Gibberellins Are Involved in Nodulation of *Sesbania rostrata*[1]

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Upon submergence, *Azorhizobium caulinodans* infects the semiaquatic legume *Sesbania rostrata* via the intercellular crack entry process, resulting in lateral root-based nodules. A gene encoding a gibberellin (GA) 20-oxidase, *SrGA20ox1*, involved in GA biosynthesis, was transiently up-regulated during lateral root base nodulation. Two *SrGA20ox1* expression patterns were identified, one related to intercellular infection and a second observed in nodule meristem descendants. The infection-related expression pattern depended on bacterially produced nodulation (Nod) factors. Pharmacological studies demonstrated that GAs were involved in infection pocket and infection thread formation, two Nod factor-dependent events that initiate lateral root base nodulation, and that they were also needed for nodule primordium development. Moreover, GAs inhibited the root hair curling process. These results show that GAs are Nod factor downstream signals for nodulation in hydroponic growth.

Legume plants develop a symbiotic interaction with rhizobia by forming root nodules in which the bacteria fix atmospheric nitrogen. Nodule formation integrates several developmental processes, such as induction of cortical and pericycle cell division and rhizobial invasion, which are coordinated in time and space. The onset of the symbiosis is marked by a complex exchange of signals, involving plant flavonoids and bacterial nodulation (Nod) factors. Recognition of specific Nod factors will switch on the nodulation program in the legume host.

The best known mode of invasion is the root hair curling (RHC) mechanism that is used by most crop legumes and the model legumes barrel medic (*Medicago truncatula*) and *Lotus japonicus*. Rhizobia induce growing root hairs to curl in the root zone I, just above the root meristem, whereby a rhizobial microcolony is entrapped. Local cell wall degradation and subsequent inward growth of the root hair plasma membrane result in the formation of an infection thread (IT) that guides the bacteria to the cortical cells. RHC is Nod factor dependent, and purified compatible Nod factors trigger several nodulation-related effects within the root hair, such as deformation, gene expression, Ca^{2+} spiking, membrane depolarization, and ion effluxes (Oldroyd and Downie, 2004). Several components of the Nod factor perception mechanism have been identified via map-based cloning of symbiotic genes (Oldroyd and Downie, 2004).

A different type of invasion, via crack entry at lateral root bases (LRBs), is observed during nodulation under flooded conditions on semiaquatic legumes, such as *Sesbania rostrata* (Goormachtig et al., 2004b). The rhizobia enter at the cracks that are generated by the protrusion of the lateral roots. Local induction of cell death creates space for bacterial colonization in intercellular infection pockets (IPs; D'Haeze et al., 2003). From these IPs, ITs guide the bacteria to the target cells for intracellular settlement. LRB invasion, as with RHC invasion, depends on proper Nod factor recognition (D'Haeze et al., 1998, 2003). The LRB mode of invasion is used also during stem nodulation at the bases of adventitious rootlets present on *S. rostrata* stems (Goormachtig et al., 1997). Developing adventitious root nodules have been very useful for transcript profiling because of their site-specific origin (Goormachtig et al., 1995; Lievens et al., 2001).

LRB nodulation is an adaptive trait to water tolerance (Goormachtig et al., 2004a) and, when *S. rostrata* roots are grown in aerated soils, rhizobial invasion occurs via the RHC process. The versatility in the invasion modes is mediated by ethylene (D'Haeze et al., 2003; Goormachtig et al., 2004b). Upon flooding,

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accumulating ethylene inhibits the RHC process, hampers the formation of zone I root hairs, promotes LRB invasion (Goormachtig et al., 2004a), and enables the switch from indeterminate (apical nodule meristem, elongated) to determinate (no meristem, round) nodules (Fernández-López et al., 1998).

