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SHORT COMMUNICATION

# Inhibition of nitrogen-fixing activity of the cyanobiont affects the localization of glutamine synthetase in hair cells of *Azolla*

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## Summary

In the *Azolla–Anabaena* association, the host plant *Azolla* efficiently incorporates and assimilates ammonium ions that are released from the nitrogen-fixing cyanobiont, probably via glutamine synthetase (GS; EC 6.3.1.2) in hair cells, which are specialized cells protruding into the leaf cavity. In order to clarify the regulatory mechanism underlying ammonium assimilation in the *Azolla–Anabaena* association, *Azolla* plants were grown under an argon environment (Ar), in which the nitrogen-fixing activity of the cyanobiont was inhibited specifically and completely. The localization of GS in hair cells was determined by immunoelectron microscopy and quantitative analysis of immunogold labeling. *Azolla* plants grew healthily under Ar when nitrogen sources, such as $NO_3^-$ and $NH_4^+$, were provided in the growth medium. Both the number of cyanobacterial cells per leaf and the heterocyst frequency of the plants under Ar were similar to those of plants in a nitrogen environment ($N_2$). In hair cells of plants grown under Ar, regardless of the type of nitrogen source provided, only weak labeling of GS was observed in the cytoplasm and in chloroplasts. In contrast, in hair cells of plants grown under $N_2$, abundant labeling of GS was observed in both sites. These findings indicate that specific inhibition of the nitrogen-fixing activity of the cyanobiont affects the localization of GS isoenzymes. Ammonium fixed and released by the cyanobiont could stimulate GS synthesis in hair cells. Simultaneously, the abundant GS, probably GS1, in these cells, could assimilate ammonium rapidly.

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## Introduction

*Azolla*, a genus of aquatic ferns distributed widely in tropical and temperate regions of the world, harbors the cyanobacterium *Anabaena azollae* in specialized cavities formed in its leaves (Figure 1). The cyanobiont in mature leaves fixes atmospheric nitrogen and releases it into the leaf cavity as ammonium ions (Peters and Meeks, 1989). The host plant efficiently incorporates and assimilates these ions via the glutamine synthetase (GS)/glutamate synthase (GOGAT) cycle (Meeks et al., 1987). In higher plants, GS is a major enzyme responsible for ammonia assimilation. Plants have two types of GS isoenzymes that are localized to different compartments: one in the cytosol (GS1) and the other in plastids/chloroplasts (GS2). Distinct roles for GS1 and GS2 have been suggested in a number of studies on organs, tissues, and developmental stages (Cren and Hirel, 1999; Ireland and Lea, 1999; Tobin and Yamaya, 2001). GS2 in leaves primarily re-assimilates ammonia released during photorespiration. GS1 is expressed differentially in various plant organs. In leaves, GS1 is scarce but is expressed abundantly in vascular tissues, particularly in the phloem companion cells. GS1 is suggested to play a role in nitrogen transport and translocation. In roots, GS1 facilitates the assimilation of ammonium taken up from the soil. From legume root nodules, nodule-specific or nodule-enhanced GS has been isolated. Analyses of the expression of GS genes have shown that the expression is regulated by symbiotically fixed nitrogen (Hirel et al., 1987; Miao et al., 1991; Carvalho et al., 2000). This isoenzyme is responsible for the rapid assimilation of ammonium excreted by the nitrogen-fixing bacteroids.

In the *Azolla*–*Anabaena* association, the cyanobiont can provide the host plant with its entire requirement of nitrogen. Thus, in this association, like in legume–Rhizobium associations, a mechanism for unidirectional and efficient nitrogen transport from the cyanobiont to the host plant is thought to be present. Specialized cells, called hair cells, protruding into the leaf cavity have been postulated to play an important role in transferring the fixed nitrogen (Peters and Meeks, 1989). Recently, it was demonstrated by immunoelectron microscopy using an anti-*Azolla* GS2 antibody that abundant labeling of GS was occurring not only in chloroplasts but also in the cytoplasm of hair cells of *Azolla* (Uheda et al., 2004). In hair cells of cyanobiont-free plants, the labeling of GS in both chloroplasts and the cytoplasm was very weak compared with cyanobiont-containing plants. These findings suggest that ammonium released by the cyanobiont is assimilated by the abundant GS found in hair cells and then transferred to other tissues and that the existence of the cyanobiont stimulates GS synthesis in hair cells. The ammonium as well as other substance(s) released by the cyanobiont and/or the cyanobiont itself may be involved in this scenario. However, exactly how the cyanobiont controls GS synthesis is unclear. In order to clarify the regulatory mechanism underlying nitrogen assimilation and GS synthesis in hair cells, the present study attempted to determine whether inhibition of the nitrogen-fixing activity of the cyanobiont affects GS localization in hair cells. Thus, *Azolla* plants were grown in an argon environment (Ar) under which the nitrogen-fixing activity of the cyanobiont was inhibited specifically and completely, and the localization of GS in hair cells of *Azolla* under Ar was determined by immunoelectron microscopy and a quantitative analysis of immunogold labeling.

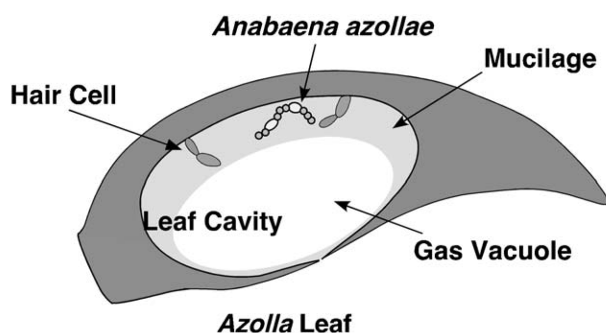


**Figure 1.** Schematic illustration of an *Azolla* leaf. The cyanobiont, *Anabena azollae*, is located in a mucilage layer of the peripheral region of the enclosed cavity. Hair cells protrude into the mucilage layer of the cavity. A gas vacuole is located centrally in the cavity.

## Materials and methods

*Azolla filiculoides* Lamarck was surface-sterilized using 10% $H_2O_2$ and 0.01% Tween 20 for 15 min. The tips were picked, washed with sterilized distilled water, and cultured aseptically in a 500-mL Erlenmeyer flask that contained 200 mL of culture medium supplemented with 5 mM MES (pH 6.0). Plants were transplanted monthly. The growth conditions and the composition of the culture medium have been described previously (Maejima et al., 2002). For examination, exponentially growing plantlets of *Azolla* (100 mg) were transferred aseptically to a new 500-mL Erlenmeyer flask (total volume 600 mL) containing 200 mL of the culture medium supplemented with or without

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