The *Rhizobium leguminosarum* biovar *trifolii* ANU794 Induces Novel Developmental Responses on the Subterranean Clover Cultivar Woogenellup

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The clover-nodulating *Rhizobium leguminosarum* bv. *trifolii* ANU794 initiates normal root-nodule development with abnormally low efficiency on the *Trifolium subterraneum* cv. Woogenellup. The cellular and developmental responses of Woogenellup roots to the site- and dose-defined inoculation of green fluorescent protein (*gfp*)-labeled cells of ANU843 (nodulation proficient) and ANU794 was investigated using light, fluorescence, and confocal microscopy. Strain ANU794-*gfp* induced three primordia types and four developmental responses at the inoculation site: true or aberrant nodules (on 5 and 25% of plants, respectively), hybrid structures (20% of plants), or lateral roots (50% of plants). The novel hybrid structures possessed nodule and lateral root-like features and unusual vascular patterning. Strain ANU794-*gfp* induces lateral root formation by stimulating pericycle cell divisions at all nearby protoxylem poles. Only true nodules induced by ANU794-*gfp* contained intracellular bacteria. In contrast, strain ANU843-*gfp* induced nodules only and lateral root formation was suppressed at spot inoculation sites. Primordium types were distinguishable by the emission spectrum characteristics of phenolic UV-absorbing and fluorescent compounds that accumulate in primordium cells. Hybrid primordia contained (at least) two fluorescent cell populations, suggesting that they are chimeric. The results suggest that ANU794 may produce both nodule- and lateral root-generating signals simultaneously.

Additional keywords: cultivar specificity, flavonoids, founder cells, Nod factor, nonheme chloroperoxidase, stem cells.

Fabaceae plants can engage in a symbiotic relationship with Rhizobiaceae soil bacteria resulting in nitrogen-fixing root-nodule formation. Nodule initiation involves two primary processes, root infection and nodule primordium induction. These processes occur predominantly in the developmentally receptive zone-of-elongation in legume roots (Bhuvaneswari et al. 1981; Mathesius et al. 1998a and b). Rhizobia gain entry into root hair cells via plant-derived infection threads that grow toward and into the primordium, carrying the rhizobia inside. On plants with indeterminate nodules, *Rhizobium*-derived Nod factors stimulate pericycle, endodermal, and inner cortical cells at proximal xylem poles to enter the cell cycle, divide, generate new symplasmic connections with phloem cells in the

stele, and form a primordium containing pluripotent stem cells (Complainville et al. 2003; Timmers et al. 1999). The infection threads meet the primordium cells and the bacteria are released from the infection threads via endocytosis into the plant cytoplasm of some meristematic-derived or submeristematic cells to form symbiosomes. The bacteria “differentiate” into bacteroids, begin to fix atmospheric nitrogen, and the nodule matures (Rolfe and Gresshoff 1998).

A feature of nodule and lateral root primordia of legumes and the main root and lateral root meristems of legumes and nonlegumes is that they accumulate fluorescent compounds during their formation (Buer and Muday 2004; Djordjevic et al. 1997; Mathesius et al. 1998a). In white clover and *Arabidopsis*, the fluorescent compounds are flavonoids (Buer and Muday 2004; Mathesius et al. 1998a), and flavonoid pathway induction is an early event in nodule and lateral root primordium formation (Djordjevic et al. 1997; Mathesius et al. 1998a). Flavonoids are the predominant accumulating fluorescent compounds in *Arabidopsis* root primordia and meristems because mutants devoid of flavonoids show negligible fluorescence (Buer and Muday 2004; Saslowsky et al. 2005). Another feature of legumes with indeterminate nodules is an overlap in the use of pericycle cells as founder cells for the formation of the lateral root and nodule primordia (Timmers et al. 1999). Nothing is known about the signals involved in the transition of nodule primordium cells to become a nodule meristem; however, the invasion of the nodule primordium by rhizobia is required for either the differentiation or the maintenance of the nodule meristem.

In this article, we investigate the cellular developmental responses of *Trifolium subterraneum* cv. Woogenellup in response to the inoculation two *Rhizobium leguminosarum* bv. *trifolii* strains (ANU843 and ANU794). Strain ANU843 forms normal nitrogen-fixing nodules on Woogenellup. In contrast, the nodulation efficiency of strain ANU794 is greatly reduced on this cultivar (de Boer and Djordjevic 1995). Our group has shown that a single recessive plant locus, *rwt-1*, determines this cultivar specificity and the “refractive-to-nodulation-by-strain-ANU794” phenotype (Lewis-Henderson and Djordjevic 1991a) and that *rwt-1* interacts with several genes in strain ANU794, including a gene involved in Nod-factor synthesis (*nodM*) and a nonheme chloroperoxidase (Lewis-Henderson and Djordjevic 1991b; Roddam et al. 2002). Nodule number can be partially increased by elevating the growth temperature or introducing *nodT* of ANU843 into strain ANU794 (strain ANU794 is naturally deleted for *nodT*), but not by the addition of ethylene synthesis inhibitors (de Boer and Djordjevic 1995; Lewis-Henderson and Djordjevic 1991a and b; Roddam et al.

