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Root Border-Like Cells of *Arabidopsis*. Microscopical Characterization and Role in the Interaction with Rhizobacteria^{1[w]}

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Plant roots of many species produce thousands of cells that are released daily into the rhizosphere. These cells are commonly termed border cells because of their major role in constituting a biotic boundary layer between the root surface and the soil. In this study, we investigated the occurrence and ultrastructure of such cells in *Arabidopsis* (*Arabidopsis thaliana*) using light and electron microscopy coupled to high-pressure freezing. The secretion of cell wall molecules including pectic polysaccharides and arabinogalactan-proteins (AGPs) was examined also using immunofluorescence microscopy and a set of anticarbohydrate antibodies. We show that root tips of *Arabidopsis* seedlings released cell layers in an organized pattern that differs from the rather randomly dispersed release observed in other plant species studied to date. Therefore, we termed such cells border-like cells (BLC). Electron microscopical results revealed that BLC are rich in mitochondria, Golgi stacks, and Golgi-derived vesicles, suggesting that these cells are actively engaged in secretion of materials to their cell walls. Immunocytochemical data demonstrated that pectins as well as AGPs are among secreted material as revealed by the high level of expression of AGP-epitopes. In particular, the JIM13-AGP epitope was found exclusively associated with BLC and peripheral cells in the root cap region. In addition, we investigated the function of BLC and root cap cell AGPs in the interaction with rhizobacteria using AGP-disrupting agents and a strain of *Rhizobium* sp. expressing a green fluorescent protein. Our findings demonstrate that alteration of AGPs significantly inhibits the attachment of the bacteria to the surface of BLC and root tip.

Many plants can produce large numbers of metabolically active root "border" cells that are programmed to separate from each other and to be released from the root tip periphery into the external environment (Brigham et al., 1995a; Hawes et al., 1998). Root border cells are defined as cells that are released into solution within seconds when root tips are placed into water (Hawes et al., 2000, 2003). In the absence of free water, these cells remain adherent to the root tips. The border cells of most species remain viable even after separation from the root tips (Hawes et al., 2000) and can survive independently from roots in vitro as well as in natural field conditions. Interestingly, border cells can undergo cell division in vitro and develop into callus tissue (Hawes and Lin, 1990).

As to the role of the border cells, Brigham et al. (1995b) have proposed that these cells are differentiated tissue of the root system that modulates the environment of the plant root by producing specific substances to be released into the rhizosphere. Proteins synthesized in border cells exhibit profiles that are distinct, qualitatively and quantitatively, from those synthesized in the root tips (Brigham et al., 1995b). These important differences in protein expression profiles are correlated with similarly distinct patterns of gene expression. These observations are consistent with the hypothesis that a major switch in gene expression occurs during border cell differentiation, as expected from cells with specialized functions in plant development. Several border cell characteristics support the hypothesis that they have the capacity to protect plant health by conditioning the environment of the growing root tips (Hawes and Brigham, 1992; for review, see Brigham et al. 1995a). For instance, they are involved in Al detection and tolerance (Miyasaka and Hawes, 2001). Release of Al-binding mucilage by border cells could play a role in protecting root tips from Al-induced cellular damages. It has also been shown that border cells have different responses to microorganism infection. These responses are plant and microorganism genotype-dependent because they can specifically

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attract certain bacteria and repel others (Hawes and Pueppke, 1989) or even produce papillae in response to pathogenic fungi infection (Sherwood, 1987). The mechanism controlling border cell-microorganism interaction is still unknown. However, it is thought that exudates, including the polysaccharides, are of high importance in these processes. Border cells can act by forming a boundary that inhibits accumulation of certain bacteria or alternatively by attracting and providing nutrients to other bacteria species (Hawes et al., 1998). Furthermore, Zhu et al. (1997) have demonstrated that chemicals released by root border cells can regulate the expression of bacterial genes required for the establishment of plant-microbe associations.

While border cells have been largely studied in species such as cotton (*Gossypium hirsutum*) or pea (*Pisum sativum*; see Hawes and Lin, 1990; Hawes et al., 1998), the characterization of border cells of the plant model Arabidopsis (*Arabidopsis thaliana*) has never been described. In this study, we report on the morphology and cell wall composition of such cells in Arabidopsis seedlings using microscopy and immunocytochemistry. We identified cells that were released from root tips as organized files and not as isolated cells as described for other species (see Hawes et al., 2000). For this reason, we termed them border-like cells. In addition, we show that BLC from Arabidopsis seedlings produce high amounts of arabinogalactan-proteins (AGPs) at their cell surface. In light of these findings, and the possible implication of border cells in the interaction between plant and microorganisms (Hawes et al., 2000), we sought to understand the function of AGPs in the interaction between BLC/root tips and a strain of rhizobacteria, *Rhizobium* sp. YAS34. To this end, we used 2 AGP-disrupting agents, namely the β -glucosyl Yariv (β -GlcY) reagent that binds and precipitates AGPs (Fincher et al., 1983) and 3,4-dehydroproline (DHP) that is known to inhibit O-glycosylation of cell wall proteoglycans, including extensins and AGPs (Cooper and Varner, 1983; Cooper et al., 1994; Baskin and Bivens, 1995). We also used a green fluorescent protein (GFP)-expressing strain of *Rhizobium* sp. as a tool to facilitate the quantification of the adhesion of the bacteria at the surface of the cells. Our results demonstrate clearly that both drugs caused strong inhibition of bacteria attachment to the surface of root tips and BLC.

RESULTS

BLC in Arabidopsis Are Released from Root Tips as Organized File

Root border cells are defined as the cells that are released into solution by simple immersion of root tips in water (Hawes and Brigham, 1992). Therefore, we sought to identify such cells in Arabidopsis using root tips from 1- to 15-d-old plants. Under our experimen-

tal conditions, roots spontaneously losing their cap was a very sporadic event observed in only 1% to 2% of seedling populations. When root tips were placed in a droplet of water on a glass slide under the microscope, BLC were frequently seen detaching from the root apex. This phenomenon seemed to be age-dependent because it was seen only in seedlings above 5 d of growth. Figure 1A shows a root tip of 15-d-old seedlings with root BLC detaching from the root tip. The pattern of release of these cells is strikingly different from what was generally described for border cells in other models such as cotton (Hawes and Brigham, 1992), wheat (*Triticum aestivum*; Zhu et al., 2003a), and pea roots (Brigham et al., 1995b). They were released from the root tip in organized cell layers rather than isolated cells.

