Specific Flavonoids Promote Intercellular Root Colonization of *Arabidopsis thaliana* by *Azorhizobium caulinodans* ORS571

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The ability of *Azorhizobium caulinodans* ORS571 and other diazotrophic bacteria to internally colonize roots of *Arabidopsis thaliana* has been studied. Strains tagged with *lacZ* or *gusA* reporter genes were used, and the principal colonization sites were found to be the points of emergence of lateral roots, lateral root cracks (LRCs). High frequencies of colonization were found; 63 to 100% of plants were colonized by ORS571, and 100% of plants were colonized by *Herbaspirillum seropedicae*. After LRCs were colonized, bacteria moved into intercellular spaces between the cortical and endodermal cell layers. Specific flavonoids, naringenin and daidzein, at 5×10^{-5} M, significantly promoted colonization by ORS571. By using a *nodC* and a *nodD* mutant of ORS571, it was shown that neither Nod factors nor NodD are involved in colonization or flavonoid stimulation of colonization. Flavonoids did not appear to be stimulating LRC colonization by their activity as nutritional factors. LRC and intercellular colonization by *H. seropedicae* was stimulated by naringenin and daidzein at the same concentration that stimulated colonization by ORS571.

Additional keywords: nitrogen fixation, *nod* genes, rhizobia.

The extension of biological nitrogen fixation to nonlegume crops would be of enormous economical and environmental value. One of the major strategies envisaged to achieve this is the establishment of an effective association between nonlegume plants and diazotrophic bacteria. From analysis of what is needed for such an association to be effective in terms of benefits to the plant, it appears that only those systems in which the diazotrophic bacteria grow and fix nitrogen within the plant can be effective (Quispel 1991). In such associations, there can be intimate metabolic exchange with the plant, and bacteria are protected from competition with other microorganisms of the rhizosphere. The most efficient systems of endophytic biological nitrogen fixation are the rhizobia-legume and *Frankia*-woody actinorhizal plant symbioses, in which bacteria fix nitrogen within specialized plant organs,

called nodules, resulting in considerable assimilation of fixed nitrogen by host plants (Mylona et al. 1995; Benson and Silvester 1993). In comparison, associative diazotrophs such as *Azospirillum* spp. colonize mainly the surfaces of plant roots, where they are in competition with other rhizosphere microorganisms, and very little of the biologically fixed nitrogen benefits the plant (Okon 1985). Associative diazotrophs have been found and characterized on roots of several of the most important nonlegume crops, such as wheat, maize, and rice. Because of the inefficiency of such associations, however, it would be beneficial to establish endophytic nitrogen-fixing associations in these cereals.

An endophytic association has recently been described in certain Brazilian varieties of sugarcane, which have been growing for many years without added nitrogenous fertilizer. In these varieties, large populations of endophytic diazotrophs, in particular *Acetobacter diazotrophicus* and *Herbaspirillum* spp., have been found (Döbereiner et al. 1993). These bacteria may colonize xylem vessels (James et al. 1994) and may be the most important contributors in sugarcane to the observed high levels of biological nitrogen fixation (Boddey et al. 1995). This indicates that endophytic biological nitrogen fixation is possible in nonlegume plants, and the example of sugarcane suggests the approach to adopt in order to achieve this in other nonlegume crops, that is, to study whether diazotrophic bacteria can internally colonize these plants. This strategy entails studying the ability of diazotrophs i) to enter nonlegume plants and ii) to multiply to sufficient levels and within appropriate plant tissue for nitrogen fixation.

Among cereals, rice has been adopted as a model monocot in studying the potential for extending endophytic biological nitrogen fixation to these plants (Ladha and Reddy 1995). For nonlegume dicots, *Arabidopsis thaliana* is an obvious choice. This model plant is easily amenable to genetic analysis and is currently by far the best plant for use in identifying and studying the role of genes involved in a given plant response.

For the choice of diazotrophic bacteria, rhizobia are good candidates because of their capacity for efficient nitrogen fixation in planta and their ability to infect root tissue of legumes, both intercellularly and intracellularly. In addition, genetic analysis of rhizobia has led to the characterization of numerous genes involved both in interactions with plants and in nitrogen

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fixation (Dénarié et al. 1996; Long 1996; Fischer 1994), and effective nitrogen-fixing nodules can be formed by certain rhizobia on species of the nonlegume genus *Parasponia* (Webster et al. 1995). However, while rhizobia are well known for their ability to induce nodules and form symbiotic associations with leguminous plants, relatively little is known about whether they can form any type of association with nonlegumes other than *Parasponia* spp. Reports have been made that certain rhizobia can enter maize, wheat, rice, and oilseed rape, but at low frequency (Cocking et al. 1992, 1994).