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2002). In addition, introducing into ANU794 additional copies of *nodL* (from ANU843) or genes from *Sinorhizobium* spp. (e.g., pRmSL26) or *R. leguminosarum* bv. *viceae* (e.g., pIJ1089, pMP180, or pIJ1095) also partially enhances the nodulation efficiency of strain ANU794 on Woogenellup (W. Lewis Henderson, L. Roddam, and M. A. Djordjevic, unpublished observations). De Boer and Djordjevic (1995) showed that ANU794 induces the early stages of nodule initiation (root hair curling, root hair penetration, and initiation of cortical cell divisions); however, the nodule ontogeny program fails to proceed in most instances and infection threads rarely extend into the cortex. Furthermore, there was no evidence of a hypersensitive (cell death) response, although peroxidase activity was elevated. A proteomic analysis of Woogenellup roots to infection by strains ANU794 and ANU843 revealed that putative ethylene-responsive proteins and α -fucosidase were differentially upregulated by ANU794 (Morris and Djordjevic 2001).

In this article, spot inoculation of green fluorescent protein (*gfp*)-labeled ANU843 and ANU794 strains, serial sectioning of roots in the immediate vicinity of the inoculated bacteria, and microscopic observation of live cells were used to follow the cellular and developmental responses induced on cv. Woogenellup roots over time. The spot inoculation technique enabled the delivery of approximately 5,000 bacteria to the zone of maximum nodule development susceptibility. The inoculum rarely spreads from the site of inoculation; therefore, the response of naïve plants can be assessed in space and time because the inoculation site can be located easily and the responses of plant cells assessed. Indeed, all the observations recorded in the article are confined predominantly to the observation of a 1-mm² section of root. We found that young primordia cells that developed in the vicinity of the spot inoculum contained distinctive fluorescent compounds that allowed primordium types to be distinguished and their genesis to be examined. The autofluorescence of these endogenous compounds was enhanced by the addition of the flavonoid-specific dye, diphenyl-boric acid-2-aminoethyl ester (DPBA) (Brown et al. 2001; Buer and Muday 2004; Mathesius et al. 1998a). Fluorescence emission spectra were captured from single live primordium cells after the addition of DPBA. We show that novel hybrid organs are formed in strain ANU794–Woogenellup interactions and that lateral root formation is stimulated at the inoculation site. We examined the genesis of the hybrid organs and show that they arise from chimeric primordia with (at least) two different cell populations.

RESULTS

Strain ANU794-*gfp* induces four developmental responses on cv. Woogenellup.

The ability of *R. leguminosarum* bv. *trifolii* strains ANU843-*gfp* and ANU794-*gfp* to affect root morphogenesis on cv. Woogenellup was determined at the spot inoculation site at 6 and 10 days postinoculation (dpi). After 10 days,

strain ANU843-*gfp* almost exclusively induced nodule formation as expected; however, lateral root formation at the inoculation site was more than 20 fold lower than in the control (Fig. 1A; Table 1). The nodules induced by strain ANU843-*gfp* at 10 dpi were sectioned and showed the typical responses of indeterminate nodule development: i) a persistent meristem, ii) an invasion zone consisting of numerous infection threads with GFP-labeled bacteria and freshly infected cells, iii) a symbiotic zone where the infected cells were tightly packed with nitrogen-fixing bacteroids with strong GFP fluorescence, and iv) the development of several tissue types (data not presented). The nodules also contained leghe-moglobin pigment.

In contrast, strain ANU794-*gfp* induced four root developmental responses, but nodule or nodule-like development was slower than on ANU843-inoculated roots by up to 4 days. In 50% of the ANU794-*gfp* inoculated roots, lateral roots formed at the inoculation site. This was a 2.5-fold higher frequency compared with the control and more than 50-fold higher than at ANU843-*gfp* inoculation sites (Table 1). Strain ANU794-*gfp* induced lateral roots at proximal and (predominantly) distal protoxylem poles relative to the spot inoculation site (Fig. 1B and C). In 25% of roots, aberrant nodules were induced (Fig. 1B to D) that showed slow development and a lack of intracellular bacteria as indicated by the absence of GFP fluorescence. Slowly developing “hybrid structures” that were apparent by 6 dpi developed into a range of structures (Fig. 1E to H) devoid of intracellular bacteria. By day 10 dpi, 20% of the spot-inoculated roots had developed hybrids. A unique hybrid structure, with the most striking features being central vascular traces linking the structure to the main root and an overall nodule-like morphology, is shown in Figure 1E. The central vascular traces branch before extending into the hybrid structure. In addition, vascular traces appear in the apical region of the hybrid, where meristematic tissue normally is located in nodules. Another unique hybrid structure that forms more frequently than the one shown in Figure 1E is shown in Figure 1F. The base of the structure appears more nodule-like as it emerges from the main root; however, the structure has unusual vascular patterning and no detectable bacterial colonization. A lateral root is seen emerging from the hybrid and the lateral root possesses a more conventional side nodule. Sections of disorganized nodule-like tissue through which a lateral roots has grown is shown in Figure 1H and G, although in these structures, the organs are distinct from each other. These structures are more akin to those in Figure 1F. Finally, approximately 5% of the roots inoculated with strain ANU794-*gfp* developed a leghe-moglobin-containing, nitrogen-fixing nodule that contained intracellular bacteria and normal development similar to that induced by ANU843-*gfp* (data not shown).

