Development of Functional Symbiotic White Clover Root Hairs and Nodules Requires Tightly Regulated Production of Rhizobial Cellulase CelC2

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The establishment of rhizobia as nitrogen-fixing endosymbionts within legume root nodules requires the disruption of the plant cell wall to breach the host barrier at strategic infection sites in the root hair tip and at points of bacterial release from infection threads (IT) within the root cortex. We previously found that Rhizobium leguminosarum bv. trifolii uses its chromosomally encoded CelC2 cellulase to erode the noncrystalline wall at the apex of root hairs, thereby creating the primary portal of its entry into white clover roots. Here, we show that a recombinant derivative of R. leguminosarum bv. trifolii ANU843 that constitutively overproduces the CelC2 enzyme has increased competitiveness in occupying aberrant nodule-like root structures on clover that are inefficient in nitrogen fixation. This aberrant symbiotic phenotype involves an extensive uncontrolled degradation of the host cell walls restricted to the expected infection sites at tips of deformed root hairs and significantly enlarged infection droplets at termini of wider IT within the nodule infection zone. Furthermore, signs of elevated plant host defense as indicated by reactive oxygen species production in root tissues were more evident during infection by the recombinant strain than its wild-type parent. Our data further support the role of the rhizobial CelC2 cell wall–degrading enzyme in primary infection, and show evidence of its importance in secondary symbiotic infection and tight regulation of its production to establish an effective nitrogen-fixing root nodule symbiosis.

Mutualistic symbioses between nitrogen-fixing rhizobia and their legume plant hosts are of critical agronomic and environmental importance, allowing successful crop production in nitrogen-limited soils without the need for chemical N fertilizers. Rhizobia invade their plant hosts through colonization of intercellular epidermal spaces, crack entry at emerging lateral roots, or penetration into the root hairs via tubular structures called infection threads (IT) (Goormachtig et al. 2004; Subba-Rao et al. 1995). The later route is the best-characterized infection pathway, elicited by a complex molecular dialogue between both symbiotic partners. During early symbiotic stages, flavonoids exuded from legume roots induce the synthesis and secretion of lipochito-oligosaccharide signal molecules or nodulation factors (NF) in their cognate rhizobia upon the transcriptional activation of the nodulation (nod) genes (Gibson et al. 2008). In turn, NF trigger specific morphogenetic responses in the plant, such as root hair deformation and dedifferentiation of the cortical cells, which reactivate their mitotic activity to divide, thereby generating the nodule primordium as the precursor of the nodular meristem (Oldroyd and Downie 2008). Primary penetration of the microsymbiont into its legume host involves the degradation of the root hair cell wall restricted to a precise location. This highly localized hydrolysis of the plant host cell wall requires cell-bound cellulolytic enzymes produced by the symbiotic bacteria sandwiched within the typical “shepherd’s crook” structures of curled root hairs (Gage 2004; Robledo et al. 2008). Concomitantly, the root hair cell wall redirects its inward growth, resulting in the formation of the tubular IT, which continues its growth to the base of the epidermal cell and then toward the nodule primordium, in which invading bacteria proliferate. During secondary infection within the infection zone of developing host nodules, rhizobia are released by endocytosis from intracellular ramifications (infection droplet) of the IT into membrane-enclosed vesicles within the cytoplasm of some nodule cells. Released bacteria undergo a process of morphological differentiation into nitrogen-fixing competent bacteroids which, in the mature nodules, remain enclosed within the membrane vesicles (i.e., the symbiosome) of host origin. Detailed ultrastructural examinations indicate that the IT are unwalled at the points of bacterial release into the host cells (Jones et al. 2007; Subba-Rao et al. 1995). However, it remains to be elucidated whether these secondary infection sites arise by arrest of the cell wall synthesis during intracellular elongation of the IT or by local degradation of the IT wall catalyzed by enzymes of bacterial or plant host origin.

Nonetheless, the process of plant cell wall degradation during the primary and secondary infection events described above is predicted to be delicately balanced in order to preserve the integrity and viability of the host cells throughout the mutually symbiotic interaction and to maintain compatibility by avoiding the plant defense responses. During early stages of rhizobial infection, legumes exhibit transient, localized defense-like responses, suggesting that the host perceives rhizobia, like phytopathogenic bacteria, as intruders. Among plant defense-like responses detected in the Rhizobium spp.–legume symbiosis are the production of root hair peroxidase, salicylic acid (SA), and reactive oxygen species (ROS). The elevation in peroxidase activity, which requires pSym nod gene expression, is of short duration and confined to the localized site of incipient penetration.
through the small portal of entry at the primary infection site in the compatible rhizobia–host legume combinations, whereas it is more intense and of longer duration in incompatible combinations (Salzwedel and Dazzo 1993). Although there is evidence that the controlled production of ROS in response to rhizobial infection might be necessary for an effective establishment of the symbiosis (Cárdenas et al. 2008; Jamet et al. 2007; Ramu et al. 2002), an intensive antimicrobial oxidative burst like the one detected in aborted IT in alfalfa plants (Vasse et al. 1993) or the accumulation of H2O2 detected in response to incompatible rhizobia (Bueno et al. 2001; Salzwedel and Dazzo 1993) could interfere with rhizobial infection.