Plant hormones control all developmental plant processes, including nodulation that is presumably initiated by a change in the cytokinin/auxin ratio within the root. Nod factor-induced inhibition of auxin transport would lead to the local accumulation of auxins needed to trigger a nodule primordium (Mathesius et al., 1998). Also, cytokinins may be involved because nonnodulating bacteria overproducing the isopentenyl transferase gene (Cooper and Long, 1994) as well as the exogenous application of cytokinin (Libbenga et al., 1973; Relič et al., 1994; Bauer et al., 1996) can provoke nodule-like structures on legume plants. Recently, in *L. japonicus*, cytokinins have been shown to accumulate within the nodule primordia by using the Arabidopsis (*Arabidopsis thaliana*) cytokinin-responsive promoter *ARR5* (Lohar et al., 2004). Ethylene exerts a positional control on nodule primordium formation. Nodules mostly arise opposite protoxylem poles because ethylene, produced in the pericycle cells opposite phloem poles would prevent cell division at those sites (Heidstra et al., 1997; Penmetsa and Cook, 1997; Nukui et al., 2004).

Cytokinin and ethylene are also involved in the invasion process. The cytokinin-responsive promoter *ARR5* is expressed in curling root hairs, and cytokinin-insensitive hairy roots of *L. japonicus* nodulate less well than control hairy roots (Lohar et al., 2004). Ethylene controls the RHC process in barrel medic, as demonstrated by the ethylene-insensitive *sickle* mutant that allows a higher number of successful infections (Penmetsa and Cook, 1997). Moreover, ethylene inhibits the Nod factor-induced Ca^{2+} response within the barrel medic root hairs (Oldroyd et al., 2001). On the other hand, ethylene is needed to initiate cell death for IP formation at LRBs of *S. rostrata* (D'Haeze et al., 2003).

Little is known about the role of GAs in the rhizobium-legume interaction. Elevated GA levels have been measured in nodules of lima bean (*Phaseolus lunatus*) and cowpea (*Vigna unguiculata*; Dobert et al., 1992). However, analysis of effects of exogenous GAs on root nodulation yielded conflicting results (Bishnoi and Krishnamoorthy, 1990; Zhang et al., 1997). Exogenous GA_3 induced the formation of nodule-like structures on the roots of *L. japonicus*, and this response was nitrogen sensitive (Kawaguchi et al., 1996) but originated from cell divisions in the pericycle and not from cortical cells. Although some free-living rhizobia have the capacity to produce GAs (Atzorn et al., 1988; Tully et al., 1998), they probably do not contribute significantly to the amount of GA within the nodule (Atzorn et al., 1988). Recently, different pea (*Pisum sativum*) lines mutated in GA synthesis or perception have been analyzed for their nodulation phenotypes (Ferguson et al., 2005). Nodulation in some mutants was hampered, resulting in fewer, nonfunctional nodules, a phenotype that could be complemented by exogenous application of GAs (Ferguson et al., 2005).

GAs comprise a large group of more than 130 diterpenoid carboxylic acids, of which most are precursors or inactivated forms, but some members, including GA_1, GA_3, GA_4, and GA_7, have an intrinsic growth-promoting activity. The biochemistry of GA biosynthesis can be subdivided into three main stages (Fig. 1; Hedden and Phillips, 2000; Yamaguchi and Kamiya, 2000). In the first stage, geranylgeranyl diphosphate is converted by two terpene cyclases to *ent*-kaurene, which, in the next stage, is oxidized by cytochrome P450 monooxygenases to yield GA_{12} and GA_{53} that, in the final stage, are converted to bioactive GAs by the 2-oxoglutarate-dependent dioxygenases, GA 20-oxidase (GA20ox) and GA 3β-hydroxylases (GA3ox). GA20ox converts GA_{12} and GA_{53} to the C_{19}-GAs, GA_9 and GA_{20}, respectively, which are converted by GA3ox to the bioactive GA_4 and GA_1. The dioxygenases involved in GA synthesis are products of small multigene families, with each family member having a specific pattern of expression, as visualized with promoter-β-glucuronidase (GUS) reporters or in situ hybridization (Phillips et al., 1995; García-Martínez et al., 1997; Carrera et al., 1999; Rebers et al., 1999).