The poor nodulation ability of strain ANU794-*gfp* was correlated with poor infection thread development. From an examination of over 100 spot inoculation sites at different times postinoculation, the number of observed infection threads that extended beyond the root hair was low (approximately 1 to 2%; Fig 1I), with most terminating in the root hair as described previously (De Boer and Djordjevic 1995). In contrast, at ANU843-*gfp* spot inoculation sites, infection threads that extended into the cortex toward foci of dividing cells were easily observed in nearly all samples.

Primordia development in response to inoculation.

In order to understand how novel hybrid organs, aberrant nodules, and lateral roots developed in response to ANU794-*gfp* inoculation, we examined the early stages of primordium formation on cv. Woogenellup inoculated with this strain, but

Table 1. Developmental outcomes at the site of inoculation at 10 days postinoculation^a

Phenotype	Outcome (%)		
	Strain ANU843- <i>gfp</i>	Strain ANU794- <i>gfp</i>	Control (sterile water)
Nitrogen-fixing nodules	99	5	0
Aberrant nodule	0	25	0
Lateral roots	<1	50	20
Hybrid structures	0	20	0

^a Data from several experiments involving the scoring of over 250 plants were pooled.

Fig. 1. Developmental responses of cv. Woogenellup roots to *Rhizobium* inoculation at 6 to 10 days post inoculation (dpi). **A**, Young nodule induced by ANU843-*gfp* with typical branched vascular traces (white arrowheads) at 6 dpi. **B** and **C**, Nodule and lateral root primordia (np and lrp, respectively) are induced by ANU794-*gfp* in the immediate vicinity of the inoculation site. Lateral root primordia are induced more frequently at protoxylem poles more distal to the inoculation site (i.e., on the opposite side of the root as in B). Original spot-inoculation site is to the left of the nodule primordium in B on the root surface. (**B**: longitudinal section, **C**: transverse section 6 dpi). **D**, Aberrant nodule (red arrow) at 6 dpi induced by ANU794-*gfp* showing slow development (i.e., same age as nodule in A). **E**, Novel ANU794-*gfp* induced hybrid structure (10 dpi) possessing central vascular traces growing from the main root that branch (red arrowhead) in the center of the hybrid. Short vascular traces occur at the apex (white arrowheads) where a nodule meristem normally would form. **F**, A second unique hybrid structure with unusual vascular patterning (red arrowheads) has emerged from the main root (10 dpi). At the apex of the structure, a lateral root has emerged which, in turn, possesses a small nodule with the typical branched traces (white arrowheads). **G** and **H**, Disorganized nodule-like growths occur at the same xylem pole as lateral roots. The lateral roots grow through the nodule-like tissue (10 dpi). **I**, Most infection threads initiated by strain ANU794-*gfp* terminate in the root hair and only rarely extend into the cortex (white arrow). Dividing cells with centrally located nuclei in an aberrant nodule primordium can be seen two cell layers from the end of the infection thread (below red arrowheads). Bars: A, 200 μ m; B, D, and E, 80 μ m; E, 150 μ m; F, 60 μ m; I, 40 μ m.

first compared this to the development of nodule primordia (on ANU843-*gfp*-inoculated plants) and lateral root primordia (on control plants). The fluorescence properties of cells in each primordium type were examined using laser scanning confocal

microscopy (LSCM) to determine whether this property could be used as a diagnostic marker. Intact cells in the thick sections remained alive for several hours and cytoplasmic streaming was readily observed (data not presented). Fluorescent nodule