We have previously reported on a chromosomally encoded cell-bound cellulase (CelC2) from *Rhizobium leguminosarum* bv. trifolii ANU843 that fulfills an essential symbiotic role in the primary infection of white clover roots (Mateos et al. 2001; Robledo et al. 2008). This enzyme was purified and characterized as a 1,4-β-D-endoglucanase with high substrate specificity for the noncrystalline cellulose as exists in clover root cell walls. These features restrict its early symbiotic activity to the transmuro erosion of the isotropic apex of growing root hairs precisely at the point of the microsymbiont penetration into the host roots. That enzyme-catalyzed reaction is enhanced by the ability of Nod factors from *R. leguminosarum* bv. trifolii to disrupt the crystallization architecture of the growing clover root hair cell wall (Dazzo et al. 1996). Consequently, an ANU843 celC2 deletion mutant was found to be unable to breach the root hair wall at the primary infection site or to form IT when inoculated onto white clover seedlings, thus eliciting nonfixing nodule structures devoid of bacteria in its host plant (Robledo et al. 2008). In work presented here, we have used a gain-of-function approach to further explore the impact of the CelC2 activity on the infection process during development of the white clover–rhizobia symbiosis. Our findings suggest that a deregulation in the production and increased localized activity of this rhizobial CelC2 cellulase disrupts the balance between the plasticity of degradation and assembly of the host cell wall architecture during both primary and secondary plant infection required for development of a canonical effective root-nodule symbiosis.

**RESULTS**

**Overexpression of the celC2 gene in *R. leguminosarum* bv. trifolii ANU843.**

The wild-type ANU843 strain (ANU843wt) and its derivative ANU843CelC2* that overexpresses the celC2 gene were compared for their ability to hydrolyze carboxymethylcellulose (CMC), as revealed by three different sensitive methods previously reported to reliably detect this enzymatic activity (Fig. 1). The double-layer plate assay using culture supernatants and sonicated cell extracts as the source of enzymes detected no differences in CMC activity between ANU843 and ANU843 empty vector control (ANU843EV) strains, and showed that trans-overexpression of celC2 in strain ANU843CelC2* resulted in an increase of its carboxymethyl (CM)-cellulase activity, as inferred from the intensity and diameter of the clearing halos resulting from substrate hydrolysis (Fig. 1A). Despite this overproduction, most of the activity detected in ANU843CelC2* remained in the sonicated extract of pelleted cells rather than in the culture supernatant fluid, consistent with the cell-bound nature of CMC-degrading enzymes in *R. leguminosarum* bv. trifolii (Mateos et al. 1992). The quantitative 2,2-bichinonic acid (BCA) assay with whole-cell extracts further confirmed this result, revealing a threefold increase of the total CM-cellulase activity in ANU843CelC2* compared with its parent strain (Fig. 1B). Finally, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) separation (Fig. 1C) of proteins in the same cell extracts followed by their activity assay resolved in gels (Fig. 1D) un-

![Fig. 1. Carboxymethylcellulase activity of *Rhizobium leguminosarum* bv. trifolii strains detected by A, double-layer plate assay of 20-µl samples; B, quantitative enzyme 2,2-bichinonic acid assay; C, sodium dodecyl sulfate polyacrylamide gel electrophoresis (*CelC2*); and D, activity stain overlay. All the cell extracts were obtained from an equivalent number of cells and adjusted to contain equal amounts of total protein.](image)

![Fig. 2. Symbiotic phenotype of *Rhizobium leguminosarum* bv. trifolii strains. A, Whole-plant phenotypes. B to D, Shoot length, dry weight, and nitrogen content per plant. Data reported in graphs are the mean of 24 replicate samples per treatment. Three replicates per treatment were analyzed. Error bars indicate the standard deviation from the mean. Values followed by the same letter are not significantly different from each other at P < 0.01 according to Fisher’s protected least significant difference test statistics.](image)
equivocally demonstrated that the observed increase of CM-cellulase activity in ANU843C2 was due to the overproduction of the 31-kDa CelC2 enzyme.

Irregular symbiotic performance of the ANU843C2+ recombinant strain.

Typical 3-month-old white clover plants that were either uninoculated or inoculated with ANU843wt or with the celC2+-overexpressing recombinant strain are shown in Figure 2. ANU843- and ANU843EV-infected plants displayed a typical Fix+ symbiotic phenotype (Fig. 2A). In contrast, most plants inoculated with the ANU843C2+ recombinant strain exhibited a Fix− phenotype characterized by stunted growth (Fig. 2A), shorter shoots (Fig. 2B), less dry weight (Fig. 2C), and less N content (Fig. 2D) than plants inoculated with strains ANU843wt or ANU843EV.

Development of clover or wounded tomato and tobacco plants grown in nitrogen-containing media and inoculated with the ANU843C2+ strain was uncompromised (not shown). Together, these findings revealed a negative influence of the overproduction of the CelC2 cellulase in the outcome of the R. leguminosarum symbiosis with its cognate legume host white clover under N-limited conditions. However, this effect is specific to the symbiotic root–nodule interaction because it was not observed either under N-supplemented conditions that inhibit nodulation in white clover or in non-legume plants.

celC2 overexpression increased the competitiveness of R. leguminosarum bv. trifolii ANU843 on clover roots.

The frequencies of nodule occupancy by ANU843pGUS3 in co-inoculation assays with ANU843EV and ANU843C2+ in greenhouse experiments were tested. Two months after inoculation, 76% of the nodules analyzed were occupied by the strain containing the pJZC2 plasmid, which overexpresses the CelC2 cellulase. This proportion was significantly higher than that obtained with the ANU843EV control strain under the same conditions (48.5%). All infected nodules (100%) stained blue when ANU843pGUS3 was inoculated alone, verifying the plasmid stability during the course of this experiment. The results of this competitive assay indicated that an increase in cellulase CelC2 production allowed ANU843 to invade and occupy a higher proportion of nodules than the control strain.

The ANU843C2+ strain induces aberrant nodules in white clover roots.