We show that GAs are involved in the intercellular invasion process at lateral or adventitious root bases

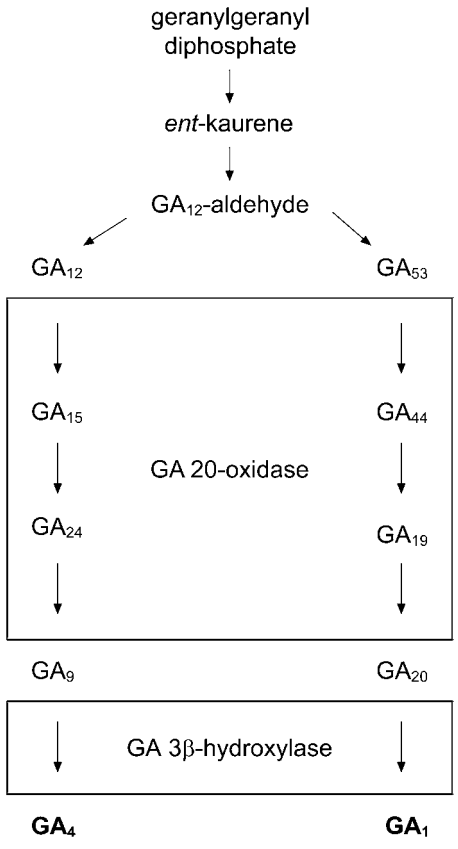

Figure 1. Overview of the GA biosynthetic pathway. The activities of the two main biosynthetic enzymes, GA20ox and GA3ox, are boxed.

of *S. rostrata*. Transcription of a gene coding for an active GA20ox (*SrGA20ox1*) was up-regulated during adventitious and lateral root nodulation in a Nod factor-dependent manner. *SrGA20ox1* transcripts were transiently detected in cells surrounding the IPs and young parts of the ITs, a pattern that depended on the local production of Nod factors. In the central tissue, *SrGA20ox1* transcripts were observed in the preinfection zone, in cells freshly delivered from the meristem that will differentiate into central tissue cells. Pharmacological approaches showed that GAs are downstream signals in the Nod factor-signaling cascade, needed for formation of IPs and ITs and for the initiation of cortical cell division and differentiation of the primordium.

RESULTS

SrGA20ox1 Codes for a Functional GA20ox

To obtain the full-length cDNA of the partial clone *Srdd16*, previously identified by differential display as an early induced tag in *S. rostrata* stem nodule development (Lievens et al., 2001), we used 5′ RACE (see "Materials and Methods"). By excluding the polyadenylation tail, the cDNA sequence was 1,365 bp long with an open reading frame of 372 amino acids. BLAST searches (Altschul et al., 1997) revealed that the deduced protein exhibited significant homology with GA20ox. The highest homology was found with *Ls20ox1* from lettuce (*Lactuca sativa*; Toyomasu et al., 1998), sharing 54% identity and 61% similarity at the amino acid level. According to the nomenclature by Coles et al. (1999), the full-length sequence was designated *SrGA20ox1*.

Alignment with amino acid sequences of GA20ox from diverse species revealed the presence of several characteristic sequence features in SrGA20ox1. The His and Asp residues involved in the binding of Fe^{2+} were present at conserved positions (−238, −240, and −294; Roach et al., 1995). The consensus sequence NYYPxCxxP was found in residues 221 to 229 and the conserved Arg and Ser residues that are involved in binding the 5-carboxyl of 2-oxoglutarate were recognized at positions 304 and 306, respectively (Valegård et al., 1998). The LPWKET motif that had been proposed to be important for GA substrate binding (Xu et al., 1995) was present in residues 140 to 145 in the *SrGA20ox1* sequence.