Fig. 2. Fluorescence microscopy of nodule, lateral root, and hybrid primordium formation on cv. Woogenellup. **A** and **B**, Nodule primordia induced by ANU843-*gfp* at 48 and 24 h, respectively. The nodule primordia span the stele and inner cortex and, at this stage, appear anatomically similar to those of lateral root primordia (**C** and **D**) at a similar developmental stage. ANU843-*gfp* has induced two individual nodule primordia in **A** and the primordium in **B** is circled. **C** and **D**, Young lateral root primordia at comparable stages of development to 48- and 24-h nodules, respectively, are shown forming on control plants. Like nodule primordia, the cell divisions forming the lateral root primordia appear to span the stele and the inner cortex (circled in **D**). **E**, Representative intracellular fluorescence emission spectra of cells from nodule (i and ii) and lateral root (iii to v) primordia. The autofluorescence spectra obtained from nodule primordium cells are characteristic and reproducible (a more bell-shaped peak and λ max of 495 nm). Fluorescence emission spectra obtained from lateral root primordia cells have a λ max of 450 nm and distinctive shape with steep left shoulder and tapered right shoulder. **F**, **G**, and **H**, Representative primordia induced by strain ANU794-*gfp* on cv. Woogenellup are shown. A 24-h nodule primordium containing centrally located fluorescent nuclei (white arrowheads) is shown in **F**. **G** shows a lateral root primordium at 24 h (circled), whereas **H** shows a hybrid-type primordium at 48 h postinoculation (hpi). **I**, Three different emission spectra were recorded from specific cell clusters within an individual hybrid primordium. Nodule-type (i and ii) and lateral-root-type intracellular spectra (iii) were found in primordium cells closest to the root surface and stele, respectively. In addition, hybrid primordium cells in between these two clusters possessed a novel emission spectrum with a λ max of 480 nm (iv and v). **J**, Very young nodule primordium (between the red arrows) induced by ANU794-*gfp* at 12 hpi showing fluorescing and dividing pericycle cells (circled) and autofluorescent endodermal and inner cortical cells. The centrally located and fluorescing nuclei in inner cortical cells (white arrowhead) suggest that they are preparing for division. (p = phloem and x = xylem). **K**, Stele and inner cortex of a root inoculated with ANU794-*gfp* is shown after staining with Toluidine Blue. The ANU794-*gfp* microcolony is located on the upper left-hand corner on the root surface (not shown). A young nodule primordium (np) is induced at the proximal protoxylem pole and a young lateral root primordium (lrp) at the distal protoxylem pole. Pericycle cells at the nodule primordium are divided, as are pericycle cells at the two remaining protoxylem poles (red arrowheads), but these do not develop into organs. **A** through **D**, **G** through **I**, and **K** are transverse sections. All images except **K** were captured using a fluorescence microscope. **K** is a light micrograph stained with toluidine blue. The spectra in **E** and **I** were obtained using the laser scanning confocal microscope.

primordia induced by ANU843-*gfp* at 2 days and 24 h are shown in Figure 2A and B, respectively. However, the early stages of nodule primordium formation appear anatomically similar to those of similar stages of lateral root primordium development as seen on control (uninoculated) plants (Fig. 2C and D). Because of this difficulty in anatomically distinguishing very young nodule and lateral root primordia, we examined the fluorescent properties of the compounds that accumulated in live cells of nodule and lateral root primordia. Young lateral root and nodule primordia can be distinguished by the fluorescence properties of the compounds accumulating within the cells (Fig. 2E). Nodule cells generated a more bell-shaped curve and a λ maximum of 495 nm, and cells in young lateral root primordia generated an emission profile with a λ max of

450 nm with a steep left shoulder and a tapered right shoulder. Although the fluorescence emission intensity varied from cell to cell in each primordia type, the peak shapes and wavelength emission λ maxima were consistent across many measurements, demonstrating that fluorescence was a good diagnostic marker to distinguish very young primordia types.

As expected, three different primordia types were detected in response to ANU794-*gfp* inoculation and the frequency of each type reflected the developmental outcomes detected (Table 1). Young nodule primordia (Fig. 2F) that occurred at protoxylem poles proximal to the inoculation site possessed cells with a λ max of 495 nm and were indistinguishable from those induced by ANU843-*gfp*. In addition, centrally located nuclei in the cells also were fluorescent (Fig. 2F). The young lateral root primordia that occurred most frequently at distal protoxylem poles (Fig. 2G) were indistinguishable from control lateral root primordia. Hybrid primordia (Fig. 2H) possessed at least two cell subpopulations in distinct clusters (Fig. 2I). Primordia cells nearest the root surface emitted a nodule primordium cell fluorescence (λ max of 495 nm), cells nearest the stele emitted a lateral root primordium cell type fluorescence (λ max of 450 nm), and cells located in between emitted a unique spectrum with a λ max of 470 nm (Fig. 2I), suggesting that these primordia were chimeric. Pericycle, endodermal, and inner cortical cells responded to the inoculation of both *Rhizobium* strains as early as 8 to 12 h postinoculation (hpi) (Fig. 2J). All three cell types developed fluorescence, and pericycle cells were observed to have divided already in the periclinal plane as early as 8 hpi (circled in Fig. 2J). In addition, the nuclei of not-yet-divided inner cortical cells were positioned centrally and were fluorescent (Fig. 2F). Another feature of plants treated with ANU794-*gfp* was the induction of pericycle cell divisions at all four protoxylem poles in the vicinity of the spot inoculation site (Fig. 2K). This was not observed in ANU843-*gfp* treated roots. Pericycle cells that divided in response to ANU794 included those nearest the spot inoculum (involved in nodule primordium formation) and cells at the distal protoxy-

Table 2. Quantitative analysis of the responses root cells to inoculation with strains ANU843-*gfp*, ANU794-*gfp*, or water (control)

Inoculum, hpi ^b	Average number of fluorescing cells ^a		
	Peri/trans	Peri/div	Cort/div
Control			
2	0.7	0	0
8	0.6	0	0
12	8.9	0.3	0
24	16.2	3.4	0.9
ANU843- <i>gfp</i>			
2	0.5	0	0
8	9.2	0.4	0
12	>20	8.4	1.2
24	>20	9.2	3.2
ANU794- <i>gfp</i>			
2	0.5	0	0
8	8.3	0.3	0
12	>20	8.3	1.3
24	>20	14.7	3.3

^a Peri/trans = pericycle cells per transverse section, Peri/div = pericycle cells per transverse section that divide, and Cort/div = inner cortical cells per transverse section that divide.