At 5 d of growth and in all seedlings observed, BLC consisted of a unique layer of several cells that remained mostly attached to the root tips after immersion in water. At 7 d of culture, 73% of the seedlings showed a single layer of BLC and 27% of the seedlings presented 2 layers of BLC (Table 1). For about 38% of 7-d-old seedlings, BLC could be easily released from the root apex by simple immersion in water. At 13 and 15 d of growth, BLC form at least 3 layers of cells surrounding the cap that were easily detachable from the root apex upon immersion (Fig. 1). At these 2 stages of development, the majority of the seedlings (i.e. more than 80%) could release BLC when immersed in water (Table 1).

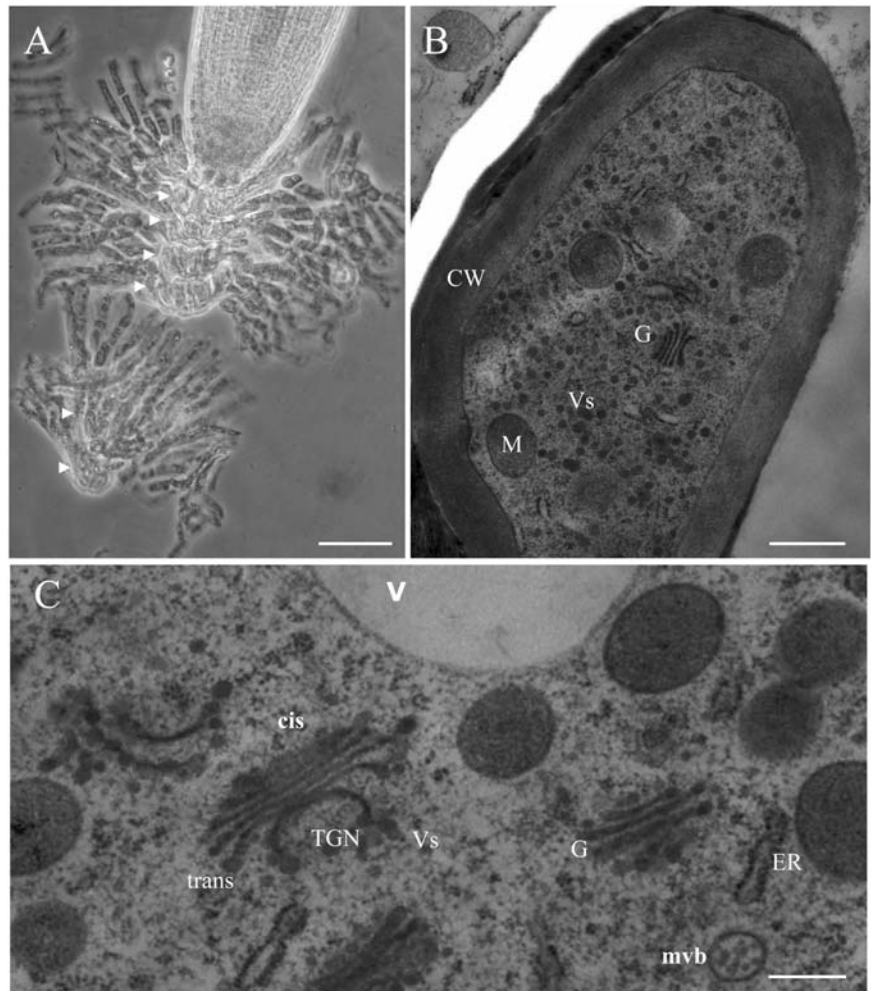
Under our conditions, BLC appeared to be spherical at the very tip of the root and become elongated during development as they moved away from the tip. At this final stage, the elongated BLC were $28 \pm 9 \mu\text{m}$ long and $6 \pm 2 \mu\text{m}$ wide. In addition, staining of root tips with Calcein-AM, a vital dye, demonstrated that freshly released BLC were viable for 100% of the seedlings and they remained viable for at least 24 h (Fig. 2).

Ultrastructural Analysis of Root BLC Using High Pressure-Freezing

To examine the ultrastructure of BLC from Arabidopsis root tips, high pressure freezing technique was used to fix the tissues followed by electron microscopic examination of ultrathin-sections. Figure 1B shows the general appearance of a BLC detaching from the root tip. All cell structures were very well cryo-preserved by this technique. A thick and electron opaque cell wall was present around the cell in a tight association with a clearly visible plasma membrane. The cytoplasmic matrix was also dense and numerous mitochondria, endoplasmic reticulum, and multivesicular bodies were observed. Small vacuoles were also observed in some, but not all, of the cells (Fig. 1C).

One major feature observed in Arabidopsis root BLC was the presence of a large number of Golgi stacks with many small Golgi-derived vesicles filled with an electron opaque material. These small vesicles accumulated all over the cytoplasm. The cis-to-trans

Figure 1. Microscopical characterization of root BLC from *Arabidopsis*. A, A root tip of 2-week-old seedlings showing BLC release. Arrowheads indicate the cell layers where border cell files came from. B, Micrograph illustrating the general morphology of a BLC from a root prepared by HPF and FS. Note the abundance of Golgi-derived vesicles filled with opaque electron material. C, High magnification view of cytoplasmic content of a BLC prepared HPF/FS. CW, Cell wall; ER, endoplasmic reticulum; G, Golgi stack; M, mitochondria; mvb, multi-vesicular bodies; TGN, trans golgi network; V, vacuole; Vs, secretory vesicles. Bars = 100 μm (A); 1 μm (B); and 300 nm (C).



polarity of Golgi stacks was clearly distinguishable as previously defined (Driouich et al., 1993) and small vesicles were frequently seen at the margins of all Golgi cisternae. The trans Golgi network compartment was seen associated with Golgi stacks. Taken together, all these observations indicate that, under our conditions of growth, root BLC were still metabolically active at the time of their release. In addition, as judged by the large number of active Golgi stacks, they may also be actively secreting materials, such as complex polysaccharides and glycoproteins, to their walls and the surrounding medium.

Detection of Homogalacturonans in BLC Walls

To check for the presence of pectins at the BLC surface, we probed root tips with the monoclonal antibody (mAb) JIM5 directed against homogalacturonans, using the immersion technique described by Willats et al. (2001). The epitope was detected at the surface of the root tip as well as at the surface of cells sloughing off from the root apex (Fig. 3A). After their complete separation from the root tips, BLC were still heavily labeled (Fig. 3B). These observations demonstrate that pectins were synthesized and secreted into

Table 1. Detachment process of BLC

Presence and release of BLC among a population of roots from 1 to 15 d old. Percentage values are means calculated from 11 to 13 seedlings.

