

## Analysis of Stem Nodule from *Sesbania rostrata* Using a New Visualization Technique of Three-Dimensional Reconstruction

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### Abstract

A three-dimensional (3-D) analysis technique was developed to visualize the whole nodule and Nitrogen fixation ( $N_2$ -fix) zone in a stem nodule from *Sesbania rostrata* infected with symbiotic rhizobium, *Azorhizobium caulinodans* ORS571. The reconstructed  $N_2$ -fix zone of the stem nodule was analyzed by arranging the ratio of opacity, by cutting at vertical and level direction, and by extraction in a personal computer. The  $N_2$ -fix zone was a ring shape, surrounding the vascular bundle. This is the first report that shows whole distribution of  $N_2$ -fix zone in *S. rostrata* stem nodule.

**Key words:** *Sesbania rostrata*, stem nodule, 3-D reconstruction.

*Sesbania rostrata* is an annual, fast-growing legume that is found in tropical climates and flooded soils of Africa, where it engages in symbiotic nitrogen fixation with *Azorhizobium caulinodans* (Dreyfus and Dommergues, 1981). *S. rostrata* is one of the few legumes that form nodules on both stems and roots. The formation of stem nodule by *A. caulinodans* is induced following crack-entry infection at the base of dormant root primordia, which are present in vertical rows along the stem (Tsien *et al.*, 1983). Nitrogen fixation ( $N_2$ -fix) is detected as early as 3 days following inoculation and a high rate of fixation is observed (Ndoye *et al.*, 1994). These features make *S. rostrata* suitable for green manure in rice field.

Among leguminous plants, two major types of nodules can be distinguished based on the presence of a persistent meristem. Determinate type nodules are round nodules, lacking persistent meristem, and associated with *Lotus japonicus* and soybean. Indeterminate type nodules are oval with persistent meristem, and associated with alfalfa, clover, and vetch (Munoz *et al.*, 1996). Nodule in *S. rostrata* appears to be intermediate between indeterminate and determinate types. Initially, nodule differentiation is comparable to that of indeterminate nodules, with the youngest meristematic cells located at the periphery and the  $N_2$ -fixing cells located at the nodule center. Two weeks after inoculation, meris-

tematic activity decreases, and nodules show the typical histology of determinate nodules (Ndoye *et al.*, 1994; Goormachtig *et al.*, 1997; Corich *et al.*, 1998). To observe the 3-D distribution of the organization in addition to the ordinary two-dimensional observation is an effective way. Therefore, 3-D reconstruction was developed to analyze *S. rostrata* nodules.

Serial sectioning methods with computer aid were applied to measure the 3-D physical structure of the central nervous system of a simple animal (Levinthal and Ware, 1972) and plant cells (Tuohy *et al.*, 1987). Recently, 3-D visualization of compound distributions for protein, starch, and lipid in a grain of brown rice (*Oryza sativa* L.) was developed (Ogawa *et al.*, 2000; Ogawa *et al.*, 2001). This 3-D imaging technique is a powerful way to analyze the complicated structure of nodules. In this study, whole and  $N_2$ -fix zone of stem nodule from *S. rostrata* were visualized and analyzed three-dimensionally.

Seeds of *Sesbania rostrata* were surface sterilized in 96%  $H_2SO_4$  for 90 min, and washed four times in sterile distilled water. Seeds were germinated on 1% agar water medium and incubated at 28 °C in the dark condition for three days. Seedlings were transferred to pots containing Norris medium, pH 7.0 (Vincent, 1970), and cultivated at 28 °C under 16 h photoperiod for 1 month. *A. caulinodans* ORS571,

grown at 37 °C in YEB medium (Fernandez-Lopez *et al.*, 1998), was inoculated on the adventitious root of *S. rostrata*. Nitrogen-fixation (N<sub>2</sub>-fix) zone in a 2-week old nodule was reddish, indicating the presence of leghemoglobin (**Fig. 1**).

Stem nodules, after 2 weeks inoculation, were harvested and processed for embedding. Nodules were fixed in a solution of 0.25% glutaraldehyde and 4% paraformaldehyde with 0.05 M Na-P buffer (0.05 M Na<sub>2</sub>HPO<sub>4</sub>, 0.05 M NaH<sub>2</sub>PO<sub>4</sub>, pH 7.2). Following aspiration for 30 min, the nodules were kept at 4 °C overnight. Samples were dehydrated and embedded in paraffin according to Angerer and Angerer (1991), and subsequently stored at 4 °C until sectioning.

Sequential sectioning (12 μm) of embedded nodules was carried out on a microtome (Leica RM2145). The obtained 62 sequential sections from a single nodule were transferred to an adhesive tape that is made of polyester, and coated with a solvent-type acrylic resin as an adhesive material (Ogawa *et al.*, 2000).