All the nodules (16 ± 2.5 per plant) induced by both the ANU843wt and ANU843EV strains on clover roots were elongated, pink, and efficient in N2-fixation (Fig. 3A; Table 1). In contrast, the ANU843C2+ strain that overproduces CelC2 induced slightly fewer nodules (13 ± 2.6 per plant), including nodule-like structures that developed a marked necrotic zone in their distal region (Fig. 3E, white arrows; Table 1). The percentage of

Fig. 3. Nodule development in white clover. Shown are portions of nodulated roots inoculated with A to D, the wild-type ANU843 or E to H, the ANU843C2+ strain. A and E, Stereophotomicrographs of whole nodules (with distal necrosis [white arrows]). B, C, and D, Plant cells infected with wild-type bacteroids. Red arrows in D indicate the release of bacteria from the infection threads. Red square and circle areas in F within the aberrant nodule induced by the ANU843C2+ cellulase-overproducing strain are enlarged in G and H to show the naked bacteria (red arrows) and exaggerated size of infection droplets terminating infection threads (red arrows), respectively. Scale bars: 1 mm (A and E); 500 µm (B and F); and 16 µm (C, D, G, and H).

Table 1. Symbiotic phenotypes in white clover after inoculation with wild-type ANU843 or the CelC2-overproducing strain

<table>
<thead>
<tr>
<th>Inoculant strain</th>
<th>Noi+Nod/plantb</th>
<th>Aberrant nodules (%)b</th>
<th>Had+Hac/cm of plantb</th>
<th>Hot/cm of plantb</th>
<th>Inf/cm of plantb</th>
<th>IT width (µmb)</th>
</tr>
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<tbody>
<tr>
<td>ANU843EV</td>
<td>16 ± 2.50</td>
<td>0</td>
<td>3.15 ± 0.69</td>
<td>0.18 ± 0.08</td>
<td>1.11 ± 0.54</td>
<td>1.13 ± 0.06</td>
</tr>
<tr>
<td>ANU843C2+</td>
<td>13 ± 2.61</td>
<td>30</td>
<td>2.18 ± 0.29</td>
<td>3.82 ± 0.66</td>
<td>0.15 ± 0.04</td>
<td>2.62 ± 0.27</td>
</tr>
<tr>
<td>Uninoculated</td>
<td>0 ± 0</td>
<td>0</td>
<td>0.0 ± 0</td>
<td>0.12 ± 0.09</td>
<td>0 ± 0</td>
<td>...</td>
</tr>
</tbody>
</table>

a Phenotype designations: Noi (nodule primordia), Nod (emerged nodules), Had (moderate root hair deformations), Hac (marked curling of root hairs, the so-called “shepherd’s crook”), Hot (hole on the tip), and Inf (infection-thread [IT] formation within root hairs).

b Results reported are the mean ± standard error (SE) of at least 12 (Hac, Hot, and Inf) or 24 (Noi + Nod, Aberrant nodules) replicate samples per treatment.

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these aberrant nodules increased from 30 to 85% when the inoculum size was tripled.

To further define the effect of CelC2 overproduction on symbiotic development, we examined embedded nodule sections induced by ANU843wt and ANU843C2+4. Bright field light microscopy showed that wild-type nodules had the typical histology of indeterminate effective nodules with differentiated meristematic, infection, and bacteroid zones (Fig. 3B). Details of representative sections of the wild-type bacteroid zone containing intact plant cells occupied with differentiated bacteroid endosymbionts are shown in Figure 3C. Host cells and the typical wild-type Bar phenotype are shown in Figure 3D. In contrast, sections through the interior of nodules induced by ANU843C2+4 (Fig. 3F) exhibited less differentiation of vegetative bacteria into bacteroid states within plant cells (Fig. 3G), presence of free bacteria (Fig. 3G), and IT that terminated with larger than normal and more irregularly shaped infection droplets in which the bacteria are released (Bar) during secondary host infection (Fig. 3H). To quantify the difference in morphology of infection droplets within nodule sections, 24 induced by the wild type and 24 by ANU843C2+ were examined further by two-dimensional CMEIAS image analysis using two measurement attributes: size (area and perimeter) and two attributes of shape (roundness [proximity to a circle] and fiber length [degree of discrete elongated protrusions along the contour of an object]). Multivariate analysis of variance (ANOVA) indicated a statistically highly significant (P = 0.0000000000000004) difference between the populations of infection droplets induced by the wild type and the CelC2-overproducing strain for all four measurement attributes combined. Univariate ANOVA indicated that the difference between the two populations for each individual size and shape measurement attribute was also statistically highly significant (Table 2). These quantitative microscopy results confirmed that the infection droplets made by the ANU843C2+ cellulase-overproducing strain were larger in size and more multilobed in shape than were infection droplets made by the wild type.

In summary, overproduction of the CelC2 cellulase impaired normal nodule organogenesis in clover roots, resulting in abnormal nodule structures that nonetheless are invaded by differentiated CelC2-overproducing bacteria.

Cellulose architecture of white clover roots.

We previously reported that the most intense cellulosic activity of the purified rhizobial CelC2 enzyme applied to white clover roots is restricted to the localized sites of the noncrystalline cellulose wall at the isotropic apex of root hairs (Hot phenotype), matching the primary portal of entry of the microsymbiont into the plant (Mateos et al. 2001; Robledo et al. 2008). To identify other potential sites for CelC2 activity, we analyzed the crystalline architecture of white clover cell walls in developing nodules upon infection with ANU843wt (Fig. 4A to D). Polarized light microscopy of epidermal and vascular cells of white clover roots confirmed the widespread distribution of plant cell wall crystallinity (Fig. 4A), except at the tips of the growing root hairs (Fig. 4B, red arrows). Polarized light microscopy of nodule sections within the infection zone showed that the IT wall synthesized de novo upon ANU843wt infection also exhibits a crystalline architecture (Fig. 4C and D, red arrows) except at localized regions, where infection droplets develop (Fig. 4C and D, dark isotropic bulges punctuated between the bright crystalline IT).