Seedling Age	Presence of BLC	Percent of Root Tips Presenting BLC Layers		Percent of Roots Releasing BLC upon Immersion in Water
		1 Layer of BLC	≥ 2 Layers of BLC	
<i>d</i>				
1 to 4	No	0	0	0
5	Yes	100	0	0
7 to 8	Yes	73	27	38
13 to 15	Yes	0	100	>80

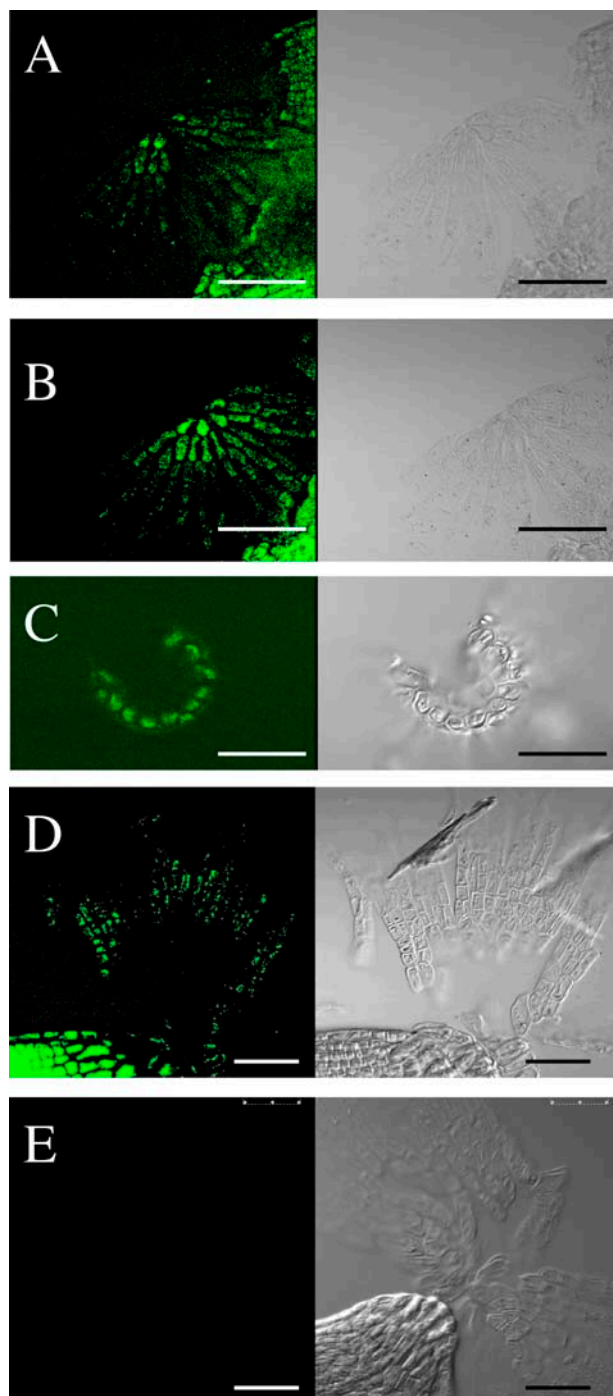


Figure 2. Micrographs of Arabidopsis BLC stained with calcein-AM. The fluorescence of the probe is indicative of the cell viability. BLC from 8-d-old seedlings stained with calcein-AM. A, At the time of root cap jettisoning. B, After 4 h in water. C, After 6 h in water. D, BLC from 13-d-old seedlings stained with calcein-AM 24 h after their release from the root apex. E, Intrinsic fluorescence of BLC from 13-d-old seedlings. Bars = 50 μ m.

the cell wall by BLC. Labeling with other antipectin antibodies such as JIM7 or LM5 confirmed this finding (data not shown; see also Willats et al., 2001). Similar observations have been made on pea using immunodot-blot assay on different fractions isolated from the root cap (Stephenson and Hawes, 1994). An increase in the level of soluble deesterified pectins recognized by JIM5 has been associated with border cell development (Stephenson and Hawes, 1994).

Expression of AGP Epitopes in Root Tips and BLC

Chemical composition of the carbohydrate moiety of AGPs from Arabidopsis roots have been described by van Hengel and Roberts (2002, 2003). Their data indicated that these AGPs have a large proportion of Gal and Ara residues in addition to some minor sugars. Furthermore, ELISA assays revealed the occurrence of various carbohydrate epitopes in Arabidopsis AGPs from roots, as detected by different antibodies including JIM13, JIM14, MAC207, and LM2 (van Hengel and Roberts, 2003). However, no data is available regarding the AGPs spatial organization in the root tips and BLC.

To investigate the occurrence of AGPs in root tips and BLC, we immunolabeled longitudinal sections of roots with 3 anti-AGP monoclonal antibodies: MAC207, JIM14, and JIM13 (Knox et al., 1991; Yates et al., 1996). As shown in Figure 4A, strong labeling with MAC207 occurred all over the root apex and BLC, except in the meristem where labeling was weaker. JIM14 also showed a high level of detection in BLC as well as in other tissues (Fig. 4B). Finally, and more interestingly, labeling with JIM13 was restricted exclusively to peripheral cells in the root cap region and BLC (Fig. 4C). No labeling was seen in meristematic, cortical, or epidermal cells with this antibody (Fig. 4C). However, up in the root, metaxylem tissue was also labeled with JIM13 (data not shown; see Dolan et al.,

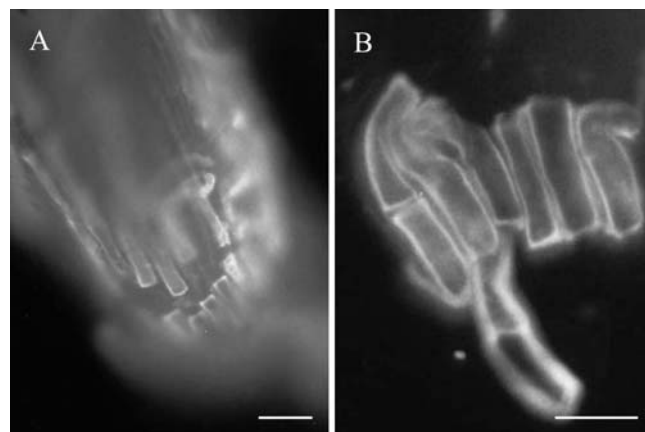


Figure 3. Immunofluorescence-staining of homogalacturonans. A, Immersion immunofluorescence showing labeling at the surface of the root apex with the mAb JIM5. B, Sloughing off BLC stained with JIM5. Bars = 20 μ m.