To confirm that *SrGA20ox1* encodes a functional GA20ox, the protein was produced in *Escherichia coli*. The cDNA fragment c8.2fl1 was inserted in sense orientation and in frame with the short N-terminal tag into a pET-3a vector (see "Materials and Methods"). Soluble extracts of *E. coli* cells producing the recombinant protein were assayed for GA20ox activity by incubation with [^{14}C]GA_{12}, and the reaction products were purified by HPLC and identified by gas chromatography-mass spectrometry (data not shown). The major peak contained [^{14}C]GA_9, [^{14}C]GA_{25}, [^{14}C]GA_{15}, and [^{14}C]GA_{24} in a ratio of approximately 4:1:1:0.1 based on the total ion intensities of the spectra (data not shown). These data demonstrate that SrGA20ox1 is an active GA20ox.

To determine the number of *GA20ox* genes in the *S. rostrata* genome, a probe spanning the open reading frame (c8.2fl1) was hybridized with genomic DNA digested with different restriction enzymes (Fig. 2A). Several bands were observed in each lane, suggesting that the *SrGA20ox1* gene is part of a small gene family. When similar filters were hybridized with the more gene-specific differential display fragment (*Srdd16*, containing the 3′ untranslated region and a short stretch of the open reading frame), a single band pattern was visible after high stringency washing, except for the lane with the *Eco*RI-digested genomic DNA, in which two bands occurred in accordance with the presence of an *Eco*RI restriction site in *SrGA20ox1* (Fig. 2B).

Up-Regulation of *SrGA20ox1* during Adventitious Root Nodulation Is Nod Factor Dependent

Reverse transcription (RT)-PCR analysis showed a very low background signal in uninfected, stem-located, adventitious root primordia (Fig. 3A). *SrGA20ox1* transcript accumulation was induced 8 h after inoculation of the root primordia with *Azorhizobium caulinodans* and increased to maximal levels after 3 and 5 d. Later, expression gradually dropped to background levels. During LRB nodulation on hydroponic roots, the gene is induced approximately 4 to 8 h after inoculation (Fig. 3B).

Two *A. caulinodans* mutants were tested for their ability to induce *SrGA20ox1* expression. One mutant that is unable to produce Nod factors because of a Tn*5* insertion in the *nodA* gene does not induce nodule primordia and does not invade the outer cortex

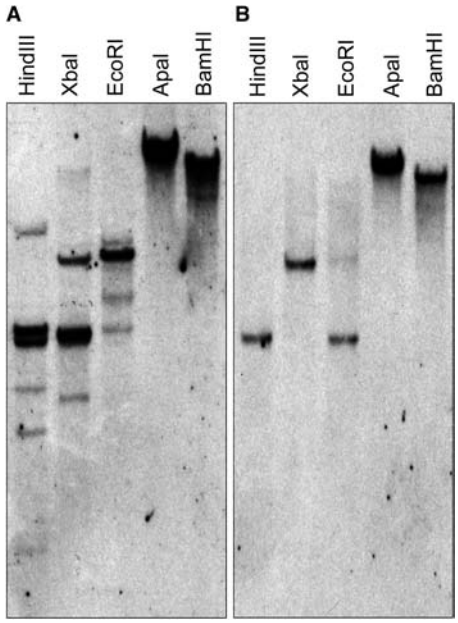

Figure 2. DNA gel-blot analysis. The different restriction enzymes used are indicated above each lane. A, Digested genomic DNA hybridized with the full-length c8.2.fl1 probe. B, Identical blot hybridized with the specific Srdd16 fragment.