Because of the restricted activity of cellulase CelC2 (Robledo et al. 2008), we predicted that only noncrystalline regions of plant walls can be hydrolyzed by this enzyme and, therefore, plant cell wall degradation by CelC2 would be restricted to localized erosion at tips of root hairs and areas of IT in which infection droplet will develop, rather than massive (systemic) plant cell wall destruction.

Effect of CelC2 overproduction on primary infection.

Root hair deformation and IT formation assays were performed on white clover plants inoculated with cells of the wild-type ANU843 on clover root hairs after 12 h of enzyme incubation. To further define the effect of CelC2 overproduction on symbiotic development, we examined embedded nodule sections induced by ANU843wt and ANU843C2+4. Bright field light microscopy showed that wild-type nodules had the typical histology of indeterminate effective nodules with differentiated meristematic, infection, and bacteroid zones (Fig. 3B). Details of representative sections of the wild-type bacteroid zone containing intact plant cells occupied with differentiated bacteroid endosymbionts are shown in Figure 3C. Host cells and the typical wild-type Bar phenotype are shown in Figure 3D. In contrast, sections through the interior of nodules induced by ANU843C2+4 (Fig. 3F) exhibited less differentiation of vegetative bacteria into bacteroid states within plant cells (Fig. 3G), presence of free bacteria (Fig. 3G), and IT that terminated with larger than normal and more irregularly shaped infection droplets in which the bacteria are released (Bar) during secondary host infection (Fig. 3H). To quantify the difference in morphology of infection droplets within nodule sections, 24 induced by the wild type and 24 by ANU843C2+ were examined further by two-dimensional CMEIAS image analysis using two measurement attributes: size (area and perimeter) and two attributes of shape (roundness [proximity to a circle] and fiber length [degree of discrete elongated protrusions along the contour of an object]). Multivariate analysis of variance (ANOVA) indicated a statistically highly significant (P = 0.0000000000000004) difference between the populations of infection droplets induced by the wild type and the CelC2-overproducing strain for all four measurement attributes combined. Univariate ANOVA indicated that the difference between the two populations for each individual size and shape measurement attribute was also statistically highly significant (Table 2). These quantitative microscopy results confirmed that the infection droplets made by the ANU843C2+ cellulase-overproducing strain were larger in size and more multilobed in shape than were infection droplets made by the wild type.

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type strain of *R. leguminosarum* bv. *trifolii* ANU843, ANU843EV, or the recombinant strain that overproduces CelC2 (Fig. 4E to Q; Table 1). As predicted, an inoculum of ANU843C2+ cells induced various alterations at the root hair tip that included i) canonical primary infection stages, as occur in the wild type or ANU843EV with deformations (Fig. 4E; Table 1), markedly curled shepherd’s crooks (Fig. 4F; Table 1), and IT formation (Fig. 4G; Table 1) and ii) the Hot phenotype, as occurs in response to purified CelC2 enzyme (Mateos et al. 2001; Robledo et al. 2008), in which the wall at the tip of some growing root hairs is degraded to produce a hole at this location (Fig. 4H; Table 1), resulting in the extrusion of the root hair protoplast or cytoplasm (Fig. 4I). Although root hairs without tips can be found at very low frequency in uninoculated plants (0.12 ± 0.09 per centimeter of root) or after inoculation with the wild-type strain (0.18 ± 0.08 per centimeter of root), this feature is 21- to 31-fold more frequent after ANU843C2+ inoculation (3.82 ± 0.66 root hairs/cm). Confocal microscopy of green fluorescent protein (GFP)-tagged bacteria showed that ANU843C2+ entered the wide opening at the tip of the dead root hairs with extensive wall degradation but they did not form normal IT (Fig. 4J). Sometimes, new cell wall material was deposited before transmural erosion of the original root hair apex, followed by redirection of new tip growth displaced at a vertex angle of 119 ± 11 degrees from the longitudinal cell axis and development of a branch in the root hair that is again susceptible to hydrolysis at its new growing tip (Fig. 4K to Q). These branched redirections of root hair growth were also frequently observed on axenic clover roots after 12 h of incubation with purified cellulase CelC2 (Fig. 4R). These features show that the CelC2 cellulase-overproducing strain evokes severe aberrant phenotypes in root hair development, disrupting the balance between biosynthesis and degradation of its host’s cell wall.

**Effect of CelC2 overproduction on secondary infection.**

Because regions of IT that develop into infection droplets exhibit a noncrystalline wall (Fig. 4D), we predicted that CelC2 may be involved in infection droplet development. To examine whether CelC2 has a symbiotic function during the secondary infection process, we monitored both ANU843wt and CelC2-overexpressing strain behavior within nodules by transmission electron microscopy (TEM). The ANU843wt strain followed the normal steps of the infection process: dissemination of bacteria within walled IT within plant cells (Fig. 5A and B), localized hydrolysis of the IT wall (Fig. 5C, arrow), movement of the vegetative bacteria into membrane-enclosed infection droplets that protrude into the host cytoplasm (Fig. 5C), and endocytosis of bacteria from infection droplets followed by their differentiation into nitrogen-fixing competent bacteroids, forming the nitrogen-fixing symbiosomes (Figs. 3C and 5J).