Among the rhizobia that have been tested is *Azorhizobium caulinodans* ORS571, which induces stem and root nodules on the tropical legume *Sesbania rostrata* (Dreyfus et al. 1988). These nodules are induced after crack entry infection; i.e., bacteria enter intercellularly between adjacent cells and not by the formation of infection threads at the tips of root hairs (de Bruijn 1989). Within the nodules, bacteria are differentiated into bacteroids and fix nitrogen (Ndoye et al. 1994), but ORS571 can also fix nitrogen without differentiation into bacteroids under free-living conditions. Moreover, ORS571 is able to fix nitrogen at relatively high concentrations (up to 12 μ M) of dissolved oxygen (de Bruijn 1989). These are unusual characteristics among rhizobia and may be useful in the context of extending biological nitrogen fixation to nonlegume plants. Other diazotrophic bacteria that are possible candidates include *Herbaspirillum seropedicae*, recently found growing endophytically in sugarcane (Boddey et al. 1995).

In this study, we investigated the ability of *A. caulinodans* ORS571 and other diazotrophic bacteria to internally colonize roots of *A. thaliana*. Bacteria were tagged with reporter genes so that we could visualize and positively identify the bacteria found associated with plant roots. In this way, we have shown reproducible, high-frequency, internal colonization at the points of emergence of lateral roots of *A. thaliana* by ORS571. We propose that *A. thaliana* can therefore be used as a model plant to study interactions between diazotrophic bacteria and nonlegume dicots in the same way that this plant is currently being exploited in the genetic analysis of plant-pathogen interactions (Davis and Hammerschmidt 1993). Colonization by ORS571 was found to be significantly stimulated by specific flavonoids at low concentrations independently of *nod* genes. The same flavonoids were also found to significantly stimulate colonization of *A. thaliana* by *H. seropedicae*.

RESULTS

Intercellular colonization by *A. caulinodans* ORS571 at lateral root cracks of *A. thaliana*.

The broad-host-range plasmid pXLGD4 was transferred into *A. caulinodans* ORS571. pXLGD4 carries a constitutively expressed fusion between the promoter of the *hemA* gene of *Rhizobium meliloti* and the *lacZ* gene of *Escherichia coli* (Leong et al. 1985). This fusion has been successfully used for studying the symbiotic behavior of *R. meliloti* mutants (Ardourel et al. 1994). Strain ORS571(pXLGD4) can be visualized on whole roots 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. Inoculated, histochemically stained roots of *A. thaliana* were found to be colonized on the surfaces of root tips and inside lateral root cracks (LRCs), the natural fissures at the bases of lateral

roots resulting from lateral root emergence. Root tip colonization was not subsequently studied because it was less intense and less reproducible than LRC colonization. The reproducibility of LRC colonization was exploited later in developing the scoring system, as was the characteristically highly localized nature of LRC colonization. In fact, there was typically very little surface colonization along the root system, except at the points of emergence of certain lateral roots. LRCs were colonized at an early stage of lateral root formation, sometimes when the emerging lateral root was only just visible (Fig. 1A and B). Then, as these lateral roots developed, bacteria moved more deeply into the cracks and from there into intercellular spaces between the cortex and endodermis in the main root (Fig. 1C and D). Intercellular colonization was confirmed by electron microscopy (Fig. 1E and F). Two sites of colonization can therefore be distinguished at the points of emergence of lateral roots: LRC colonization, mainly observed with young lateral roots, and intercellular colonization, seen at older lateral root emergence sites.

These observations indicate that LRCs of *A. thaliana* provide points of entry into the root system for ORS571. Unlike the response to pathogen root infection, however, no obvious signs of plant defense were seen. No signs of plant cell wall degradation were visible at the electron microscopic level, and no accumulation of callose or phenolic compounds could be detected (data not shown). Occasionally, epidermal or endodermal cells were entirely filled with bacteria, indicating that such cells were no longer viable. No other evidence of intracellular colonization was found, nor was evidence found of bacteria in the vascular system, although this was studied only 1 and 2 weeks after inoculation. Finally, *A. thaliana* plants that had been inoculated with ORS571(pXLGD4) did not show any obvious differences in root morphology compared with uninoculated control plants.

Quantification of LRC colonization.

To analyze LRC colonization in more detail and, in particular, to compare bacterial strains and mutants as well as plant mutants, it was necessary to develop a quantitative assay. This was possible since LRC and intercellular colonization were reproducible and occurred at higher frequencies than in previous reports, probably because of the sensitivity of the *lacZ* reporter gene to visualize bacteria. For purposes of quantification, LRC and intercellular colonization were grouped together as LRC colonization, since they can be considered different stages of the same process. Between 63 and 100% of *A. thaliana* plants were found to have LRCs colonized by ORS571(pXLGD4), and those plants that showed LRC colonization averaged one or two sites per plant. A more detailed scoring system was developed, which consisted of counting the colonized LRCs (any visible signal was counted as positive) and expressing this as a percentage of the total number of LRCs per plant (counting one LRC per lateral root, there were an average of 15 to 20 LRCs per plant). The mean result for four independent experiments was that 10.8% of LRCs were colonized per plant.

