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XYLEM COLONIZATION OF TOMATO BY *AZORHIZOBIUM CAULINODANS* ORS571*

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Tomato seedlings growing aseptically in Murashige and Skoog Medium were inoculated with *Azorhizobium caulinodans* ORS571 (pXLGD4), carrying the *lacZ* reporter gene. By microscopic analyses of inoculated tomato roots, it has been demonstrated that the xylem of tomato roots can be colonized by *Azorhizobium*. We discuss whether this colonization of the xylem of tomato roots by diazotrophic azorhizobia might provide a suitable niche for endophytic nitrogen fixation.

Keywords: Tomato – *Azorhizobium caulinodans* – xylem colonization

INTRODUCTION

Our finding that the xylem of the roots of the legume *Sesbania rostrata* is colonized when inoculated with *Azorhizobium caulinodans* ORS571 [14] has led us to investigate whether the xylem of the roots of other non-legumes could also be colonized by this nitrogen-fixing (diazotrophic) bacterium. If so, colonized xylem might provide a non-nodular niche for endophytic nitrogen fixation in various non-legume crops such as rice and tomato and in the model plant *Arabidopsis thaliana*. It seems logical that nitrogen-fixing bacteria such as ORS571 located within the plant in the xylem (endophytic diazotrophs) will be more likely to fix N_2 and transfer fixed N to the host plant with much enhanced efficiency than diazotrophs occurring outside the plant in the plant rhizosphere. Rhizobia, such as *Azorhizobium caulinodans*, appear to secrete their own pectinases and cellulases to facilitate their entry into the root system by crack entry invasion, that is, bacteria enter intercellularly between adjacent cells; there is no need to add extra cell-wall degrading enzymes known to remove barriers to rhizobial-host specificity in legumes [4].

Recent experiments, using ORS571 (pXLGD4) tagged with the *lacZ* reporter gene, along with microscopic histochemical analyses of sections of roots of inocu-

*Dedicated to Professor Lajos Ferenczy on the occasion of his 70th birthday.

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lated aseptically grown seedlings, have shown that the xylem of rice [8] and *Arabidopsis* [3] roots is colonized by ORS571. Plants inoculated with ORS571 did not exhibit disease symptoms, suggesting that a non-pathogenic interaction occurs. In tomato seedlings inoculated with the bacterial wilt pathogen *Ralstonia solanacearum* microscopic studies demonstrated intercellular infection and protoxylem invasion of tomato roots by this bacterium [17] and significantly also by a non-pathogenic strain of this bacterium [7].

In this study, the ability of *A. caulinodans* ORS571 to colonize the xylem of tomato seedlings has been investigated. Bacteria were tagged with the *lacZ* gene so that any bacteria in the xylem could be visualized and positively identified. In this way colonization of the xylem of both primary and lateral roots of tomato plants by ORS571 has been detected and conditions established for the vascular colonization of tomato seedlings grown aseptically under controlled conditions and inoculated with ORS571 (pXLGD4). Whether this colonization of the xylem of tomato roots by azorhizobia could provide a suitable niche for endophytic nitrogen fixation is discussed.

MATERIALS AND METHODS

Plant growth

Tomato seeds variety Ailsa Craig were surface sterilized in 15% (v/v) Domestos bleach (Lever Industrial Ltd, Runcorn, UK) for 15 min. They were rinsed with sterile water and shaken in sterile water for 3 days at 28 °C in the dark to remove germination inhibitory materials and to initiate germination. Germinated seeds were transferred aseptically to 50 ml of hormone-free Murashige and Skoog medium [13] (3% w/v sucrose) solidified with 0.8% (w/v) agar (Sigma, Poole, UK) in 175 ml screw-capped Powder Round glass jars (Beatson Clarke, Rotherham, UK), either with or without naringenin (Sigma) at 5×10^{-5} M. The flavonoid naringenin is the aglucon of naringen, which is found in the fruits of grapefruit trees. The roots of the germinating tomato seeds were pushed into the sterile agar using forceps and incubated in the screw-capped glass jars under Daylight fluorescent tubes (37 μ EM^{-2} S^{-1} illuminance) in a growth room (25 °C day, 22 °C night) with a 16-hour photoperiod.