Secondary host infection by the recombinant strain ANU843C2+ included steps that were similar to and others that were different from ANU843wt. Bacterial progression through the IT was similar to the wild type (Fig. 5D), except for more frequent areas of localized hydrolysis resulting in an increase in diameter of the lumen and infection droplets that were larger, with prominent multilobed protrusions (Fig. 5E, G to I), consistent with results obtained by light microscopy (Fig. 3H; Table 2). Oftentimes, vegetative cells of the CelC2-overproducing strain were released into the host cells without being surrounded by a peribacteroid membrane or bacteroid differentiation (Fig. 5F and K). The cell wall of nodule cells containing naked vegetative bacteria showed significant deformation accompanied by thinning and breakage, indicating significant disruption of this host barrier (Fig. 5K). Thus, invasion of host nodule cells by the CelC2-overproducing strain was uncontrollable, with plant cell wall disorganization and absence of well-defined symbiosome formation (Fig. 5K) leading to senescent-like nodules that do not fix nitrogen. In summary, these observations indicate that the CelC2 cellulase could intervene in bacteria liberation from IT and that its in situ concentration and activity must be critically regulated to advance successful symbiotic development. Thus, an excess of CelC2 production leads to an aberrant, exaggerated Bar phenotype of bacterial release without peribacteroid membrane enclosure, followed by induction of nodule premature senescence and eventual abortion of the symbiotic nitrogen-fixing process.

**CelC2-overproducing strain of *R. leguminosarum* bv. *trifolii* induces higher accumulation of ROS than does the wild-type strain.**

Sensing of cell wall integrity may be one of the mechanisms used by plants to detect pathogen attack and, in response, activate a variety of defenses such as the production of ROS or SA aimed at limiting pathogen ingress (Hemati et al. 2009). Our findings described above suggest that the overproduction of the CelC2 cellulase by *R. leguminosarum* bv. *trifolii* would sufficiently disrupt plant cell wall integrity to activate severe plant defenses that, in turn, impair the establishment of an effective symbiosis. To test this hypothesis, SA and ROS accumulation were measured at different time intervals following inoculation of *Trifolium repens* with *R. leguminosarum* bv. *trifolii* ANU843wt, ANU843EV, or ANU843C2+ interactions. No significant differences in SA accumulation were detected in clover plants inoculated with the CelC2-overexpressing strain compared with those inoculated with the control strain harboring the empty vector. However, fluorescence microscopy using the ROS-sensitive dye 2′,7′-dichlorodihydrofluorescein-diacetate (H2DCFDA) detected a higher percentage of root hairs showing fluorescence indicative of this defense response marker in plants inoculated with the CelC2-overexpressing strain than in those treated with ANU843EV at different time points and locations (Fig. 6J).

We observed that ANU843wt can elicit soft signs of oxidative burst on clover at the early and late stages of symbiotic development (24 h and 25 days after inoculation, respectively).

**Table 2.** Differences in morphology of infection thread (IT) droplets made during secondary host infection of white clover nodules by wild-type (wt) ANU843 and the CelC2-overproducing strain ANU843C2+.

<table>
<thead>
<tr>
<th>Attribute</th>
<th>ANU843wt</th>
<th>ANU843C2+</th>
<th>Fold difference (C2+:wt)</th>
<th>Interpretation (C2+)</th>
<th>Significance (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (µm²)</td>
<td>69.34</td>
<td>540.88</td>
<td>↑ 7.80</td>
<td>Larger</td>
<td>5.250 x 10⁻⁹</td>
</tr>
<tr>
<td>Perimeter</td>
<td>36.9</td>
<td>137.9</td>
<td>↑ 3.74</td>
<td>Larger</td>
<td>2.000 x 10⁻⁹</td>
</tr>
<tr>
<td>Roundness</td>
<td>0.6355</td>
<td>0.3729</td>
<td>↑ 0.59</td>
<td>Less circular</td>
<td>1.013 x 10⁻¹¹</td>
</tr>
<tr>
<td>Fiber length</td>
<td>14.79</td>
<td>61.28</td>
<td>↑ 4.14</td>
<td>More multilobed</td>
<td>4.550 x 10⁻¹¹</td>
</tr>
</tbody>
</table>

a Number of droplets = 24 from wt nodule sections and 24 from CelC2+ nodule sections. Data extracted from digital images using CMEIAS image analysis.

b Area and perimeter measure size, roundness measures the degree of circularity for an object, and fiber length measures the degree that discrete elongations protrude along the contour of an object.

c Univariate analysis of variance significance.
The early time course is similar to the transient elicitation of peroxidase activity that occurs after inoculation of clover with strain ANU843wt (Salzwedel and Dazzo 1993). Moreover, this biphasic oxidative burst matches the already described response in the compatible Sinorhizobium meliloti–Medicago truncatula interactions, in which ROS transiently and weakly accumulate during the initial steps of the interaction between those symbionts (Santos et al. 2001). Therefore, ROS are thought to con-
tribute to the infection process (Salzwedel and Dazzo 1993), linked to the NF’s signaling transduction pathway (Ramu et al. 2002). However, the time course of ANU843C2+-activated ROS production increases to a higher level than after inoculation with ANU843EV or the uninoculated control (Fig. 6J). This increase in ROS activity has been also reported in incompatible symbiosis between rhizobia and non-host legumes (Salzwedel and Dazzo 1993).