Optimization of the LRC colonization bioassay.

The levels of LRC colonization of *A. thaliana* by ORS571(pXLGD4) were compared 1 and 2 weeks after inoculation,

and no significant difference was found (data not shown). Because under the experimental conditions used (16 h of light per day), *A. thaliana* started to produce flowers 2 weeks after inoculation (at which time the plants were 3.5 weeks old),

longer time periods were not tried. However, in order to delay the onset of flowering, experiments were performed in a growth room with shorter day lengths (9 h of light per day). Flowering was thus delayed by 1 week, but the level of LRC

Fig. 1. Light (A–D) and electron (E and F) microscopy of colonization of *Arabidopsis thaliana* by *Azorhizobium caulinodans* ORS571(pXLGD4). A–D, Lateral root crack (LRC) colonization sites visualized by histochemical staining of bacterial β -galactosidase activity (blue color). Bacteria can be seen entering the LRC mainly on the left of the lateral root in A and on both sides in B. In C and D, bacteria can be seen inside at the bases of the lateral roots (intercellular colonization). Bars = 50 μ m in A and 100 μ m in B–D. E and F, Bacteria in the intercellular space between cortical (left in each photo) and endodermal (right) cells. Bars = 1 μ m.

colonization progressively decreased for time points longer than 2 weeks after inoculation (data not shown). The developmental process of flowering is known to be a drain on photosynthesis and plant reserves, which may affect the level of bacterial colonization that can be supported by the root system. All subsequent experiments were scored 1 week after inoculation on plants grown with 16 h of light.

Several ecotypes of *A. thaliana* were tested (Col-0, Wei-0, Ct-1, Be-0, Ts-1, Sf-2, Ws-0, and Nd-0) to determine whether there was any variation among ecotypes in LRC colonization by ORS571(pXLGD4). Since no significant differences were found in the level of LRC colonization among the different ecotypes (data not shown), the widely used ecotype Columbia (Col-0) was chosen.

The possible effect of nitrate on the level of colonization was considered, because combined nitrogen is known to inhibit infection and nodulation in rhizobia-legume symbioses. *A. thaliana*, because of its small seed size, is particularly dependent on a source of nitrogen, such as nitrate, and when no nitrate was added to the medium, plants did not grow well. In addition, without added nitrate, there was abundant surface colonization of the root system by ORS571(pXLGD4). This can be explained by the fact that increased levels of root exudates are produced by plants under stressful conditions, such as growth in the absence of nitrate (Clarkson 1985), which would favor bacterial colonization. Of the tested nitrate concentrations, there was no significant difference in the level of LRC colonization between the 0, 0.25, and 1 mM nitrate treatments (Fig. 2A). Although the development of the plants was better at 2.5 mM than at 0.25 mM, the level of LRC colonization was lower, and 0.25 mM nitrate was chosen for all subsequent experiments.

Stimulation of LRC colonization by specific flavonoids at low concentrations.

To find out whether the same plant signals that induce rhizobial *nod* genes (and by doing so, start the process of infection in rhizobia-legume associations [Dénarié et al. 1992]) are also involved in rhizobia-nonlegume interactions, we investigated whether flavonoids could affect the level of LRC colonization of *A. thaliana* by ORS571(pXLGD4). For this, flavonoids were incorporated into the plant growth medium, and LRC colonization was quantified. One of each of the four families of flavonoids shown to have *nod* gene-inducing activity (Dénarié et al. 1992), luteolin (flavone), naringenin (flavanone), myricetin (flavonol), and daidzein (isoflavone), was tested. Acetosyringone, a plant phenol that induces virulence genes of *Agrobacterium tumefaciens*, was also tested. Of these compounds, naringenin and daidzein at 5×10^{-5} M were both found to significantly ($P = 0.01$) stimulate LRC colonization (Fig. 2B). None of the other molecules had any significant effect on the level of colonization, and none of the tested compounds had any significant effect on the number of lateral roots present per plant (data not shown).

Nod factors are not involved in LRC colonization.

In rhizobia-legume symbioses, the induction of rhizobial *nod* genes by flavonoids leads to the production of specific lipo-chitooligosaccharides, the so-called Nod factors. These in turn act as signals to the plant and are required for infection, nodulation, and the control of host specificity (Dénarié and Cullimore 1993). Since naringenin is one of the most efficient inducers of *nod* genes in ORS571 (Goethals et al. 1989), we investigated whether flavonoids were stimulating LRC coloni-