^b Hours postinoculation (hpi).

Fig. 3. Detection of fluorescent compounds from cv. Woogenellup roots. C18 reverse-phase chromatography was used to separate and detect UV-absorbing (not shown) and fluorescent compounds extracted from Woogenellup root segments exposed to ANU843, ANU794, or a water control for 48 h. This exposure time was chosen because the time course conducted showed that primordium formation is established by this time. The most highly fluorescent and UV-absorbing material extracted from roots eluted between 3 and 5 min; however, late-eluting peaks were apparent at 46 and 48 min that did not differ between the samples. The spectrum for ANU843-treated roots is shown over 50 min. There was a reproducible difference in the ratio of the first and third eluting peaks in the treated samples that could account for the fluorescence difference seen using microscopy. The insets show a zoom of the early eluting peaks (3 to 5 min) for A, ANU843, B, ANU794, and C, water-treated samples. Hydrolysis of the sample resulted in an almost complete loss of the 3-to-5-min fluorescing material in all samples and an increase in peaks eluting between 43 and 48 min.

lem pole (engaged in the earliest stages of lateral root primordium formation); pericycle cells at the two remaining protoxylem poles were divided but did not form an organ (Fig. 2K). In some instances the entire pericycle layer was duplicated (data not presented).

Quantitative analysis of the responses of root cells to inoculation.

A quantitative analysis of the responses of root cells to inoculation was done. The spot inoculation site was limited to an area of approximately 1 mm², and the boundary of the microcolony of rhizobia on the root surface could be defined easily by the GFP fluorescence of the bacteria. This allowed spot-inoculated roots to be sectioned serially (basipetal to acropetal) and the sections of root specifically exposed to the bacteria to be observed in the order that they occurred in the root. These sections (approximately 10 per plant) were observed and three measurements were taken at 2, 8, 12, and 24 hpi: the average number of fluorescing pericycle cells per plant per section, the average number of fluorescing pericycle cells per plant per section that divided, and the average number of fluorescing and dividing inner cortical cells per plant per section. The results (Table 2) show that pericycle cells at proximal protoxylem poles could be induced to fluoresce and divide within 8 to 12 h of the inoculation of either *Rhizobium* strain. At 8 hpi, there was a 14- to 15-fold increase in pericycle cell fluorescence in *Rhizobium*-treated roots compared with control treatments and a 28-fold increase in the number of fluorescent and dividing pericycle cells at 12 h compared with controls (Table 2). Because transverse sections were observed, pericycle cell division in the periclinal (second division) plane only could be detected (Fig 2J). The increased incidence of stimulation of pericycle cells by rhizobia was maintained at the 24-h time-point. As expected, inner cortical cell fluorescence and cell division was enhanced by the inoculation of either strain, especially at 12 hpi (Table 2).

Analysis of root fluorescence compounds using high-performance liquid chromatography.

To determine the nature of the developmentally related fluorescent compounds in cv. Woogenellup root primordia, root segments exposed for 48 h to a flood inoculation (1 µl) of ANU843, ANU794, or a water control were excised and extracted. Some plants that remained unharvested were grown for a further 8 to 10 days. Examination of these roots confirmed that one to five nodules were induced on ANU843-treated plants in the harvested zone but few visible nodules were present on ANU794-treated plants. Lateral roots also were present on all treated plants in the harvested zone and had emerged by day 7. The extracted material was examined using high-performance liquid chromatography (HPLC; C18 reverse-phase column) (Fig. 3) and compared with the migration of a series of phenolic standards run under the same conditions. UV-absorbing and fluorescent peaks were analyzed. Although many peaks were detected, the majority of the fluorescence eluted in two broad areas the first 5 min and between 45 and 50 min of the separation, where the fluorescent flavonoid aglycone standards eluted (Fig. 3). There was no correlation with intensity of UV absorbance and fluorescence intensity in the peaks detected. There was no detectable unique fluorescence peak in the treatments, although the relative ratios of the early eluting peaks consistently differed between the treatments (Fig. 3 and insets). We reasoned that the fluorescent intracellular compounds were vacuole-located glycosides, as had been found previously (Mathesius et al. 1998a) and this was consistent with the early elution times recorded. To confirm this, the material was hydrolyzed, and this resulted in the disappearance of

major UV-absorbing and fluorescence peaks that eluted early; these peaks were replaced in the spectrum by an increase in the intensity of a heterogeneous and complex mix of late-eluting (between 40 and 50 min) UV-absorbing and fluorescing peaks (not shown). Again, there was no correlation between UV-absorbing intensity and fluorescence intensity. There was too much intra- and intersample variation to identify a unique peak in the hydrolyzed samples. However, the elution times for the late-eluting materials prior to and after hydrolysis was consistent with but not identical to those of several flavonoid aglycone standards.

DISCUSSION

Strain ANU794-*gfp* induced novel developmental responses on cv. Woogenellup roots.