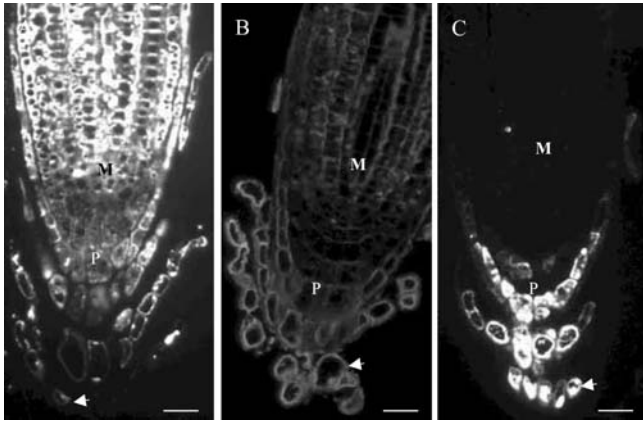


Figure 4. Immunofluorescence staining of AGPs on longitudinal section of Arabidopsis root tip. A, Staining with the mAb Mac207 antibody. B, Staining with the mAb JIM14. C, Staining with the mAb JIM13. Labeling was exclusively associated with peripheral and root BLCs. BLC, Arrowheads; M, meristem; P, peripheral cells. Bars = 20 μm .

1995; Seifert et al., 2004). The mAb LM2 also labeled BLC as strongly as MAC207 (data not shown). These results demonstrate that BLC produced significant amounts of AGPs, reflecting an active Golgi-based synthesis and secretion of these glycoproteins.

Role of AGPs in the Interaction between Root BLC and Microorganisms

To gain information on the role of BLC and AGPs in the recognition/adhesion mechanism between root cells and microorganisms, we examined the effects of β -GlcY reagent known for its ability to bind and precipitate AGPs (Fincher et al., 1983), and DHP, a reagent that can inhibit the O-glycosylation of cell wall proteoglycans, including AGPs (Cooper and Varner, 1983; Cooper et al., 1994; Gateau and Driouch, unpublished data). Alpha-mannosyl Yariv reagent (α -ManY), which is a nonreactive analog of β -GlcY, was used as a control. Arabidopsis root tip morphology, BLC release, and the binding of a GFP-expressing strain of *Rhizobium* sp. YAS34 to the root cap and BLC were monitored in these experiments.

Morphological Effects

Arabidopsis seedlings grown in medium containing β -GlcY reagent presented reduction in root growth and increase in radial expansion of root epidermal cells (Willats and Knox, 1996). Similar effects were observed under our experimental conditions (Figs. 5 and 6). When grown on 10 μM Yariv-containing medium, roots from 7-d-old seedlings presented a red color and were less than 40% and 48% of the length of α -ManY-treated ($P < 0.05$) and nontreated ($P < 0.01$) plants, respectively (Fig. 5). Swelling of epidermal cells was also seen as reported previously (Willats and Knox, 1996). BLC, with a more rounded shape, were

frequently seen detaching from the root tip and forming layers (Fig. 6B).

When seedlings were grown on 5 μM DHP for 7 d, there was no statistical difference in root length ($P > 0.05$) as compared to control plants. In contrast, a slight but significant reduction of root length (11%, $P < 0.01$) was observed for 15-d-old seedlings grown under the same conditions. Nonetheless, under both time conditions, BLC were produced and frequently seen (Fig. 6C).

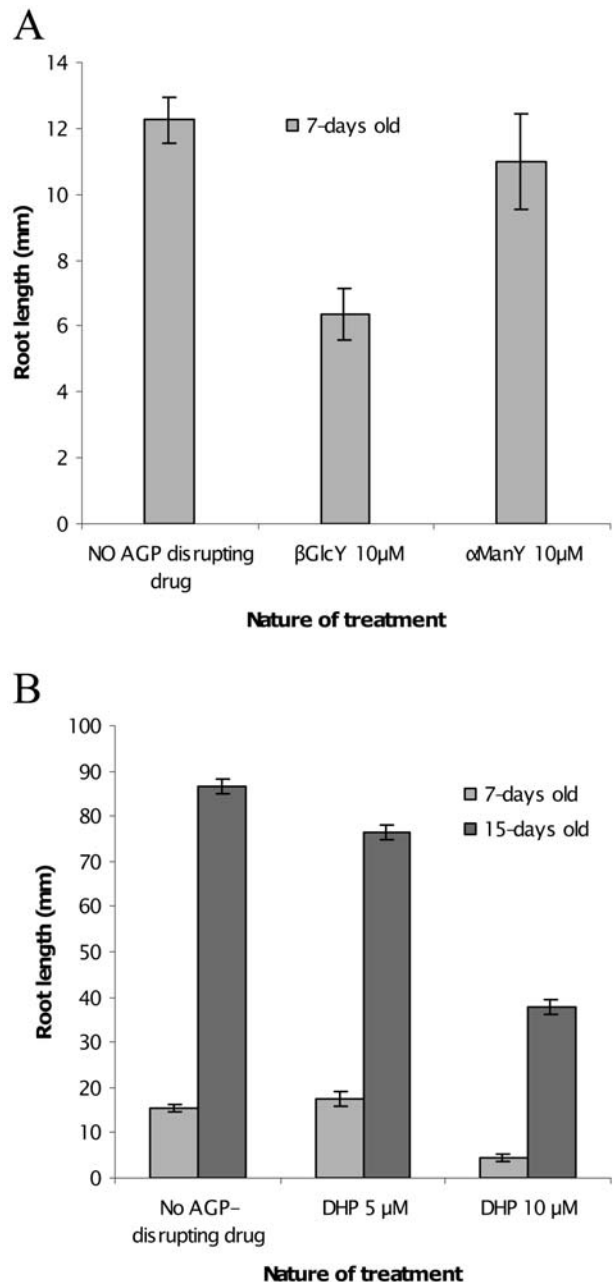


Figure 5. Bar graphs showing the effects of Yariv reagents and DHP on root length from 7-d-old (gray bars) and 15-d-old (dark bars) Arabidopsis seedlings. A, Treatments with 10 μM β -GlcY and 10 μM α -ManY. B, Treatments with 5 μM and 10 μM DHP. $n = 30$ to 100 and confidence intervals at 95% (LSD).