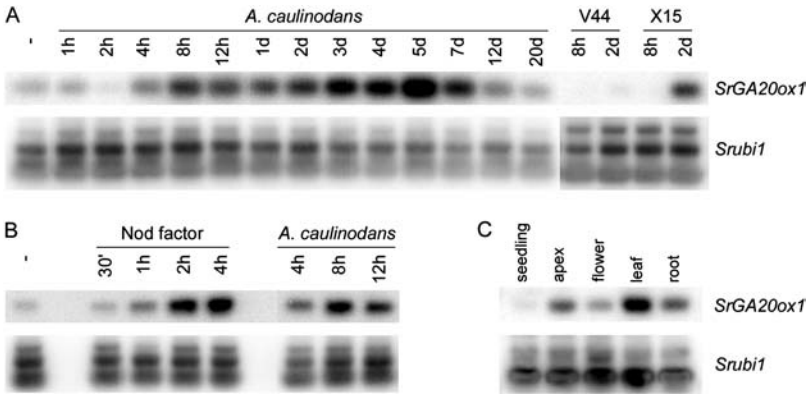

Figure 3. *SrGA20ox1* expression analysis determined by RT-PCR analysis. A, *SrGA20ox1* expression levels in dormant adventitious root primordia before (−) and at different time points from 1 h (1h) to 20 d (20d) after infection with *A. caulinodans* ORS571. *SrGA20ox1* expression levels in adventitious root primordia 8 h (8h) and 2 d (2d) after inoculation with a Nod factor-deficient strain, ORS571-V44 (V44), or with a surface polysaccharide-deficient strain ORS571-X15 (X15). As a constitutive control, an ubiquitin gene (*Srubi1*) was amplified. B, *SrGA20ox1* expression levels in uninfected roots (−), in roots 30 min (30′), 1 (1h), 2 (2h), and 4 h (4h) after treatment with Nod factors, and in developing hydroponic root nodules, at 4 (4h), 8 (8h), and 12 h (12h) after infection with *A. caulinodans* ORS571. C, *SrGA20ox1* RNA levels in seedlings (seedling), apices (apex), flowers (flower), leaves (leaf), and roots (root).

(ORS571-V44; Van den Eede et al., 1987; Mergaert et al., 1993; D'Haeze et al., 1998). Its effect on *SrGA20ox1* expression was examined by RT-PCR. Samples, taken at 8 and 48 h after inoculation of root primordia, showed no accumulating transcripts (Fig. 3A). The second mutant (ORS571-X15; Goethals et al., 1994) is fully functional in Nod factor production but is affected in its surface polysaccharide composition, resulting in the arrest of invasion at the stage of IP formation and slower growth (D'Haeze et al., 1998). Application of these bacteria to root primordia resulted in an induction of the *SrGA20ox1* gene after 48 h, to expression levels similar to those after wild-type inoculation (Fig. 3A). Hence, Nod factor-producing bacteria are required for *SrGA20ox1* induction.

To test whether the bacterial signaling molecule alone is sufficient for *GA20ox* gene induction, roots were inoculated with 10^{-8} M Nod factors and harvested after several time points in two independent experiments. RT-PCR analysis indicated that *SrGA20ox1* transcripts started to accumulate 1 to 2 h after Nod factor addition and peaked after 8 h (Fig. 3B; data not shown).

In all plants in which several members of the *GA20ox* gene family have been identified, individual members show a differential tissue specificity (e.g. Phillips et al., 1995; García-Martínez et al., 1997; Carrera et al., 1999; Rebers et al., 1999). To test the specificity of *SrGA20ox1* expression, RT-PCR analysis was applied on RNA samples taken from different plant tissues (Fig. 3C). In samples prepared from vegetative shoot apices, flowers, leaves, and roots, various levels of expression were observed.