Clover plants inoculated with ANU843C2+ also displayed a stronger and more prolonged emission of ROS-mediated fluorescence in its cortical, meristematic, and root hair cells (Fig. 6B and E) than when inoculated with ANU843EV (Fig. 6A and D) or uninoculated (Fig. 6C and F). Quantitative color segmentation and image analysis of in situ cumulative area-weighted densitometry indicated that the intensity of ROS fluorescence was 1,167-fold higher in plant roots inoculated with the CelC2+-overproducing strain. One week after inoculation, the oxidative burst was especially localized at the root hair tip matching the distribution of cellulase CelC2 substrate, suggesting that the localized action of this rhizobial enzyme may possibly contribute to the elicitation of the oxidative burst by the bacteria on the host cell (Fig. 6E).

It has been reported that abundant H₂O₂ associated with degrading bacteroids is localized in the nodule senescent zone (IV), reflecting the oxidative stress that ensued during nodule senescence (Rubio et al. 2004). We have observed signs of a weak oxidative burst in senescent zones of 2-week-old nodules on clover plants inoculated with the wild-type strain (Fig. 6G); however, it seems that there is a massive production of ROS in the invasion zone of nodules occupied by the ANU843C2+ recombinant (Fig. 6H), showing that the uncontrolled hydrolysis of the noncrystalline zone of the IT cell wall in which the bacteria are released is concomitant with ROS production.

In both treatments, the Rhizobium spp.-induced fluorescence was inhibited by diphenylene iodonium (DPI) (Fig. 6I), suggesting that the detected fluorescence is the consequence of ROS accumulation after activation of a plant NADPH oxidase (Mazel et al. 2004), and that ANU843C2+ induces higher accumulation of ROS as a defense response than does ANU843EV.

Taking into account these elevated ROS responses in plants inoculated with the ANU843C2+ overexpression recombinant strain, it seems probable that this strain is recognized as an invasive pathogen by clover plants at certain stages of the nodulation process.

**DISCUSSION**

Root hairs and pollen tubes are the best-studied examples of plant cells that elongate through the process of tip growth. The polar growth during the elongation phase occurs through the loosening of cell wall components in specific zones of the growing root hair (i.e., just behind the root hair apex). In this way, root hair growth can be limited to only the tip, with the majority of the cell wall at the flanks of the root hair reinforced by crystallization to prevent expansion (Knight 2007). Infection threads develop from growing root hairs and are also thought to be tip-growing structures and, therefore, are most likely to elongate by recruiting at least some of the machinery involved in root hair growth before infection (Gage 2004). Time-lapse microscopy supports this hypothesis by indicating that root hairs stop elongating when IT form, and elongation of both structures ceases when the hair nucleus is displaced from the growing tip (Fähraeus 1957; Nutman et al. 1973). The tip of the developing IT is a site of new wall and membrane synthesis, and has been proposed to involve inversion of the tip growth that is normally exhibited by the root hair and to be the result of the reorganization of cellular polarity (Gage 2004). Intracellular infection of legume host cells begins when rhizobia become entrapped between adjacent root hair cell walls within the shepherd crook structure. The entrapped microcolony of rhizobial cells apparently generates, inter alia, a high local concentration of cellulase that may be required in order to reach a threshold concentration for localized degradation of the cell wall, generating a point of intrusion. At the same time, the continued growth and division of entrapped bacteria generates an inward pressure counteracting the turgor pressure of the host plant cell and avoiding the extrusion of its protoplast. Elevation of peroxidase activity at that same location of incipient penetration likely cross-links some residual wall polymers of the host root hair cell, thereby aiding to “heal the wound” and maintain protoplast integrity to avoid its lysis (Salzwedel and Dazzo 1993). Entrapped live bacterial cells are apparently a requirement for the initiation of an inwardly growing IT at the center of a curled root hair cell (Gage and Margolin 2000). It is still not clear how an IT is initiated but several elements in the process can be identified as a result of genetic and cytological analysis (Brewin 2004). Our results show how one of these steps, plant cell wall rupture, must be tightly regulated in order to generate a successful intracellular infection via the IT. The absence of CelC2 enzyme activity blocks initiation of IT (Robledo et al. 2008) whereas an excess of this cellulase activ-

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**Fig. 6.** Confocal microscopy showing reactive oxygen species (ROS) production (green fluorescence) in *Trifolium repens*. Images showing root meristem and root hairs of plants 8 days after inoculation with strains A and D, ANU843 empty vector control (ANU843EV); B and E, ANU843C2+; or C and F, uninoculated; and nodules induced by G, ANU843EV and H, ANU843C2+ in plants 15 days after inoculation. I, Involvement of NADPH oxidase in ROS production of seedlings inoculated with ANU843C2+ was indicated by decreased fluorescence brightness following addition of diphenylene iodonium (DPI) inhibitor. Representative experiment from three with similar results is shown. J, Percentage of root hairs expressing the ROS response at different time intervals after inoculation.
ity reduces the number of epidermal IT. This tight regulation seems crucial not only in primary infection within root hairs but also in secondary infection within the nodule. When the bacteria reach the nodular infection zone, they must be internalized within nodule cells and establish their N-fixing niche. In a target plant cell, each bacterium is endocytosed in an individual, unwalled membrane compartment that originates from the infection droplet outgrowth of the IT. Once the released bacteria have been engulfed within host cell membranes, they must survive within the symbiosome compartment and differentiate into the N2-fixing bacteroid form. An overproduction of CelC2 likely leads to an elevated loosening of cell wall at the tip of the IT, forming larger, protruding, multilobed infection droplets that cannot retain the pressure of the IT lumen and eventually rupture. As a consequence, naked bacteria are released into the interior of nodule cells. Without surrounding peribacteroid membranes, bacteria cannot complete the bacteroid differentiation program that includes expression of the nitrogenase complex enzymes and, thus, cannot begin to fix N2. Furthermore, these naked bacteria presumably continue to overproduce CelC2 cellulase, causing extensive destruction to the host cell wall and ultimate lysis from within, eventually inducing a premature senescence of the nodule.