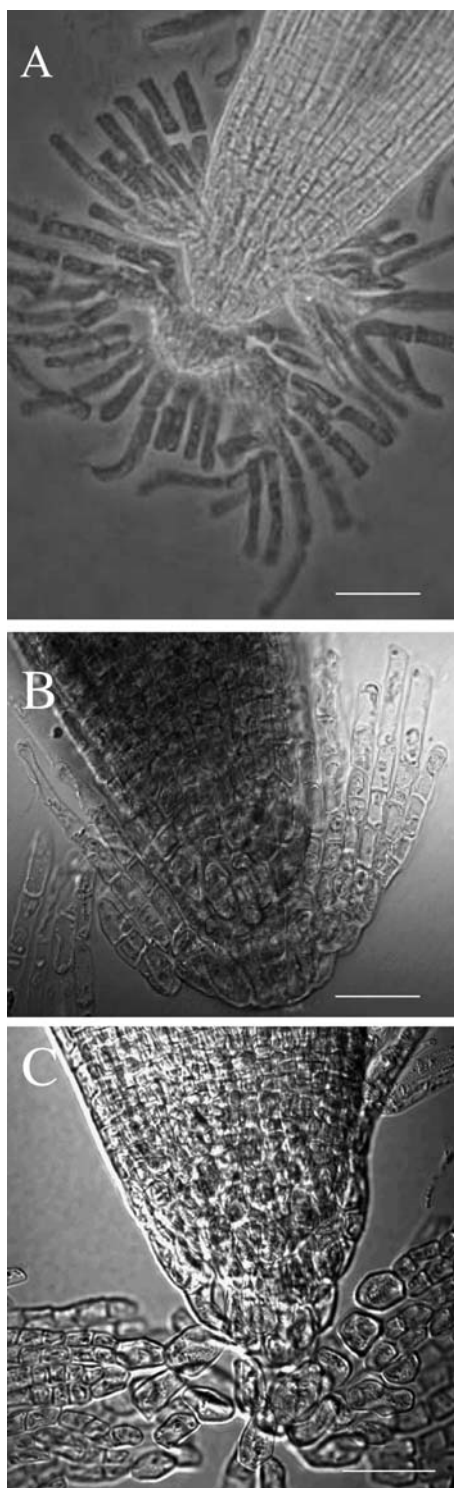


Figure 6. Micrographs of Arabidopsis root tips treated with AGP-disrupting agents. A, Control. B, 10 μM $\beta\text{-GlcY}$. C, 5 μM DHP. Bars = 100 μm (A) and 50 μm (B and C).

Seedlings treated with 10 μM DHP showed a severe inhibition of their root elongation (70% and 56% less than in nontreated plants at 7 and 15 d, respectively; $P < 0.01$; Fig. 5B). These same plants also had much less BLC formation. Therefore, for rhizobacteria adhesion experiments, we used the lower concentration of DHP (i.e. 5 μM) not only because BLC release was still occurring at this concentration, but also because it was possible to test longer periods of treatment (i.e. 15 d; see below).

Rhizobium sp. YAS34 Binds to BLC and Root Tip of Arabidopsis

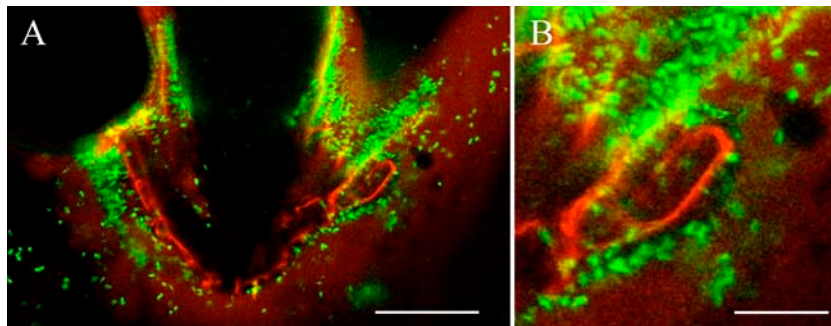
First, we selected a suitable model of recognition between bacteria and Arabidopsis root and we found that *Rhizobium* sp. YAS34 strain, isolated from the rhizoplane of the sunflower (Alami et al., 2001), was able to colonize Arabidopsis roots and BLC. Using a constitutive GFP-expressing YAS34 strain, referred to as YAS34GFP, we were able to localize the bacteria and quantify adhesion/colonization process by following the GFP-fluorescence with a confocal microscope. In addition, the use of mAb JIM5 antibody, which can effectively stain root tips (Willats et al., 2001; this work), allowed the visualization of colonized root BLC. Thus, by using these staining methods simultaneously we could visualize root-bacteria interaction. Figure 7 shows the colocalization of root cap/BLC with the mAb JIM5 and YAS34GFP. The association between the root tip cells and the bacteria YAS34GFP was clearly visible and, more interestingly, released BLC could also be seen with bacteria adhering to their surface (Fig. 7B).

Effect of $\beta\text{-GlcY}$ and DHP on *Rhizobium* sp. YAS34 Adhesion

Seeds were inoculated with YAS34GFP and germinated on culture medium supplemented with 10 μM $\beta\text{-GlcY}$, 10 μM $\alpha\text{-ManY}$, or 5 μM DHP in absence of Suc to limit overproliferation of the bacteria. To check the effect of AGP-disrupting drug on bacterial growth, the growth rates of bacterial populations in the presence of 10 μM $\beta\text{-GlcY}$ or 10 μM DHP or in the absence of drugs were estimated by serial dilution and plating. At these concentrations, these drugs did not have any effect on bacterial growth (data not shown).

The estimation of the effect of the AGP-inhibitors drugs was determined by calculating the ratio between the occupied surface by the fluorescent bacteria (Sb) and the surface occupied by root tip and surrounding BLC (Sr). The region of the root enclosed in a volume of 100- μm length from the tip, about 200- μm width, and 15- μm depth from the root surface, where BLC were frequently present, was optically sectioned by steps of 1 μm . The shadow projections of the optical sections were analyzed using the software Image J (<http://rsb.info.nih.gov/ij/>). An ANOVA test was performed on the variable ratio (Sb/Sr) consisting of

Figure 7. Double localization showing the association between BLC and bacteria YAS34-GFP. A, BLC, labeled with JIM 5 antibodies, present a red fluorescence. The GFP-expressing rhizobacteria appear green. B, Higher magnification showing the association between BLC and rhizobacteria. Bars = 50 μm (A) and 20 μm (B).



the rate of Sb over Sr and statistical analyses on all collected data were done using StatGraphic.

We found that plants grown in presence of 10 μM β -GlcY or 5 μM DHP for 7 or 15 d had significant impact on the variation of the Sb/Sr ratio ($P < 0.01$ and $P < 0.05$, respectively). The results are presented in Figure 8 and show that both β -GlcY and DHP caused a significant decrease in the surface area occupied by the bacteria YAS34GFP in comparison with both controls, α -ManY (10 μM) or no treatment at all ($P < 0.05$). Figure 9 illustrates colocalization of fluorescent bacteria (YAS34GFP) and root BLC/cap cells in the presence of both AGP-disrupting agents.