Strain ANU794-*gfp* induced four developmental responses on cv. Woogenellup roots: lateral roots, hybrid structures, aberrant nodules, and true nodules. These structures arise from the formation of three types of primordia that can be distinguished by the fluorescent compounds that accumulate in their cells. To our knowledge, this is the only *Rhizobium*-legume interaction where this diversity of developmental responses occurs. Although aberrant nodules occur on flood-inoculated plants amidst true nodules, it is most unusual for a naïve plant to hinder the progress of spot-inoculated rhizobia. For example, with ANU843 spot inoculations, close to 100% nodulation success occur at spot inoculation sites (Mathesius et al. 1998a). Nevertheless, the likely distinction between aberrant and true nodules at ANU794 spot-inoculation sites may be simply the inability of the infection threads induced to penetrate through to the nodule primordium to permit bacterial release into the dividing cells as occurs commonly on aborted infections on flood-inoculated plants. This would result in the interruption of the nodule developmental program because invasion of the nodule primordium is required for full nodule development to occur. Therefore, one conclusion is that the inability of most infection threads to reach the induced primordia contributes to the generation of the aberrant nodule primordia that stall in their development. The inability of infection threads to penetrate the cortex and to reach the primordium is correlated with the low numbers of true nodules.

The analysis of primordium-specific fluorescent compounds indicates that the hybrid structures generated by ANU794 result from nodule and lateral root primordia being generated at the same protoxylem pole, forming at least two distinct primordium cell subpopulations—a chimeric primordium. This would explain why hybrids develop into structures with lateral root and nodule-like features, such as the central and peripheral vascular patterning or unusual vascular patterning seen in Figure 1E to H. The detection of the development of two population subtypes in young primordia has not been described previously. We could not determine whether some nodule primordia cells “transform” into lateral root primordium cells or whether the two subpopulations are generated simultaneously or independently by separate signals; however, the developmental destiny of each primordium correlates with the types of fluorescent compounds that they contain, and this may suggest that developmental commitment is determined early in primordium formation. It is possible that lateral roots arise sequentially at the same site as a result of the stalling of nodule formation and that lateral root development is normal from this point onward.

Does ANU794 secrete a lateral root-inducing signal?

It is clear that ANU794 cells, but not ANU843 cells, also can stimulate pericycle cells distal to the spot-inoculation site to divide and form lateral root primordia at high frequency on

the opposite side of the root. In some cases, complete duplication of the pericycle layer was observed in the immediate vicinity of the spot-inoculation site. One possibility is that ANU794 cells can generate a separate, unknown lateral root-inducing signal. If so, then the novel developmental programs that are induced in response to ANU794 may result from the interaction of two competing signals: Nod factors and a lateral root-generating signal. However, structures somewhat similar to the hybrids described here were observed by Ferraioli and associates (2004) on *Phaseolus vulgaris* roots in response to *R. etli* mutants. Their results suggest that determinate nodule primordia may be able to transform into lateral root primordia and, subsequently, a lateral root upon infection abortion and interruption of the complete nodule developmental program.

An obvious candidate for a strain ANU794 lateral root-generating signal is microbial auxin because soil bacteria are known to secrete auxin (Theunis et al. 2004). However, so far no known function for auxin production by rhizobia has been uncovered (Costacurta and Vanderleyden 1995). Auxin production by strain NGR234 is known to be regulated by genes controlled by a *nodD*-dependent *nod* box promoter and the resulting auxin accumulates in nodule tissues (Theunis et al. 2004). However, NGR234 mutants defective in auxin synthesis did not show a detectable nodulation phenotype. Interestingly, a mutation in a putative nonheme chloroperoxidase gene of ANU794 (mutant W78) partially restores nodulation on cv. Woogenellup (Roddam et al. 2002). These enzymes are known to chlorinate indole compounds and it is intriguing to speculate that chloro-IAA derivatives (highly potent auxins) might be synthesized by strain ANU794 and contribute to its ability to induce lateral roots. This possibility remains to be tested.

Pericycle cells are founder cells of both lateral-root and nodule-primordium formation.

We have shown, in subterranean clover, that pericycle cells as well as adjacent endodermal and inner cortical cells are founder cells for nodule-primordium formation, and that cell divisions that span the stele and the inner cortex contribute to the generation of pluripotent nodule primordia. Most models of nodule formation show that cortical cells contribute only to nodule primordium formation. Complainville and associates (2003) also showed that nodule-primordium generation in *Medicago truncatula* was similar to subterranean clover but slower than that observed in cv. Woogenellup. In nodule primordium generation in Woogenellup, pericycle cells fluoresced and divided as early as 8 to 12 hpi in the periclinal plane and prior to cortical cell division. Pericycle cell divisions (and fluorescence) induced by endogenous cues in control plants that generate lateral root primordia occurred more slowly than pericycle cell fluorescence and division triggered by rhizobia (Table 2). This suggests that the targeting of the pericycle cells near protoxylem poles is an important step in nodule-primordium generation. Indeed, a race between nodule-inducing Nod factors and lateral root-inducing endogenous signals may occur, and nodule induction may have to occur prior to the influence of endogenous signals for nodules to form efficiently. The pronounced suppression of lateral root formation by ANU843 at the inoculation site in favor of nodule formation (Table 1) supports this hypothesis, and this result has not been reported previously. The first pericycle division giving rise to lateral roots is anticlinal in a plane that cannot be observed in transverse sections (Casimiro et al. 2001); therefore, it is possible that the first periclinal divisions observed in nodule-primordium formation are the second division. If this is true for clover species, this indicates that pericycle cells respond even earlier to *Rhizobium* inoculation than detected here and that pericycle cells must enter and complete the cell cycle rapidly.