To deparaffinize the samples for staining, the nodule sections on the adhesive tape were transferred to 100% xylene for 30 min (two times), 100% xylene : 100% Ethanol = 1:1 for 10 min, and a graded ethanol series (from 100 to 80%) for 10 min each. Fast green staining (0.5% fast green FCF with 90% ethanol) was done for 1 min and the samples were washed with 90% ethanol for 10 min two times, and dehydrated in 90% ethanol to 100% xylene. The temperature during the entire procedure was 25 °C.

Infection cells inside the stem nodule were stained blue by fast green FCF (**Fig. 2A, B**). Same observation is shown in the nodules of *Lotus japonicus* and clover (M. Abe, personal communication). After 2 weeks inoculation, mature nodules of *S. rostrata* is filled with bacteroids that fixed nitrogen in infection cells (Tsien *et al.*, 1983; Ndoye *et al.*, 1994). N<sub>2</sub>-fix zone of the 26th section image was oval shape (**Fig. 2A**), however section of the 35th section showed two round shapes separated with a vascular bundle, which existed in the center of stem nodule (**Fig. 2B**; Tsien *et al.*, 1983). This shape of nitrogen fixation zone depended on the position that cut the stem nodule of *S. rostrata*. Morphology of N<sub>2</sub>-fix zone was neither a round shape as seen in determinate nodules nor an oval shape as observed in indeterminate nodules, therefore the morphology is different from determinate and indeterminate type nodules.

The 2-D image analysis of stem nodules is limited to whole N<sub>2</sub>-fix cells because of different shapes at the position of cutting (**Fig. 2A, B**).

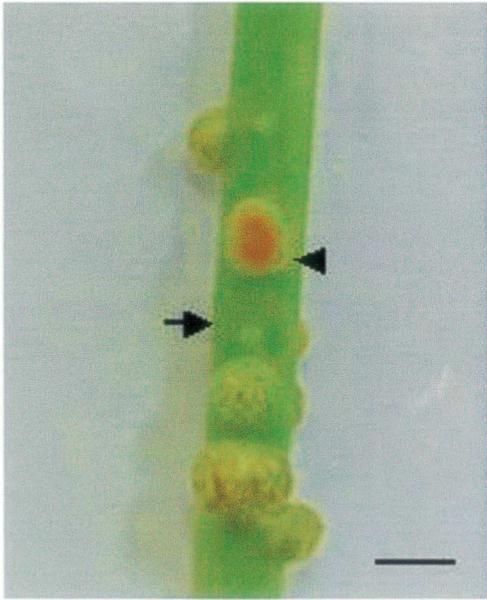
Therefore, 3-D image by reconstruction was needed and applied to observe the whole stem nodule and N<sub>2</sub>-fix cells in the nodule.

The 2-D digital images were converted to 24-bit RGB scale for 3-D reconstruction in a personal computer. The obtained 2-D digital images were reconstructed to produce a 3-D data set by the volume rendering method, which can be plotted on the display for 3-D visualization, using the software Voxel Viewer (Toshiba Machine Co. Ltd.). Therefore, the voxel data can represent the position of nitrogen fixation zone in a reconstructed nodule.

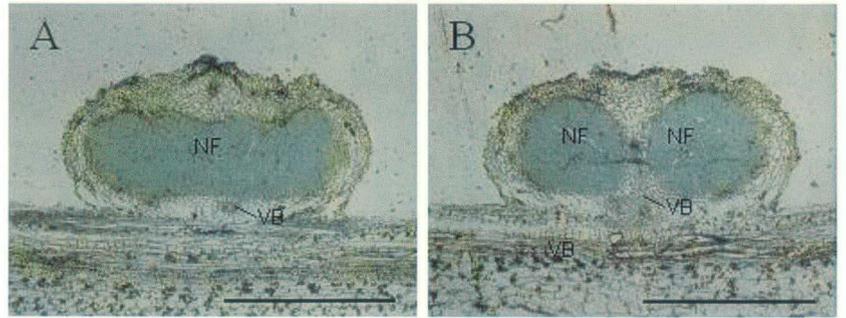
Sixty-two 2-D images of sequential stem nodule sections composed by pixel data were adjusted on the position and color. These 2-D images were piled up to produce a 3-D image of the stem nodule (**Fig. 3A-D**). For clear 3-D representation, an opaque algorithm, which can set up the opacity ratio of the 3-D plotting image to allow the backside to shine through the front side, was applied to the 3-D data set. The ratio of the opacity was 3/10 in this image.