Fig. 2. Comparison of lateral root crack (LRC) colonization of *Arabidopsis thaliana* by *Azorhizobium caulinodans* ORS571(pXLGD4) in the presence of different compounds added to the plant growth medium. **A**, Effect of different nitrate concentrations on LRC colonization. **B**, Effect of flavonoids and acetosyringone on LRC colonization. Compounds were added at 5×10^{-5} M. **C**, Effect of succinate, naringenin, and daidzein on LRC colonization. Compounds were added at 10^{-5} M (white columns) or 5×10^{-5} M (black columns). For all graphs, values with the same letter are not significantly different at $P = 0.01$, and analysis of variance was conducted with Fisher's test (Snedecor and Cochran 1980). Data (including standard deviation values) are representative of several (A) or the average of two (B and C) independent experiments. Sixteen plants were scored per treatment in each experiment.

zation of *A. thaliana* via the production of Nod factors. A *nodC* mutant of ORS571 (Geelen et al. 1993) was used for this assessment. *nodC* is a structural gene involved in the synthesis of the core chitooligosaccharide backbone of Nod factors (Geremia et al. 1994; Kamst et al. 1995). The *nodC* mutant was just as able as the wild type to colonize LRCs of *A. thaliana*, and this colonization was still stimulated by naringenin and daidzein (Fig. 3A).

Flavonoid stimulation of LRC colonization is not mediated by NodD.

The flavonoid induction of rhizobial *nod* genes is mediated by the regulatory NodD proteins. These proteins are activated by specific flavonoids, and once they are activated, they induce the transcription of *nod* operons (Dénarié et al. 1992). It cannot be excluded that activated NodD proteins could induce the transcription of genes other than *nod* genes, and we considered this possibility by testing a *nodD* mutant of ORS571 (Goethals et al. 1990). Although many rhizobia contain multiple copies of *nodD*, ORS571 has only a single copy. Quantification of LRC colonization by the *nodD* mutant showed that this strain was just as able to colonize as the wild type and, moreover, that LRC colonization by the *nodD* mutant was still stimulated by naringenin and daidzein (Fig. 3B).

Flavonoids have no detectable effect on growth of ORS571.

We considered that naringenin and daidzein might be acting as carbon sources and compared LRC colonization in the presence of these flavonoids and in the presence of succinate, an appropriate carbon source for ORS571 (Dreyfus et al. 1988). In spite of the fact that daidzein contains nearly four times more carbon than succinate does, daidzein at 10^{-5} M significantly ($P = 0.01$) stimulated LRC colonization, while succinate at 5×10^{-5} M did not (Fig. 2C). This indicates that daidzein is not stimulating LRC colonization of *A. thaliana* via a simple growth effect.

Another possible explanation was that low concentrations of naringenin and daidzein were increasing the growth rate of ORS571. It has been reported, for example, that luteolin at

10^{-5} M can reduce the doubling time of *R. meliloti* by a factor of five and that this effect is independent of *nodD* genes (Hartwig et al. 1991). However, we were unable to show any effect of low concentrations of flavonoids on the growth rate of ORS571 (data not shown).

Bacteria were isolated from inoculated plants grown in the presence or absence of flavonoids, and their numbers, as well as their associated β -galactosidase activity, were determined. No significant differences were found in either bacterial counts or associated β -galactosidase activity between treatments. In addition, inoculation of *A. thaliana* plants with 10 times as much inoculum of ORS571 did not reproducibly increase the level of LRC colonization and had no effect on the level of LRC colonization in the presence of flavonoids (data not shown).

Finally, it was verified that naringenin and daidzein at 5×10^{-5} M had no effect on the level of β -galactosidase activity exhibited by the strains ORS571(pXLGD4), ORS571::*nodC*-(pXLGD4), or ORS571::*nodD*(pXLGD4) grown in pure culture.

Flavonoid stimulation of LRC colonization by *H. seropedicae*.

First, in order to find out whether another species of rhizobia could colonize LRCs of *A. thaliana*, *R. meliloti* (pXLGD4) was tested (Table 1 and Materials and Methods). The level of colonization of *A. thaliana* by *R. meliloti* was not significantly different from that of ORS571 (data not shown). Given that LRC colonization is independent of *nod* genes, we tested non-rhizobial diazotrophic bacteria: two strains of the plant-associated diazotroph *Azospirillum brasilense* and an endophytic diazotroph, *H. seropedicae* (Table 1), containing a *lacZ* and a *gusA* reporter gene construct, respectively (Materials and Methods). Neither strain of *A. brasilense* was able to colonize *A. thaliana* better than ORS571 did (data not shown), but plants inoculated with *H. seropedicae* had a level of LRC colonization (23.6%) approximately twice that of plants inoculated with ORS571 (10.8%), and 100% of plants were colonized by *H. seropedicae*. All of the tested bacteria colonized LRCs of *A. thaliana* in preference to other root sites, and after LRC

Fig. 3. Comparison of lateral root crack (LRC) colonization of *Arabidopsis thaliana* in the presence of naringenin and daidzein by **A**, the wild type and *nodC* mutant of ORS571 and **B**, the wild type and *nodD* mutant of ORS571. All strains contained the plasmid pXLGD4. Compounds were added to the plant growth medium at 5×10^{-5} M. Control = black columns; naringenin = hatched; and daidzein = white. For both graphs, values with different letters differ significantly at $P = 0.01$, and analysis of variance was conducted with Fisher's test (Snedecor and Cochran 1980). Data (including standard deviation values) are representative of several independent experiments. Sixteen plants were scored per treatment in each experiment.

colonization, bacteria appeared to colonize intercellular spaces adjacent to LRCs (shown for *H. seropedicae* in Figure 4). After 2 weeks, none of these bacteria had progressed farther into the roots than the area immediately adjacent to the LRCs.