DISCUSSION

Whereas root morphology has been previously well described in *Arabidopsis* (Benfey et al., 1993; Dolan et al., 1993), border cell characterization has not been reported in this species. Although Brigham et al. (1995a) reported that border cells were not detected in seedlings of *Arabidopsis* growing under their conditions, they could not eliminate the possibility that border cell separation may occur under different conditions.

Our findings demonstrate that such cells can be produced and released by *Arabidopsis* root tips as organized components of several viable cells. They do not get dispersed individually into suspensions as found for border cells from other species (Hawes et al., 2000, 2003). For this reason and to distinguish them from the classical border cells, we propose to call them border-like cells (BLC).

It is difficult to analyze structural features of these border cells because they are loosely attached to the root tip and are often lost during preparation for microscopy. Our electron microscopy data of high-pressure frozen BLC showed that these cells present characteristics of metabolically active cells with a very well-developed secretory apparatus (e.g. Golgi stacks and transport vesicles) and a large number of mitochondria. The abundance of active Golgi stacks and vesicles indicate that these cells still remain engaged in the secretion of molecules, such as complex polysaccharides and glycoproteins after their release. Our data are consistent with the previous published stud-

ies on root border cells of many species (see Hawes et al., 2000). For example, border cells from pea root synthesize cell-specific proteins that are absent in other cells of the root tips (Brigham et al., 1995b). Furthermore, border cells from other models have been shown to produce specific metabolites such as antibiotics, shikonin, or enzymes including a low-pH galactosidase and a rhizobium-induced peroxidase (Brigham et al., 1999; Hawes et al., 2000).

To date, all the studies have been carried out on species that release large amounts of root border cells such as pea (Brigham et al., 1995b), cotton (Hawes, 1991), or wheat (Zhu et al., 2003a). By contrast, *Arabidopsis* border cells have been much less investigated, hampered probably by the fact that *Arabidopsis* root tips release relatively few cells depending on experimental conditions. Our results show that immersion of *Arabidopsis* root tips in water does not

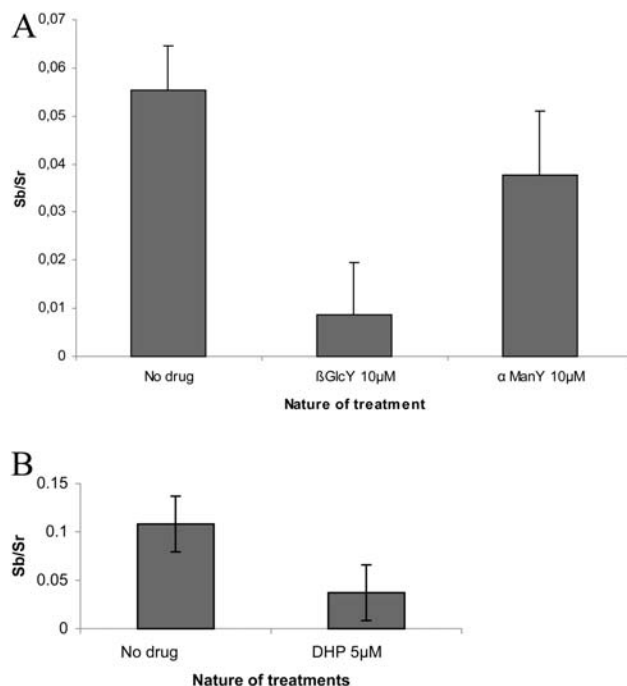


Figure 8. Effect of Yariv reagents and DHP on the association between root tip cells and *Rhizobium* sp. YAS34GFP. A, Effect of β -GlcY 10 μM and α -ManY 10 μM ; 7-d-old plants. B, Effect of DHP 5 μM ; 15-d-old plants. $n = 3$ to 15. Average and confidence intervals are at 95.0% (LSD).

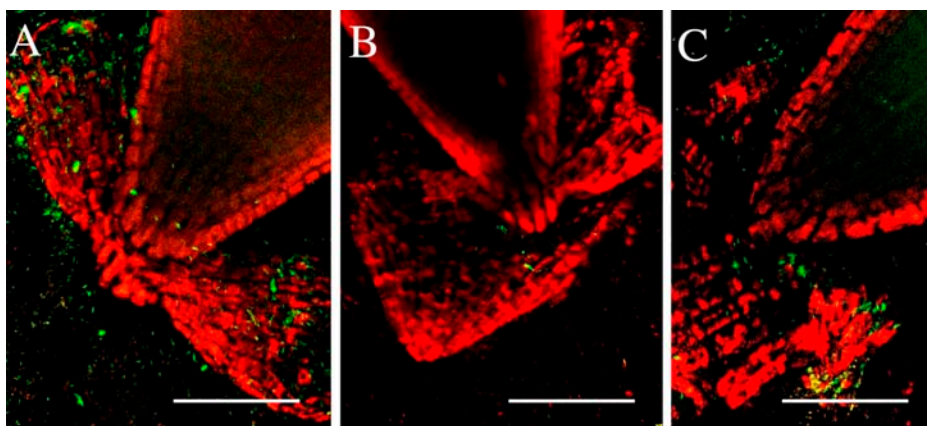


Figure 9. Effects of AGP-disrupting agents on the association between Arabidopsis root tips and *Rhizobium* sp. YAS34GFP. Images represent the projection of Z-sections over 15- μm depth (1- μm step). Root tips are stained in red with Nile red and the expressing-GFP bacteria appear green. A, Control. B, 10 μM Yariv. C, 5 μM DHP. Bars = 50 μm (A and C) and 30 μm (B).

result in the release of a large number of isolated cells as is the case in pea (Brigham et al., 1995b) or cotton roots (Hawes et al., 2000). However, we found that Arabidopsis cells present a very unusual characteristic, because they tend to remain clustered together as files rather than being released individually as isolated cells. Thus, BLC of Arabidopsis may not be programmed to separate from each other. This suggests that either they might possess specific interconnecting molecules at their cell surface or might be insensitive to cell wall-degrading enzymes involved in the release of border cells. In this context, the occurrence of JIM5-containing pectins (see Fig. 3B) is of importance as these polysaccharides could serve as a glue to cement these cells together. AGPs could also serve as adhesive molecules between such cells; however, we could not observe any separation of BLC under conditions where these proteoglycans would be altered by β -GlcY reagent.