The 3-D plotting image of the compound distribution of a stem nodule was shown in **Fig. 3A**. The 3-D stem nodule of this opacity showed the surface of nodules similar to those taken on a scanning electron microscopy. To visualize the N<sub>2</sub>-fix zone of the stem nodule, the reconstructed stem nodule was cut at the vertical and level direction (**Fig. 3C**). The distribution of N<sub>2</sub>-fix zone could be observed in all directions. To visualize the internal N<sub>2</sub>-fix zone in uncut condition, the ratio of the opacity was set at 3/100. The N<sub>2</sub>-fix zone was shown as light blue (**Fig. 3B**). Because of the unique form of the meristem, *Sesbania* stem nodules develop uniformly around a central axis (Ndoye *et al.*, 1994). Furthermore, only N<sub>2</sub>-fix zone was colored with blue using image-processing software (Photoshop 5.5™, Adobe System, Inc.) and reconstructed (**Fig. 3D**). From the results that the vascular bundle existed in the center of stem nodule (**Fig. 2B**) and 3-D observation, the zone was surrounding the vascular bundle and had a ring shape like a doughnut. Determinate type nodule and indeterminate type nodule form globular N<sub>2</sub>-fix zone (Munoz *et al.*, 1996). As the result of 3-D reconstruction, N<sub>2</sub>-fix zone of *Sesbania* stem nodule was clearly different from that of determinate type nodule and indeterminate type nodule. This suggests that the stem nodule of *S. rostrata* could be classified as a new type of nodule.

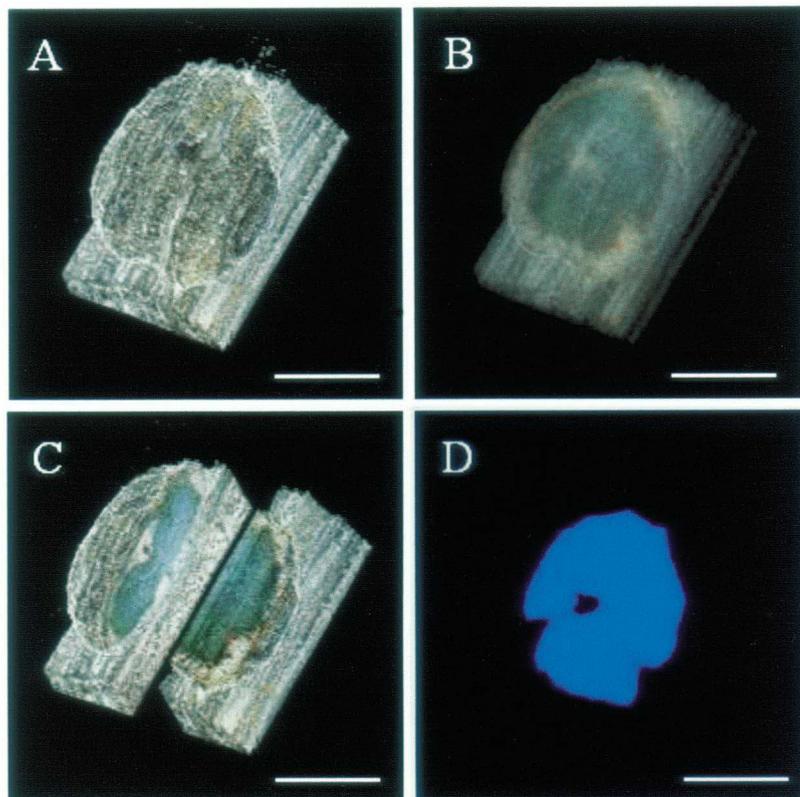
This is the first report to show the whole distribution of N<sub>2</sub>-fix zone in the stem nodule of *S. rostrata*. This advanced 3-D technique could be applied to observe the infection process of rhizo-



**Fig. 1** Stem nodule of *Sesbania rostrata* 2 weeks after inoculation with *Azorhizobium caulinodans* ORS571. Arrow shows root primordia and arrowhead indicates the cut stem nodule. Bar = 2.0mm



**Fig. 2** Two-dimensional images of stem nodules. (A) the 26th section image of all sequential sections; (B) the 35th section of all sequential sections. NF, nitrogen fixation zone; VB, vascular bundle. Bar = 1.0mm



**Fig. 3** Three-dimensional reconstruction images of stem nodules. (A) surface of reconstructed stem nodule; (B) whole stem nodule by more transparent condition; (C) divided image from **Fig. 3A**; (D) extracted nitrogen fixation zone. NF, nitrogen fixation zone. Bar = 1.0mm

bium into nodule, and the formation of vascular bundles in nodule and nodule primordia. In addition, this technique might be useful for the study of more complicated organs such as flower and shoot apical meristem.

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