H. seropedicae, the best colonizer of the newly tested bacteria, was chosen to test the effect of flavonoids on LRC colonization. We tested the same flavonoids that had been tested on ORS571, as well as acetosyringone, and found that naringenin and daidzein at 5×10^{-5} M again significantly ($P = 0.01$) stimulated LRC colonization (Fig. 5). In the presence of naringenin or daidzein, LRC colonization by *H. seropedicae* reached very high levels; approximately 60% of the LRCs were colonized per plant. Luteolin and myricetin did not affect the levels of ORS571 colonization, but in the presence of acetosyringone, *H. seropedicae*, unlike ORS571, colonized LRCs of *A. thaliana* at a significantly lower level.

DISCUSSION

In this paper, we have presented data showing the reproducible, high frequency, intercellular root colonization of *A. thaliana* by *A. caulinodans* ORS571 and *H. seropedicae*. The use of the *lacZ* and *gusA* reporter gene systems facilitated detection of bacteria at points of emergence of lateral roots in the same way that reporter gene fusions have proved very effective as a sensitive means of detecting both bacteria, as well as bacterial gene expression, in numerous other plant-microbe interactions. The visualization of rhizobial colonization of nonlegume plants by more traditional methods, such as sectioning, probably explains the previously reported low frequencies of colonization (Cocking et al. 1992, 1994).

LRCs of *A. thaliana* were the preferential first sites of colonization by all the bacteria tested. By scanning electron microscopy, LRCs of *A. thaliana* have been shown to be fissures in the otherwise smooth root surface (Dolan et al. 1993), and this, together with the presence of root exudates, probably makes them suitable niches for bacterial colonization. Sites of lateral root emergence are also among the primary sites of colonization of wheat by *A. brasilense* (Vande

Broek et al. 1993), of sugarcane by another diazotrophic bacterium, *Acetobacter diazotrophicus* (James et al. 1994), and of tomato by the pathogen *Ralstonia solanacearum* (Vasse et al. 1995). In a parallel study on an important cereal crop, wheat, a similar frequency of LRC and intercellular colonization by ORS571 was reported (Gough et al. 1996; Webster et al., in press). The demonstration of quantifiable intercellular rhizobial colonization in different nonlegume plants, including a monocot and a dicot, represents an important advance in studies of interactions between diazotrophic bacteria and nonlegume plants. In particular, these results overcome the principal bottlenecks that existed previously in this field, i.e., the rarity of reported internal colonization and the difficulty in obtaining reproducible and quantitative data.

By showing that *A. thaliana* can be colonized by diazotrophic bacteria, we have shown that this model dicot can be used for studying interactions between diazotrophs and nonlegume plants. Numerous resources that use *A. thaliana* for genetic and molecular genetic studies can therefore be exploited. The monocot rice is also being used to study interactions between diazotrophic bacteria and nonlegume plants (Cocking et al. 1992; Ladha and Reddy 1995). Rice is one of the most important nonlegume crops in the world, and one of the strategies envisaged to achieve the higher rice yields needed to feed the ever-increasing world population is to extend biological nitrogen fixation to rice (Khush and Bennett 1992). Among cereals, rice has a relatively small genome, and the order of genes is well conserved among major cereal genomes, providing good genetic reasons to use rice as a model monocot (Shimamoto 1995).

For nonlegume dicots, one of the main advantages of *A. thaliana* as a model plant is the ability to use molecular genetics to identify and understand the mechanisms by which plant genes are involved in these interactions. Numerous mutants of *A. thaliana* have now been characterized, so the role in colonization of plant secondary metabolites or plant growth regulators, for example, can be studied by using appropriate mutants (Shirley et al. 1995; Chan et al. 1993; Boerjan et al. 1995). Another advantage of *A. thaliana* is the detailed knowl-

