bacteria to eukaryotes has been discussed almost since the discovery of nitrogen fixation genes, but the hypothetical constraints for nitrogen fixation in eukaryotes has not yet been analyzed in depth. In this work, we showed that bacteria-independent nodules could have the physiological requirements of the nitrogenase complex, suggesting that eukaryotic cells have the ability to produce a microenvironment adequate for a nitrogenase activity.

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