The induction and accumulation of fluorescent compounds in pericycle cells is correlated with the ability to generate primordia. We did not observe the cell division of any nonfluorescent pericycle cells. We could not determine whether fluorescence was necessary for primordium and organ formation; however, this would seem unlikely because *Arabidopsis* mutants devoid of fluorescent endogenous flavonoid molecules still are capable of forming lateral roots, although they do display developmental defects (Buer and Muday 2004). In addition, there were many more fluorescent pericycle cells present than lateral organs generated (Table 2). We propose that the accumulation of fluorescence compounds in pericycle and other root cells is a useful and distinguishing endogenous marker, indicating a highly responsive state for pericycle and other primordium-forming cells; however, it does not reliably indicate that a primordium or an organ will develop. In addition, the results in Figure 2 show that the accumulation of distinctive fluorescent compounds is an early step in the formation of different primordia.

Are the fluorescent compounds accumulating in Woogenellup primordium cells flavonoids?

Several data suggest that the distinctive fluorescent compounds observed in primordia are flavonoids. First, the natural autofluorescence of these compounds is greatly enhanced by the addition of DPBA, a dye thought to be flavonoid specific, and DPBA causes a shift of the endogenous cellular autofluorescence to higher wavelengths, which is characteristic of flavonoids (data not presented) (Mathesius et al. 1998a; Peer et al. 2001). The emission spectrum of plant cells was strongest in the 400- to 600-nm range, which is typical for flavonoids (Agati et al. 2002). Finally, we also used HPLC analysis to determine the nature of the intracellular fluorescing compounds accumulating in the different organs observed using microscopy. The major UV-absorbing and fluorescing compounds extracted from roots exposed to bacteria or the control treatment eluted early from the C18 reverse-phase column. This strongly suggested that they were low-molecular weight phenolic glucosidic derivatives. Mathesius and associates (1998a) found similar findings in white clover treated with rhizobia. There was a consistent difference in the relative ratio of the early eluting UV-absorbing and fluorescing compounds that could account for the differences in intracellular fluorescence found using LSCM (Fig. 3). There was no consistent difference in the late-accumulating compounds between the extracts of the different treatments. The hydrolysis of this sample strongly suggested that the early eluting fluorescing compounds were glucosides because there was a substantial shift in elution times for these peaks as they became more hydrophobic after hydrolysis (data not shown). The UV-absorbing and fluorescent properties and elution times were consistent with them being flavonoid or other phenolic compounds; however, we could not pinpoint which derivatives these might be because the fluorescing material was a complex, heterogeneous mixture. We concluded from these results that plant roots contain a complex mixture of UV-absorbing and fluorescing phenolic compounds. It is likely that the amount of material accumulating in specific cells in organ primordia was a small proportion of the overall total pool of fluorescing compounds found in roots. Therefore, to identify those compounds specifically accumulating in the cells of individual organs would require a specific microdissection strategy using, for example, laser microdissection instead of the approach used here. In addition, the fluorescence and UV-absorbing properties of the intracellular compounds measured with the LSCM in vivo and HPLC in vitro could not be compared reliably because these properties change depending upon the solvent and pH conditions used.

In *Arabidopsis*, intracellular and nuclear fluorescence has been shown to be due to de novo flavonoid synthesis in different subcellular compartments (Saslow et al. 2005). Mutants defective in chalcone synthase in *Arabidopsis* show greatly diminished cellular fluorescence and abolition of nuclear fluorescence (Buer and Muday 2004; Saslow et al. 2005). We also found that nuclei in primordia were fluorescent. The accumulation of fluorescent compounds in the primordia of several legumes and the nuclei of the main root meristem suggests that flavonoid accumulation in dividing cells might be a universal phenomenon in plants (Mathesius et al. 1998a). The observation of nodule primordium induction is relatively easy to pinpoint using spot inoculation. However, the same cannot be said of lateral root primordium induction. Where, how, and when specific pericycle cells are “selected” to become lateral roots remains one of the mysteries of root developmental biology. The induction and accumulation of fluorescence in pericycle cells destined to become primordia might be a useful tool to aid the study of lateral root formation in plants.

MATERIALS AND METHODS

Microbial and plant preparation.

Strains ANU794-*gfp* and ANU843-*gfp* were constructed using a triparental mating system with HB101(pTB93F Sp^r Cm^r) as a donor (Gage et al. 1996) and maintained on BMM agar plates (Rolfe et al. 1980) containing spectinomycin (100 mg liter⁻¹) at 28°C. Certified cv. Woogenellup seed (Seedgrowers Co-op Ltd, Hilton, South Australia) were germinated and grown as described previously (Morris and Djordjevic 2000).

Spot inoculation and embedding and sectioning of roots.