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Characteristics	Reference
Bacterial strain		
<i>Azorhizobium caulinodans</i>		
ORS571	Wild type, Nod ⁺ Fix ⁺ on <i>Sesbania rostrata</i>	Dreyfus et al. 1988
ORS571::nodC	nodC ⁻ Nod ⁻ Fix ⁻ on <i>S. rostrata</i>	Geelen et al. 1993
ORS571::nodD	nodD ⁻ Nod ⁻ Fix ⁻ on <i>S. rostrata</i>	Goethals et al. 1990
<i>Rhizobium meliloti</i>		
RCR2011	Wild type, Nod ⁺ Fix ⁺ on <i>Medicago sativa</i>	Rosenberg et al. 1981
<i>Azospirillum brasilense</i>		
SP7	Wild type, isolated from <i>Digitaria decumbens</i> rhizosphere soil	Baldani et al. 1987
SP245	Wild type, isolated from surface-sterilized wheat roots	Baldani et al. 1987
<i>Herbaspirillum seropedicae</i>		
Z67	Wild type, isolated from rice roots	Baldani et al. 1986
Plasmid		
pXLGD4	pGD499 prime (IncP) carrying a <i>hemA-lacZ</i> fusion	Leong et al. 1985
PLA-lacZ	pLA2917 (IncP) carrying a <i>kan-lacZ</i> fusion	Arsène et al. 1994
pCAM121	mTn5SgusA21 (<i>Paph-gusA-trpA</i> transcriptional terminator translational fusion) in pUT/mini-Tn5	Wilson et al. 1995
pRK2073	Helper plasmid for mobilization of IncP and IncQ plasmids	Leong et al. 1982
pRG290-12::T20	IncP plasmid containing a Tn3-HoHo1 <i>lacZ</i> fusion with <i>nodA</i> of <i>Azorhizobium caulinodans</i>	Goethals et al. 1989

edge we have of the cellular organization of its roots, including the root development pattern and the precise number, position, and morphology of root cells (Dolan et al. 1993, 1994). This should be an important help in describing intercellular colonization of roots of nonlegume dicots, as well as in describing any effects that intercellular colonization could have on roots. Thus, the same features of *A. thaliana* currently being exploited in the molecular genetic analysis of plant-pathogen interactions should also enable rapid progress in understanding the mechanisms involved in nonlegume-diazotroph interactions.

Specific flavonoids at low concentrations were found to significantly stimulate LRC colonization of *A. thaliana* by ORS571. By using *nod* mutants of ORS571, it was shown that neither Nod factors nor NodD are involved in colonization or the flavonoid stimulation of colonization. Flavonoids were also found to stimulate colonization by *H. seropedicae*, which supports this conclusion, as does the fact that daidzein stimulates colonization but does not induce *nod* genes of ORS571 (Goethals et al. 1989). Naringenin also stimulates LRC colonization of wheat by ORS571 independently of *nod* genes (Gough et al. 1996; Webster et al., in press). Different mechanisms therefore appear to be operating in the flavonoid induction of nodulation in legumes and the flavonoid stimulation of

colonization of nonlegumes. However, while nodulation of *S. rostrata* is Nod factor dependent, it has recently been observed that crack entry is not (K. O'Callaghan and E. C. Cocking, *unpublished*). A *nodC* mutant of ORS571 is, in fact, able to form intercellular pockets of bacteria in the cortex of *S. rostrata*, although the number of crack entry sites is lower than that of the wild type. The fact that crack entry by rhizobia into both nonlegumes and host legume plants is independent of Nod factors could explain the low specificity of this phenomenon regarding the range of nonlegume plants for which it can be observed.

With the present evidence, we cannot determine whether plants or bacteria are the targets of the flavonoid effect. In this respect, both the colonization of *A. thaliana* by two different bacteria (ORS571 and *H. seropedicae*) and the colonization of two different plants (*A. thaliana* and wheat) can be promoted by flavonoids. Certain differences were also noted: daidzein stimulates colonization of *A. thaliana*, but not of wheat, by ORS571 (Webster et al., in press) and acetosyringone decreases the level of colonization of *A. thaliana* by *H. seropedicae*, but not by ORS571 (this work).

In considering that bacteria may be the target of the flavonoid effect, the possible direct role of flavonoids as bacterial carbon sources or growth factors was examined. Rhizobia

Fig. 4. Light microscopy of colonization of *Arabidopsis thaliana* by *Herbaspirillum seropedicae* Z67(pCAM121) visualized by histochemical staining of bacterial β -glucuronidase activity (blue color). **A**, Lateral root crack colonization and **B–D**, intercellular colonization. Bars = 50 μ m in **A** and 100 μ m in **B–D**.

have been shown to be able to catabolize *nod* gene-inducing flavonoids, which may be degraded to be used as carbon and energy sources or to detoxify them (Rao and Cooper 1994), and low concentrations of flavonoids can increase the growth rate of *R. meliloti* (Hartwig et al. 1991). Two lines of evidence, however, suggest that the specific flavonoids do not stimulate LRC colonization by their activity as nutritional factors: i) succinate at five times the concentration of daidzein did not promote colonization, and ii) flavonoids at 5×10^{-5} M did not detectably increase the growth rate of ORS571. Other characterized responses of bacteria to flavonoids, such as the inhibitory effect on bacterial growth of phytoalexins (Dakora and Phillips 1996) and the chemo-attractant effect of certain flavonoids (Caetano-Anollés et al. 1988), do not provide satisfactory explanations either.