Inoculation with Azorhizobium caulinodans *ORS571*

After 4 days growth in jars, seedlings (one per jar) were inoculated with 0.5 ml (10^8 ml^{-1}) of *Azorhizobium caulinodans* ORS571 (pXLGD4) cultured in TGYE liquid medium [15]. Strain ORS571 (pXLGD4) can be visualized by the blue pigment resulting from degradation of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) by the *lacZ*-encoded β-galactosidase [1]. Uninoculated controls were inoculated with 0.5 ml of sterile water. After inoculation plants were grown in the growth room for a further 11 days.

Detection of azorhizobial β-galactosidase activity

Plants, both uninoculated and inoculated, were fixed in 2% v/v glutaraldehyde in 0.1 M sodium phosphate buffer pH 7 with vacuum infiltration for 30 min and incubation at 28 °C for 1 h to inactivate endogenous plant β-galactosidase activity [16], prior to thorough rinsing in buffer only and incubation with X-Gal [2].

Microscopic examination

After overnight incubation with X-Gal, plants were washed 3 times in 0.1 M phosphate buffer and the intact root system examined microscopically to detect regions with blue pigment. No blue pigment was seen in the controls (uninoculated). Because of the transparency of the young root system it was possible to observe the xylem directly by placing the roots under a cover-slip. Root explants were processed for sectioning and further light microscopic observations as described previously [6].

RESULTS

Blue pigment resulting from the degradation of X-Gal by the *lacZ*-encoded b-galactosidase was visible in the xylem of *Azorhizobium* ORS571 (pXLGD4) inoculated tomato plants. Blue bands of xylem colonized by ORS571 were readily detected by direct microscopic examination of intact roots (Fig. 1). No blue bands were detected in the uninoculated controls. Tomato plants inoculated with ORS (pXLGD4) in the presence of 5×10^{-5} M naringenin also showed the presence of blue bands of xylem colonized by ORS571. No blue bands were detected in the uninoculated controls, containing 5×10^{-5} M naringenin. Approx. 5% (n = 90) of tomato plants inoculated showed blue bands in the xylem, either with our without naringenin.

To further confirm the presence of blue histochemically stained bacteria of *Azorhizobium caulinodans* in the xylem elements of the root vascular system of inoculated tomato plants, regions of the roots with blue pigment were dehydrated through a graded ethanol series and embedded in LR White resin (The London Resin Co., Basingstoke, UK). Sections for light microscopy (1 μm thick) were observed following staining with dilute Safranin in water (approx. 0.001%). This enabled detection of the blue regions of bacteria and pink stained cell walls of the lignified xylem elements when sections were examined at high magnification using oil immersion objectives. The colonization of the xylem by the azorhizobia was clearly visible (Fig. 2).

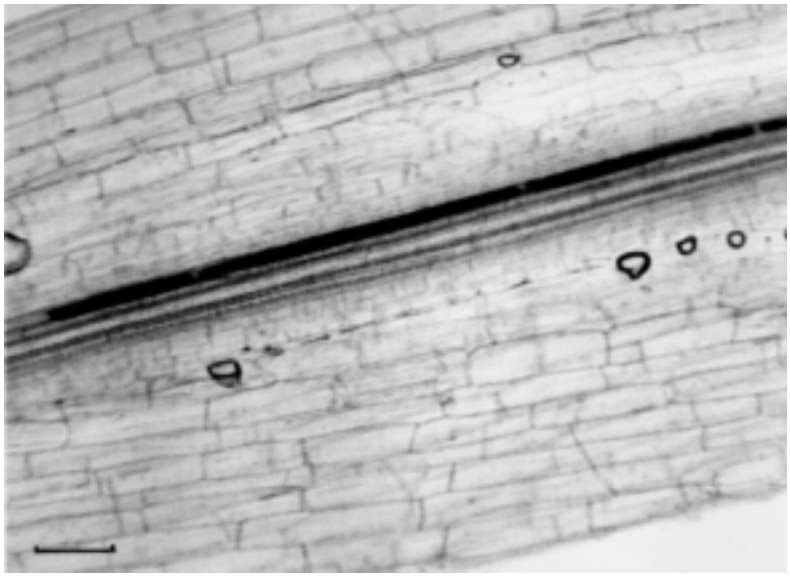

Fig. 1. Colonization of the xylem of tomato 11 days after inoculation with ORS571 (pXLDG4). Root treated with X-Gal (direct observation) showing a blue band in the xylem. Bar = 50 μm