SrGA20ox1 Transcripts Accumulate Around the Bacterial Invasion Track and in the Preinfection Zone

The spatio-temporal pattern of *SrGA20ox1* expression during stem-located, adventitious root nodulation was examined by in situ hybridization. Sections (10 μm) of adventitious root nodules harvested at different phases of development were hybridized with an antisense probe derived from the gene-specific *Srdd16* cDNA fragment. No expression could be detected in uninfected root primordia (data not shown). Consistent with the results of the RT-PCR analysis, the earliest time point at which *SrGA20ox1* induction could be perceived was 8 h after inoculation with *A. caulinodans* (Fig. 4, A and D). At this early stage, no morphological changes occur in the root primordia, and the bacteria proliferate in the fissure that surrounds the base of the root primordium (Tsien et al., 1983; Duhoux, 1984; Goormachtig et al., 1997). *SrGA20ox1* transcripts were highly abundant in the outermost cortical cell layers that enclose the epidermal crack (Fig. 4, A and D). The first cell divisions, which will give rise to the nodule primordium in the mid-cortex, usually become noticeable approximately 1 d postinoculation (dpi), and at the same time the rhizobia in the fissure start penetrating the intercellular spaces of the outer cortex. Approximately 2 dpi, large, heavily colonized IPs could be seen with strong *SrGA20ox1* expression in the neighboring plant cells (Fig. 4, B and E). Then, azorhizobia move from the IPs toward the nodule primordium that develops deeper into the cortex through intercellular and intracellular ITs (Tsien et al., 1983; Duhoux, 1984; Goormachtig et al., 1997). In 3-d-old developing stem nodules, *SrGA20ox1* transcripts were found in the cells neighboring deeper IPs and in cells containing the growing ITs (Fig. 4, C and F). The expression in the outer cell layers had mostly disappeared by this time.

Approximately 4 dpi, the ITs enter the first cells of the central tissue, and a fixation zone, an infection zone, and a distal meristem are established. The ITs grow in the direction of the meristem, and bacteria are released in the differentiating meristem descendants (Goormachtig et al., 1997). *SrGA20ox1* was expressed