Despite this, there is evidence that bacterial traits are involved. In particular, ORS571 and *H. seropedicae* do not respond in the same way to acetosyringone, and *H. seropedicae* colonizes *A. thaliana* much better than ORS571 does. The fact that colonization is *nod* gene independent does not exclude the fact that naringenin and daidzein could be inducing other bacterial genes by a NodD-independent mechanism. The existence of such genes in *Rhizobium* sp. NGR234 has been suggested (Fellay et al. 1995), and a recognition process in *Bradyrhizobium japonicum* is specifically activated by isoflavonoids and does not involve *nodD* genes (Parniske et al. 1991). Moreover, phenolic compounds can induce a variety of genes in plant-associated bacteria (Peters and Verma 1990). Finally, it has been reported that the efficiency of the *R. meliloti*-alfalfa symbiosis can be enhanced by naringenin, although naringenin does not induce *nod* genes in *R. meliloti* (Jain et al. 1990).

We have shown that after LRC colonization, ORS571 can colonize intercellular spaces, hence gaining access to internal root tissue. This work therefore provides some clues as to how diazotrophic bacteria can internally colonize nonlegume plants. LRCs are one of the principal entry sites into roots for soilborne fungal and bacterial plant pathogens (Huang 1986). Advances in the understanding of how rhizosphere bacteria colonize plant roots, and especially LRCs, are therefore necessary to develop effective biocontrol strategies against soilborne pathogens of plants, and the importance of genetic approaches to this work has recently been stressed (Handelsman and Stabb 1996). Our quantitative study of LRC colonization, which enables the genetic analysis of this process, could therefore be applied to study mechanisms of biocontrol as well as those of root colonization by plant growth-promoting rhizobacteria. Interestingly, it has recently been shown that a mutant of *Pseudomonas fluorescens*, which is affected in its ability to induce systemic resistance in tomato against Fusarium wilt disease, is also affected in its ability to intercellularly colonize root tissues at sites of lateral root emergence (Duijff et al. 1997).

Despite the high level of LRC colonization, especially by *H. seropedicae*, once the bacteria reached the intercellular spaces of *A. thaliana*, multiplication appeared to be limited. The induction of plant defense reactions in response to bacterial colonization may contribute to the observed decrease in intercellular colonization over time, although no obvious signs of defense were detected. In addition to the low numbers of bacteria, we found no evidence that the bacteria progress

farther into plant roots than the area immediately adjacent to LRCs. However, it has been suggested by James et al. (1994) that xylem vessels are potential sites of nitrogen fixation by diazotrophic bacteria because they provide both the low pO_2 for nitrogenase activity and a site for metabolic exchange. *Acetobacter* and *Azoarcus* spp. have recently been observed in xylem vessels of sugarcane and rice, respectively (James et al. 1994; Hurek et al. 1994). Bacteria presumed to be *Azorhizobia* have also been observed in xylem vessels of wheat roots (Sabry et al. 1997). In the case of *A. thaliana*, what is now needed is an approach that will both increase the ability of diazotrophic bacteria to better exploit plant resources for colonization and enable them to colonize xylem vessels. Preliminary results suggest that *R. solanacearum*, the causal agent of bacterial wilt of tomato, is able to colonize xylem vessels of *A. thaliana* (Y. Marco, *personal communication*), and we were able to show LRC colonization of *A. thaliana* by this bacterium (data not shown). As a plant pathogen, *R. solanacearum* also has the ability to exploit plant nutritional resources for multiplication in the apoplast (Alfano and Collmer 1996). Therefore, one possible approach could be to transfer these abilities from *R. solanacearum* by transferring a gene library of *R. solanacearum* into ORS571 or *H. seropedicae* and selecting genes that confer the ability for *A. thaliana* vascular colonization.

MATERIALS AND METHODS

Bacterial strains.

The bacterial strains used in this study are listed in Table 1. Strains of *A. caulinodans* and *R. meliloti* were grown on TY medium (tryptone, 5 g/liter; yeast extract, 3 g/liter; and $CaCl_2 \cdot 2H_2O$, 0.88g/liter; pH 7); *Azospirillum* strains on YEP medium (Vanstockem et al. 1987); *H. seropedicae* on GGPY

Fig. 5. Effects of flavonoids and acetosyringone on colonization of lateral root cracks of *Arabidopsis thaliana* by *Herbaspirillum seropedicae* Z67(pCAM121). Compounds were added to the plant growth medium at 5×10^{-5} M. Values with different letters differ significantly at $P = 0.01$. Analysis of variance was conducted with Fisher's test (Snedecor and Cochran 1980). Data (including standard deviation values) are the average of two independent experiments. Sixteen plants were scored per treatment in each experiment.