In addition to pectins, AGPs were also present in the walls of root tips as well as in root BLC. Furthermore, JIM13 epitope was exclusively associated with the periphery of the root tips and BLC. Similar results have also been reported by Dolan et al. (1995). These data also suggest that cell differentiation into peripheral cells and BLC in Arabidopsis root is accompanied by an active synthesis and secretion of significant amounts of AGPs. The precise function of these AGPs has yet to be determined. JIM13 epitope-containing AGPs have been suggested to fulfill many functions. For instance, studies in carrot and radish roots have shown that the expression of JIM13 epitope was correlated with xylem maturation (Casero et al., 1998). Also, it has been reported that JIM13 and JIM14 staining were associated with cell dissociation in maize (*Zea mays*) coleoptiles (Schindler et al., 1995), suggesting that AGPs may serve as developmental markers.

It is now known that root border cells are not only cells in the process of sloughing off but they are involved in many processes such as protection against pathogens attacks and can even divide under appropriate growing conditions (Hawes and Pueppke, 1986). The considerable importance of root border

cells to interactions with microorganisms has been reviewed by Hawes et al. (2000), who reported that border cells can regulate this interaction by producing either chemo-attractants or repellents. Park et al. (2002) identified in border cells and root exudates a novel protein, PAP-H, that has a role in depurination of fungal ribosomes, contributing to its antifungal activity against soil-borne fungi. Interestingly, immunofluorescence studies on pea roots indicated that this enzyme was located in the cell wall of root border cells and released from the root tip as the root grew (Park et al., 2002).

Our results show that Arabidopsis BLC were able to recognize and bind the rhizobacteria *Rhizobium* sp. YAS34. Moreover, when seedlings were grown in the presence of the β -GlcY reagent, the colonization, which involves adhesion and cell division, of the rhizobia to the root tip cells was altered, implying an involvement of AGPs in this process. Indeed, AGPs have previously been suggested to play an important role in several plant symbiotic associations. Scheres et al. (1990) reported that *Enod 5*, a gene encoding an AGP-like protein, was induced during nodule development in the *Rhizobium* spp.-legume symbiosis. van Buuren et al. (1999) identified and characterized plant cDNAs representing genes induced in the mycorrhizal symbiosis in *Medicago truncatula* including one encoding a putative AGP. Furthermore, in situ hybridization revealed that the expression of this AGP was specifically restricted to the cortical cells containing arbuscules. The authors suggested that this protein might be involved in mediating the interaction between the plant cortical cell and fungal hypha during arbuscule development.

Using immunolabeling, Balestrini et al. (1996) detected an abundant epitope associated with arabinosylated β -(1-6)-galactan of AGPs and rhamnogalacturan I (Puhlmann et al., 1994), which accumulated in maize root cells infected with arbuscular mycorrhizal fungi. The AGP epitope occurred near the penetration point of the hyphae and within vesicles inside infected cells. These results indicate that the distribution of the epitope was affected by mycorrhizal fungi infection, but no direct link between AGPs and fungi attachment

was established. Our data show that the colonization (i.e. recognition and binding) of BLC by *Rhizobium* sp. YAS34GFP was strongly modified upon DHP treatment. DHP is known to inhibit Pro-hydroxylation and to specifically induce the synthesis and the secretion of structurally abnormal Hyp-rich glycoproteins, including AGPs (Cooper and Varner, 1983; Cooper et al., 1994; Gateau and Driouch, unpublished data). The effect of DHP on the association of the rhizobium to BLC is probably due to alterations of the structure of AGPs or other Hyp-containing wall proteins such as extensins and repetitive-Pro-rich protein. But support for the involvement of AGPs in the interaction of root cells with soil bacteria can be found in the study of Nam et al. (1999) who identified and partially characterized *Arabidopsis* mutants that are resistant to transformation by *Agrobacterium tumefaciens* (*rat* mutant). The authors found that the *Agrobacterium*-mediated transformation process was blocked at a very early stage with the mutants *rat1* and *rat3* as the bacteria could not bind to the roots. The affected genes in the mutant *rat1* and *rat3* were shown to encode an AGP and a cell wall protein, respectively (Zhu et al., 2003b), thus supporting the role of these proteins in the process of interaction between root cells and the bacteria.

In summary, our findings provide evidence for the occurrence of BLC in *Arabidopsis* and strongly support the hypothesis that AGPs are involved in recognition and binding between root cells of this model plant and the rhizobacteria *Rhizobium* sp. YAS34. How such recognition occurs and which signals are involved from both sides are not known yet and need further research.

MATERIALS AND METHODS

Plant Material

Arabidopsis (*Arabidopsis thaliana*) L. Heynh cv Columbia seeds were germinated on agar-solidified nutrient medium in petri dish plates as described by Baskin et al. (1992). Plates with seeds were placed vertically to avoid roots penetrating the agar, which would result in the loss of border cells (Baskin and Bivens, 1995). The required amount of inhibitor was added to the plates after autoclaving the media. The β -GlcY, α -ManY reagents, and DHP were used at a final concentration of 10 μ M or 5 μ M. Four days after plating, seedlings were transferred to freshly prepared plates containing the inhibitors. Control plants were transferred on plates containing only freshly prepared medium. Alternatively, seeds were sown, inoculated with the bacteria, and grown directly on half-strength Hoagland medium (Arnon and Hoagland, 1940) solidified with 0.7% w/v Gelrite (Sigma, St. Louis) and containing either no drugs or the required amount of AGP-inhibitors.

Root lengths were measured using analySIS software and data are reported as mean \pm 95% confidence intervals (LSD). Prior to examination under a Leica or Olympus microscope, seedlings were mounted on a microscopic slide and a droplet of water was added on the root apex to visualize the presence of root border cells.

Ultrastructural Analysis by Electron Microscopy

Root tips were prepared using the high-pressure freezing (HPF) and freeze-substitution (FS) method and embedded in Epon resin as previously described (Staehelin et al., 1990). Thin sections (90 nm) were mounted on copper grids. The sections were stained with 2% uranyl acetate for 10 min and

lead stain for 30 to 60 s and observed at 80 kV with a TEM (Tecna 12, Bio-Twin; Philips).

Staining with Calcein-AM

Root tips were immersed in a drop of 5 μ M calcein-AM (Sigma) in water. For time-course viability studies, BLC were detached from the root tips by addition of water in a glass slide and stored at room temperature. Labeling was performed about 10 to 15 min before acquisition of the images. Pictures were obtained in Kalman acquisition mode (2 scans) at low energy of the laser beam to allow the survival of the cells and the observation of the same BLC over time.