Bacteria from BMM plates were resuspended in water to an optical density at 600 nm (OD₆₀₀) of 0.6. The roots of 3-day-old seedlings were spot inoculated with 10 nl of bacteria using a microsyringe (depositing approximately 5,000 bacteria onto the developmentally susceptible zone of root elongation). The inoculum was confined to an approximately 1-mm² area of the root and all developmental responses reported occurred at this site at different times postinoculation (8 and 12 hpi and 1, 3, 6, and 10 dpi). Control roots were inoculated with sterile water. Plants then were transferred to a controlled-environment chamber (140 μmol m⁻² s⁻¹, 16 h of light, 70% relative humidity).

For every timepoint, a minimum of 30 roots was examined for each treatment and the experiment was repeated in triplicate. In addition, inoculated and uninoculated plants were set aside for 2 to 3 weeks in each experiment to assess the reliability of spot inoculation. One nodule usually resulted at the site of spot inoculation of strain ANU843-*gfp*, indicating minimal inoculum spread. Root segments (approximately 1 cm) that included the point of inoculation and tissue above and below the inoculation point were embedded in 3% agarose (Bio-Rad, Hercules, CA, U.S.A.) and sectioned (110 μm thick) using a vibratome (Lancer series 1000; Technical Products, International, St. Louis). For transverse sections, the root was sectioned progressively from the basipetal end toward the acropetal end of the root segment and the order maintained. Approximately 10 root sections spanned the spot-inoculation site (identified easily by the presence of a *gfp*-labeled bacterial microcolony on the root surface). The average number of pericycle or inner cortical cells that were fluorescent or divided was calculated by totaling the number of fluorescing cells in each plant and dividing by the number of sections cut to give the values in Table 2.

Microscopy and photography.

For light and fluorescence studies, live cells in fresh root sections were examined using a Zeiss microscope equipped

with epifluorescence optics, Zeiss Plan-NeoFluar UVFL series objectives, and a HBO50W mercury arc lamp. The filter set used was a UV-G365 comprising a G-365 exciter filter, FT395 dichroic mirror, and LP420 barrier filter. Images were captured on Fujichrome Provia 400 35-mm slide film. Spectral analysis of fluorescent cell contents was obtained on a Leica TCS SP2 laser scanning confocal microscope (Leica Microsystems, Heidelberg, Germany). Fluorescence was excited by an argon laser (Enterprise II-651; Coherent, Santa Clara, CA, U.S.A.) at 364 nm. Emission was scanned using a 10-nm bandwidth in 5-nm steps over an emission range of 400 to 700 nm with LambdaScan software (Leica Microsystems). For every cell type and timepoint, spectra were recorded from at least six different comparable cells per primordium. Sections were incubated for 5 min with DPBA (0.5% in 10 mM phosphate buffer, pH 6, containing 2% dimethyl sulfoxide and 1% sucrose) (Vogt et al. 1994) to enhance flavonoid-specific fluorescence in the cells within the root sections. For every timepoint, a minimum of 30 roots was examined. Cell walls of cortical cells showing high cell wall fluorescence were counter-stained with Toluidine Blue (pH 4.4) (O'Brien and McCully 1981) to diminish the high autofluorescence of the cell wall.

Extraction and analysis of fluorescent compounds from cv. Woogenellup.

Germinated seedlings of cv. Woogenellup with root radicles of 10 to 15 mm in length were placed on nitrogen-free Fahraeus medium plates (Morris and Djordjevic 2000) and inoculated at the root tip with 1 μl of ANU794 or ANU843 culture resuspended in sterile water to an OD₆₀₀ of 0.6, or with sterile water as a control. The inoculum was observed to flood the entire root tip region by capillarity. The position of the root tip was marked at the time of inoculation (0 time). After 48 h of growth (the same conditions as for spot inoculation above), the roots had grown 30 to 40 mm and a 10-mm section of root was excised below the position of the root tip at the time of inoculation, frozen immediately in liquid nitrogen, and crushed in a mortar and pestle. This root segment contained an unknown number of *Rhizobium*-induced primordia. Crushed tissue (100 mg) was extracted with 300 μl of 100% acetone. Representative plants on several plates were retained and grown for a further 8 to 10 days to score the level of nodulation in the harvested zone.

Extracts were centrifuged and the supernatant (40 μl) was analyzed by HPLC in duplicate. HPLC was performed with a Shimadzu LC-10 VP series HPLC on a C18 column (Alltima, 250 by 4.6 mm i.d.). The column was eluted (0.8 ml min⁻¹) with an acetonitrile (1% acetic acid) gradient from 10 to 40% (35 min) and from 40 to 88% (10 min). The column eluent was monitored for absorbance by diode array (190 to 800 nm) and for fluorescence (excitation, 365 nm; emission 450 nm). An extract (100 μl) was hydrolyzed in 6% HCl for 1 h in a boiling water bath and re-analyzed by HPLC as described above. Phenolic standards used included 3 hydroxy flavone, 5 hydroxy flavone, 6 hydroxy flavone, 4',7 dihydroxy flavone, 5,7 dihydroxy flavone, biochanin A, catechol, coumestrol, daidzein, ferulic acid, formononetin, genistein, liquiritigenin, luteolin, medicarpin, naringenin, and vanillin.

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