medium (glucose, 2 g/liter; glutamic acid, 1.5 g/liter; peptone, 1.5 g/liter; K_2HPO_4 , 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 0.5 g/liter; and yeast extract, 2 g/liter; pH 6). Except for *A. brasilense* SP7(pLA-lacZ) and SP245(pLA-lacZ), for which the donor strain of *E. coli* (S17-1) carried the transfer functions (Arsène et al. 1994), bacterial strains carrying reporter genes were constructed by triparental mating with the helper plasmid pRK2073 (Leong et al. 1982): *A. caulinodans* ORS571-(pXLGD4), ORS571::nodC(pXLGD4), ORS571::nodD-(pXLGD4), and *R. meliloti* RCR2011(pXLGD4). *H. seropedicae* Z67(pCAM121) was kindly provided by J. K. Ladha (IRRI, Philippines). Transconjugants were selected with 10 µg of tetracycline per milliliter.

Plant growth conditions and inoculation.

A. thaliana seedlings were grown in tubes 22 cm long and 20 mm wide (four plants per tube) containing soft agar Fåhræus medium (Fåhræus 1957) with 0.25 mM KNO_3 at 21°C with 16 h of light per day. Different ecotypes of *A. thaliana* were obtained from the Nottingham Arabidopsis Stock Centre, England. Flavonoids, succinate, and acetosyringone were incorporated into the agar medium while it was still liquid. Flavonoids and acetosyringone were dissolved at 4 mg/ml in 0.02 M NaOH (K. O'Callaghan and E. C. Cocking, unpublished), and controls containing the same volume of added 0.02 M NaOH were always included. In order to minimize the conversion of naringenin to the chalcone form, which is favored in the presence of alkali, we restricted the pH of the final naringenin solution to 9.8. At this value, the solution is barely yellow; the yellow color at higher pH values indicates the presence of the chalcone form. In addition, naringenin dissolved in alkali was unaffected in its ability to induce a *nodA-lacZ* fusion of ORS571, as determined by the level of β-galactosidase activity induced by the strain ORS571(pRG290-12::T20) in pure culture (Table 1) (Goethals et al. 1989). Ten-day-old plants were inoculated with suspensions of bacteria in water by adding approximately 10^8 bacteria per tube directly onto the agar medium. Once inoculated, plants were generally left for 1 week before staining and observation. The root systems of these plants were 1 to 2 cm long.

Plasmid stability, enumeration of bacteria, and measurement of β-galactosidase activity associated with roots.

Although the plasmid pXLGD4 has been successfully used to study the symbiotic behavior of *R. meliloti* mutants (Ardourel et al. 1994), we verified the in planta stability of pXLGD4 in ORS571(pXLGD4) by scoring for the tetracycline resistance of bacteria removed from the agar immediately next to inoculated roots. At least 80% of isolated bacteria were still tetracycline resistant 1 week after inoculation. To count the bacteria associated with roots, bacteria were extracted from roots of inoculated *A. thaliana* by grinding plants in Z buffer (Miller 1972) devoid of β-mercaptoethanol, and then appropriate dilutions were plated onto selective growth medium. β-Galactosidase activity of these bacteria was measured according to Arsène et al. (1994) and expressed in Miller's units per minute per milligram of plant protein. Protein concentration was determined with the Bio-Rad (Richmond, CA) protein assay reagent.

Microscopic methods.

Whole roots of inoculated *A. thaliana* plants were stained for β-galactosidase (plasmids pXLGD4 and pLA-lacZ) or β-glucuronidase (pCAM121) activity. For plants inoculated with bacteria expressing β-galactosidase activity, endogenous plant β-galactosidase activity was first inactivated according to Vasse et al. (1995). Histochemical staining of β-galactosidase activity of bacteria associated with roots of inoculated plants was then performed with X-Gal according to Boivin et al. (1990). For plants inoculated with bacteria expressing β-glucuronidase activity, roots were stained by incubating them overnight at 37°C in 0.1 M phosphate buffer (pH 7) containing 0.5 mg of X-Gluc (5-bromo-4-chloro-3-indolyl-β-glucuronide) per milliliter, 10 mM EDTA, 0.5 mM $K_3(Fe(CN)_6)$, and 0.5 mM $K_4(Fe(CN)_6)$. Uninoculated plants were routinely included as controls. Plants were observed by bright-field microscopy with an Axiophot light microscope (Zeiss, Jena, Germany), and bacterial colonization was visualized on whole roots by the blue pigment resulting from degradation of X-Gal or X-Gluc. Sections for electron microscopy were prepared and observed as described by Vasse et al. (1995).

To look for the accumulation of phenolic compounds, we soaked roots in potassium permanganate (0.04% in 0.2 M sodium cacodylate, pH 7.2) for 1 h, rinsed them in the same buffer, and observed them by light microscopy. For callose detection, roots were soaked in aniline blue (0.01% in 0.12 M sodium cacodylate, pH 7.2), for 1 h, rinsed in the same buffer, and observed by fluorescence microscopy.

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