There are two host-plant physical defense barriers: the cell wall (in direct contact with external bacteria) and the cytoplasmic membrane (surrounding the inward-growing IT). An effective symbiosis needs to maintain integrity of the cytoplasmic membrane surrounding the IT. However, overt root hair cell wall hydrolysis could lead to loss of turgescence and destruction of its cell membrane. Without the presence of the overlapping shepherd crook, the hole made by CelC2 cellulase at a root-hair tip leads to extrusion of a large portion of the protoplasm (the Hot phenotype) which ultimately lyses, killing the root hair cell. However, during bacterial release from IT (secondary infection), overt hydrolysis of the noncrystalline cell wall leads to bacterial “intrusion” in the nodule cells. Both effects abort the infection process: the first one blocks normal development of functional IT and the second one induces premature nodule senescence.

Our results document the crucial role of the Rhizobium cell-bound CelC2 enzyme in the root-nodule symbiosis with legumes, and identify these enzymes as biotechnological targets to optimize the balance between endophytic, endosymbiotic, and pathogenic states in rhizobial biofertilizers. Confocal microscopy of roots showed that ANU843C2

DNA methods and construction of ANU843 derivative strains.

Plasmid DNA preparation and recombinant DNA techniques were performed according to standard procedures (Sambrook et al. 1989). To generate the recombinant ANU843C2

### Table 3. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Description*</th>
<th>Source or reference</th>
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<tr>
<td><strong>Rhizobium leguminosarum</strong> bv. trifoli</td>
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</tr>
<tr>
<td>ANU843</td>
<td>Wild-type Nod* Fix*</td>
<td>Rolfe et al. 1980</td>
</tr>
</tbody>
</table>
| ANU843Ev | Control derivative of wild-type ANU843 containing the empty vector pBBR1MCS-2; Km
| This work |
| ANU843C2 | ANU843 containing plasmid pJZC2 expressing celC2; Km
| This work |
| ANU843pgus | ANU843 containing plasmid pGUS3; Km
| This work |
| ANU843GFp | ANU843 containing pH60; Tc
| This work |
| ANU843C2pGFp | ANU843C2 containing pH60; Km
| This work |
| *Escherichia coli* | | |
| DH5α | Host for cloning | Sambrook et al. 1989 |
| S17.1 | thi pro hasR– hasD+ recA RP4 2-2c::Mu- Km::Tn7 (Sp'Sm') | Simon et al. 1983 |
| **Plasmids** | | |
| pGEM-T easy | Amp', M13ori pBR322ori, linear T overhangs vector | Promega Corp. |
| pBRR1MCS-2 | Km', lacPOZ mob, cloning vector | Kovach et al. 1994 |
| pJZC2 | CelC2 encoding fragment cloned into the Xhol/Sall site of pBRR1MCS-2 | Robledo et al. 2008 |
| pGUS3 | pnfE-gusA translational fusion in pB1101; Km
| Garcia-Rodríguez and Toro 2000 |
| pH60 | pH41 derivative containing the gfp gene constitutively expressed; Tc
| Cheng and Walker 1998 |
| pRK213 | Helper plasmid for mobilization with replicon ColE1; Km
| Figurski and Helinski 1979 |

* Km', Tc', Sp', Sm', and Amp' indicate resistance to kanamycin, tetracycline, spectinomycin, streptomycin, and ampicillin.
was mobilized into *R. leguminosarum* bv. trifolii ANU843 by a triparental mating using pRK2013 as a helper, GFP- or β-glucuronidase (GUS)-tagged bacteria were similarly constructed by conjugation of plasmids pHC60 or pGUS3, respectively, into the corresponding recipient strains (ANU843 or ANU843C2*). These plasmids have been shown to be stable in long-term rhizobia–legume symbiotic experiments (van Dillewijn et al. 2001).

**Enzyme assays.**

Cellulase activity in sonicated extracts of centrifuged bacterial cultures standardized to cell density was examined by a double-layer plate enzyme assay using Congo red staining as previously described (Mateos et al. 1992). Briefly, a bottom layer containing 15 ml of 0.7% agarose in water was overlaid with 5 ml of 0.2% (wt/vol) CMC and 0.5% agarose in 50 mM potassium phosphate-citric acid buffer (pH 5.2). Plates were inoculated by adding 20 µl of samples containing equal amounts of total protein and were incubated at 37°C overnight. To detect CM-cellulase activity, plates were flooded with 0.1% Congo red solution for 30 min and then rinsed several times with 1 M NaCl. This procedure revealed clear areas of cellulase-catalyzed substrate hydrolysis. Cellulase overexpression was confirmed by a reducing sugar assay using BCA and SDS-PAGE followed by activity stain overlay (Jiménez-Zurdo et al. 1996; Mateos et al. 1992). One unit of enzyme activity (U) is defined as the amount releasing 1 nmol of reducing sugar equivalent per minute at 40°C using CMC as substrate.