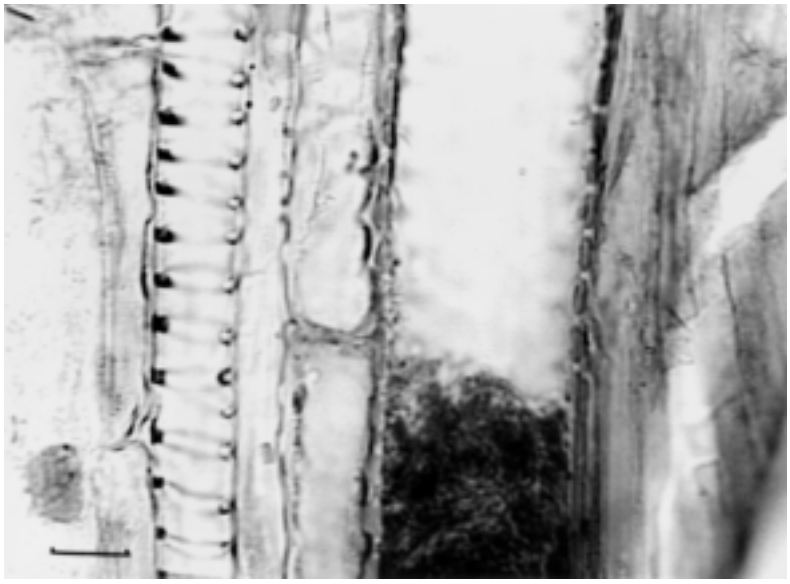

Fig. 2. Colonization of the xylem of tomato 11 days after inoculation with ORS571 (pXLDG4). Section of a root (as in Fig. 1) showing blue histochemically stained bacteria in the xylem. Bar = 10 μm

DISCUSSION

This is the first report that *Azorhizobium caulinodans*, which is a rhizobial strain forming nitrogen fixing root and stem nodules on the legume *Sesbania rostrata*, will colonize the xylem of the roots of tomato plants. Xylem colonization of the roots of *S. rostrata* by azorhizobia has been observed [14] and also xylem colonization of rice [8] and *Arabidopsis* [3]. However, because of the extensive use of tomato in plant physiological and plant microbe-interaction investigations a more detailed quantitative study of the factors influencing xylem colonization and of the pathways involved will be particularly timely. A large number of plant species exhibit xylem colonization by bacteria without disease symptoms and xylem colonization is increasingly being seen as a common aspect of plant-microbe interactions [10]. Interestingly, in this study no adverse effects of inoculation and xylem colonization were observed in the inoculated plants as compared with the uninoculated controls.

At present little is known as to how bacteria such as azorhizobia reach and invade the xylem. Previous studies have shown that diazotrophic bacteria, including ORS571, have an ability to internally colonize the cortex of root systems by entry at lateral root cracks. It has also been shown that specific flavonoids promote intercellular root colonization [9]. The non-rhizobial diazotroph *Acetobacter diazotrophicus* has been shown to penetrate sugarcane roots intercellularly at the root tip and at cracks in lateral root junctions and to colonize xylem vessels following inoculation of aseptically grown plants [11]. It has been suggested that xylem elements are possible sites of nitrogen fixation by diazotrophs such as azorhizobia, since xylem elements could provide the low pO_2 and a site for exchange of metabolites necessary for nitrogen fixation. Interestingly, *Azorhizobium caulinodans* ORS571 is able to fix nitrogen in the free living state without differentiation into bacteroids in up to 3% oxygen [12]; this report of tomato root xylem colonization by ORS571 may be a first step towards achieving non-nodular nitrogen fixation with a non-legume such as tomato of benefit to its growth and development. As I have recently discussed, energy and environmental concerns arising from the overuse of nitrogenous fertilizers have highlighted the need for plants, especially non-legumes, to obtain more of their nitrogen from the air by biological nitrogen fixation [5].

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