Staining of Fresh Roots with Nile Red Dye

Roots were stained 10 to 15 min with Nile Red (5H-benzo [α] phenoxazin-5-one 9-diethyl amino; Sigma) solution. A stock solution of Nile Red was prepared in dimethyl sulfoxide (8 mg mL⁻¹) and then 10 μ L was diluted in 5 mL of CPW buffer (250 mM KCl, 5 mM CaCl₂, and 1 mM MES, pH 5.5). A total of 20 μ M of this solution were added on the root apex.

mAbs

The mAb used in this study have all been described previously. The mAb JIM5 binds to low ester of the homogalacturonan regions of pectin (Willats et al., 2001). The mAb JIM14 is directed against carbohydrate portion of AGP (Knox et al., 1991) and the epitope of AGPs recognized by the mAb JIM13 is a β -D-GlcP-A-(1 \rightarrow 3)- α -D-GalpA-(1 \rightarrow 2)-L-Rha (Yates et al., 1996). Mac 207 is an anti-AGP mAb believed to recognize an Ara-containing AGP epitope (Yates et al., 1996).

Immunolabeling of Homogalacturonans

Roots from 7- to 15-d-old seedlings were fixed for 30 min in 4% paraformaldehyde in 50 mM PIPES, 5 mM MgSO₄, and 5 mM EGTA and immunolabeled according to Willats et al. (2001). Roots were washed in phosphate-buffered-saline (PBS) and then incubated for 1 h in JIM5 mAbs diluted in PBS containing 5% (w/v) fat-free milk powder. Roots were washed in PBS (5 changes, 2 min/wash) and incubated in anti-rat-IgG (dilution 1/50) coupled to fluorescein isothiocyanate (Sigma). After washing in PBS, roots were mounted in anti-fade agent (Citifluor, Agar Scientific, Dover, UK) and examined using epifluorescence with a Zeiss Axioscope microscope. Unfixed material was used when JIM5 immunolabeling was performed on roots inoculated with GFP-expressing bacteria (YAS34GFP).

Immunofluorescence Detection of AGPs

Roots of 7-d-old seedlings were harvested and small fragments (1–2 mm) were fixed, dehydrated, and embedded in methacrylate resin (Baskin et al., 1992; Andème-Onzighi et al., 2002). Thick sections (0.5 μ m) were cut and mounted on glass microscope slides. Sections were incubated in a blocking solution of 3% low-fat dried milk in 10 mM sodium phosphate, 500 mM NaCl, and 0.1% Tween 20, pH 7.2 (PBST) for 30 min. Sections were then incubated for 4 h at 30°C with the primary antibodies Mac 207 (diluted 1:5 in PBST), JIM 14 (diluted 1:30 in PBST), or JIM 13 (diluted 1:15 in PBST). Sections were carefully washed with PBST containing 0.5% Tween for 1 h before being incubated in a droplet of goat anti-rat IgG conjugated to Texas Red isothiocyanate or fluorescein isothiocyanate at a concentration of 1:50. Epifluorescence of the immunostained tissue sections was observed on a Zeiss filter set (excitation filter 550 nm, barrier filter 750 nm). Control experiments were performed by omission of primary antibodies.

Bacterial Strain and Growth Conditions

The bacterial strain *Rhizobium* sp. YAS34 (Alami et al., 2001) was grown at 30°C in 10-fold diluted tryptic soy broth (TSB/10) (DIFCO Laboratories, Detroit). For growth on plates, the medium was solidified with 15 gL⁻¹ Bacto-agar (TSA/10; DIFCO Laboratories). *Escherichia coli* strains were grown at 37°C on TSB medium. Bacteria were supplemented with antibiotics when required at 15 μ g mL⁻¹ tetracycline, 50 μ g mL⁻¹ nalidixic acid, and 50 μ g mL⁻¹ kanamycin.

Rhizobium sp. YAS34 expressing constitutively the GFP was obtained by triparental mobilization of plasmid pHC60 (Chen and Walker, 1998) with the conjugative plasmid pRK2013 in *E. coli* LE392. A spontaneous nalidixic acid resistant mutant of *Rhizobium* sp. YAS34 served as the recipient strain. *Rhizobium* cells expressing constitutively the GFP were selected on TSA/10 containing 15 $\mu\text{g mL}^{-1}$ tetracycline and 50 $\mu\text{g mL}^{-1}$ nalidixic acid. The green fluorescent derivative was designated *Rhizobium* sp. YAS34GFP.

Effect of AGP-Disrupting Drugs on *Rhizobium* sp. YAS34GFP Cell Growth

A suspension of $10^4 \pm 10^3$ cells of a *Rhizobium* sp. YAS34GFP grown up to late exponential phase was suspended in TSB/10 medium containing no-AGP disrupting drug, 10 μM β -GlcY, or 10 μM DHP and incubated at 30°C, under shaking (150 rpm), for 96 h. The bacterial population growth rate was determined over time by plating serial dilutions from the cultures on TSA/10 and counting of the colony-forming units.

In Vitro Root Colonization

Rhizobium sp. YAS34GFP was grown on TSB/10 medium supplemented with tetracycline (SIGMA; 15 $\mu\text{g mL}^{-1}$). Cell concentration was estimated by testing samples with direct plate counts and adjusted in such way as to get a volume containing 10^7 bacteria/mL. Bacterial cells were collected by centrifugation and washed twice with sterile KCl (8.5g L⁻¹) to remove residual medium and then resuspended with 1 mL of sterile ultrapure water. Seeds were each inoculated with a volume of 5 μL of the bacterial suspension. This suspension was used to estimate the size of the population by serial dilution plating on a selective TSA/10 medium containing tetracycline (15 $\mu\text{g/mL}$) and a nonselective medium TSA/10. Control plants were inoculated with 5 μL of sterile ultrapure water.

Plants were examined using a confocal laser scanning microscope (Olympus, Tokyo) equipped with krypton-argon lasers (488 nm, 568 nm, and 647 nm lines). GFP was excited with 488 nm (green channel) and Nile Red (red channel) with 568 nm wavelengths. Emissions were observed with appropriate filters (510–560 nm on green channel, 585–640 nm on red channel). Samples were examined in the fluorescence and in the transmission mode. Z-projections and optical sections were generated using the Fluoview software package. Images were further processed for display by using Photoshop software (Adobe, Mountain View, CA) and Image J (<http://rsb.info.nih.gov/ij/>) software. For the effect of AGP-inhibitors, 1 to 4 independent experiments were performed on 3 to 15 plants for each condition ($n = 15$ and 4 for no-AGP-disrupting drugs for 7 and 15-d-old plants, respectively; $n = 13$ for β -GlcY treatment; $n = 3$ for α -ManY treatment; and $n = 5$ for DHP treatment).

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