**Plant growth conditions.**

White clover (*T. repens* L.) seeds were surface sterilized, germinated, and grown gnotobiotically in tube culture as previously described (Robledo et al. 2008). For long-term greenhouse experiments, surface-sterilized white clover seed were germinated for 2 days in humid air on inverted plates of nitrogen-free Fähraeus agar medium (Fähraeus 1957) at room temperature in the dark. Seedlings with straight roots were transferred to pots containing sterile vermiculite as support. After 48 h, each plant was inoculated with 1 ml of a bacterial suspension (optical density at 600 nm [OD600] of 0.5; 4 × 10⁸ bacterial cells) in nitrogen-free Fähraeus medium (Fähraeus 1957). Pots were watered as needed and fertilized once every week with either nitrogen-free Fähraeus nutrient solution or half-strength Hoagland’s number 2 (Sigma-Aldrich) complete plant growth medium. Plants were harvested 3 months after inoculation to determine the shoot length, dry weight, and nitrogen content of the aerial parts according to standard AOAC methods (Johnson 1990). Data were analyzed by one-way ANOVA, with the mean values compared using Fisher’s protected least significant difference (*P* = 0.01). For competition assays, germinated clover plants were inoculated with bacterial suspensions containing 1:1 mixtures of ANU843EV/ANU843pGUS3 or ANU843C2*/ANU843pGUS3. Two months after inoculation, plant roots from each of the three replicates per treatment were pooled and stained to detect GUS activity in nodules after incubation of roots in X-Gluc solution (1 mM 5-bromo-4-chloro-3-indolyl-D-glucuronic acid [X-Gluc], 1% [wt/vol] SDS, and 50 mM sodium phosphate buffer [pH 7.5]) at 37°C in the dark (van Dillewijn et al. 2001). Nodule occupancy was assessed by blue (ANU843pGUS3 occupied) and white (ANU843EV or ANU843C2* occupied) nodule counting.

For the analysis of symbiotic phenotypes plus ROS and SA responses, seedlings with straight roots were transferred to the surface of 1.5% agar plates containing nitrogen-free Fähraeus medium and overlaid with Whatman number 1 sterile filter paper wetted with 4 ml of sterile water. The plates were incubated vertically at room temperature in the dark for 48 h and then transferred to a plant-growth chamber programmed for a 16:8-h photoperiod. After 5 days, plants were inoculated with a suspension (OD₆₀₀ of 0.5, 80 μl/plant) of 5-day-old *Rhizobium* culture grown on YMA for 40 days.

**Microscopy.**

Root hairs were examined by phase-contrast and Nomarski interference-contrast light microscopy to assess the Had (root hair deformation), Hot (hole on the tip), and Inf (IT formation) phenotypes of primary infection events (Robledo et al. 2008). Confocal spectral microscopy was carried out with a Leica confocal microscope equipped with a krypton-argon laser using a blue excitation filter (excitation maximum 488 nm; 530-nm long-pass filter), allowing simultaneous visualization of GFP and propidium iodide fluorescence. Roots and root hairs were stained with 10 μM propidium iodide (Sigma-Aldrich). Projections were made from adjusted individual channels in the image stacks using Leica software.

Roots and nodules were excised from 40-day-old plants and processed for light microscopy using Bright field, cross-polarized, and phase-contrast optics, and TEM and scanning electron microscopy as previously described (Dazzo et al. 1996; Mateos et al. 2001; Velázquez et al. 1995). In the latter case, samples were coated with osmium instead of gold. The internal morphological features of clover nodules were examined by microscopy after Toluidine blue staining. Nodules were fixed in 4% formaldehyde in 50 mM phosphate buffer (pH 8), dehydrated in an increasing ethanol series, and embedded in paraffin. Toluidine blue-stained sections (2 μm) of embedded nodules were examined by light microscopy.

The morphology of infection droplets in nodule sections was measured using CMEIAS computer-assisted microscopy and image analysis (Liu et al. 2001).

**In situ detection of ROS production in clover roots.**

The ROS-sensitive dye H₂DCFDA (Sigma-Aldrich) was used to detect in situ ROS production (Ubezio and Civilli 1994). Uninoculated and inoculated clover roots were harvested at different times and submerged in 10 μM dye solution dissolved in dimethyl sulfoxide. After 15 min, the plants were gently washed with sterile water; then, their root hairs and nodules were examined for fluorescence by confocal microscopy as described above, using a different excitation filter (excitation maximum 514 nm). In situ densitometry of ROS fluorescence was measured using CMEIAS image analysis (Liu et al. 2001). The percentage of root hairs showing fluorescence present in zone II (Gage 2004) was determined. To corroborate that fluorescence was specific for ROS production, a treatment with the NADPH oxidase inhibitor DPI was performed (Mazel et al. 2004). Briefly, DPI (20 μM) was added at the onset of *Rhizobium* inoculation and again 30 min before observations to test for inhibition of *Rhizobium* spp.-induced ROS production. At least three different plants were analyzed for each time point and treatment.

**Analysis of SA content.**

SA production was measured by high-performance liquid chromatography as previously described (Stacey et al. 2006). Briefly, root tissues from white clover plants were collected at different time points after inoculation with ANU843EV or ANU843C2* rhizobia strains, weighed, and frozen in liquid nitrogen. Between 100 to 150 plants were used for each SA measurement. For each sample, 0.3 to 0.6 g of the frozen tissue was extracted and quantitated for total SA, free, and SA β-glucoside, essentially as described previously (Bowling et al. 1994; Enyedi et al. 1992). In all, 2 μl from a total volume of 80 μl of root methanolic extracts was injected into a 3-μm Waters
Spherisorb S3 ODS2 column (4.6 by 150 mm). SA was separated isocratically with 30% (vol/vol) methanol containing 1% (vol/vol) acetic acid at a flow rate of 1.1 ml min⁻¹. The temperature of the oven was 35°C. SA was detected with a Varian Prostar fluorescence detector using an excitation wavelength of 313 nm and an emission wavelength of 405 nm.

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LITERATURE CITED