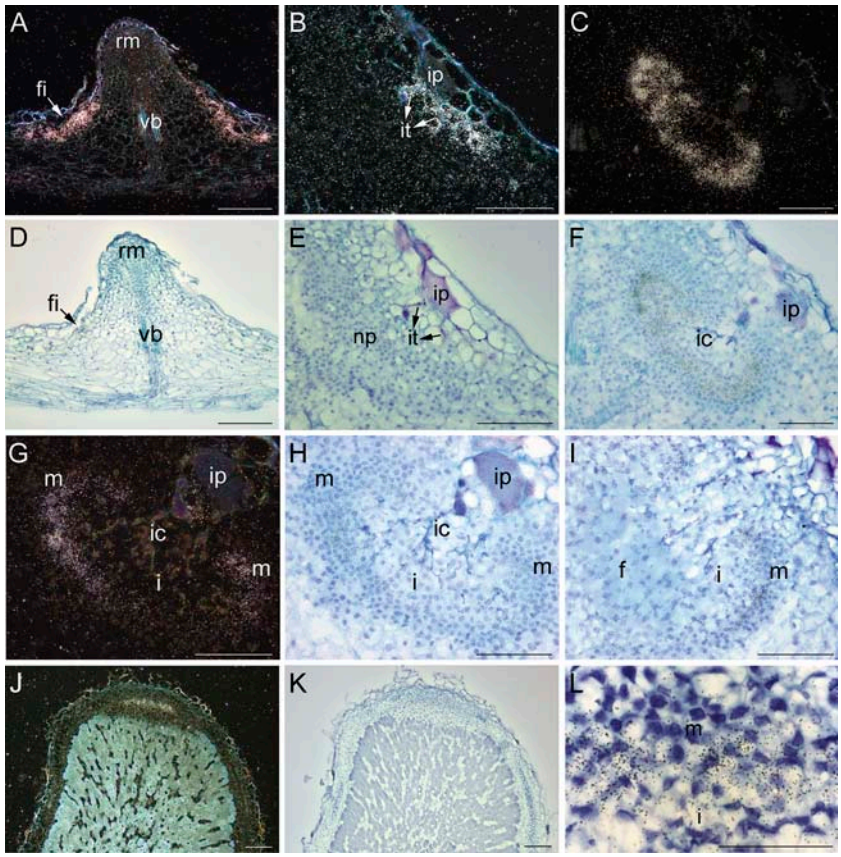

Figure 4. In situ localization of *SrGA20ox1*. Signals are detected as white spots or dark spots using dark-field (A–C, G, and J) or bright-field (D–F, H, I, K, and L) optics, respectively. A to C, Longitudinal sections through an adventitious root primordium, 8 h after infection, a developing adventitious root nodule, 2 d after bacterial inoculation, and developing adventitious root nodule, 3 d after infection, respectively. D to F, Bright-field pictures of A to C, respectively. G, Longitudinal section through a developing adventitious root nodule, 4 d after infection. H, Bright field of G. I, Longitudinal section through a developing adventitious root nodule, 5 d after infection. J, Longitudinal section through an indeterminate root nodule (25 dpi). K, Bright-field picture of J. L, Enlargement of the meristematic and infection zone of K. f, Fixation zone; fi, fissure; i, infection zone; ic, infection center; ip, infection pocket; it, infection thread; m, meristem; np, nodule primordium; rm, root meristem; vb, vascular bundle. Bars = 100 μm.

in a very narrow region at the distal end of the infection zone (Fig. 4, G–I), in cells that were still small and contained a large nucleus and dense cytoplasm, but stopped dividing and were committed to enlarge (Goormachtig et al., 1997). In adventitious root nodule development of *S. rostrata*, the meristem is not persistent. At approximately 8 dpi, meristematic activity ceases and shortly afterward the complete central tissue consists of infected cells containing nitrogen-fixing bacteria, interspersed with a few noninfected cells. *SrGA20ox1* expression completely disappeared, and no transcripts could be detected in mature, 15-d-old nodules (data not shown). Similar expression patterns were observed during LRB nodulation on hydroponic roots (data not shown). The specific expression in the narrow region of meristem descendants was also detected in sections through indeterminate root nodules (Fig. 4, J–L) that develop under aeroponic conditions (Fernández-López et al., 1998).

The Expression That Colocalizes with the Invasion Track Depends on Locally Produced Nod Factors

To analyze whether local Nod factor perception or distant Nod factor signaling were responsible for *SrGA20ox1* gene induction, in situ hybridizations were performed on developing nodules induced after co-inoculation of ORS571-V44 and ORS571-X15 strains. The latter strain produces Nod factors but stays in outer-located IPs, and can complement the Nod factor-deficient strain ORS571-V44, giving rise to complementation nodules in which only ORS571-V44 resides in ITs and, later on, in the infected cells (D'Haeze et al., 1998). As shown in Figure 5, in the complementation nodules, transcripts were not detected around the IPs or the ITs. On the other hand, *SrGA20ox1* transcripts were still seen in the preinfection zone of the developing nodule (Fig. 5, C and D, arrow). Because only ORS571-V44 resides in the ITs, these results demonstrate that *SrGAox1* gene expression around the invasion track during wild-type invasion depends on Nod factors produced locally by bacteria within the ITs.

SrGA20ox1 Transcripts Do Not Accumulate after Pathogen Attack

Because IP formation involves controlled death of a few cells (D'Haeze et al., 2003), we wondered whether *SrGA20ox1* would be induced upon pathogen attack. Two different approaches were followed. First, *S. rostrata* leaves were infected with spores of the pathogenic fungus *Botrytis cinerea*. After 48 h of spore application, macroscopic lesions were visible (data not shown). β-1,3-Glucanase gene expression was strongly induced from 4 h after inoculation on. However, no *SrGA20ox1* induction was detected by RT-PCR (Fig. 6A).

Secondly, the bacterial pathogen *Ralstonia solanacearum* was applied to adventitious rootlets on the *S. rostrata*

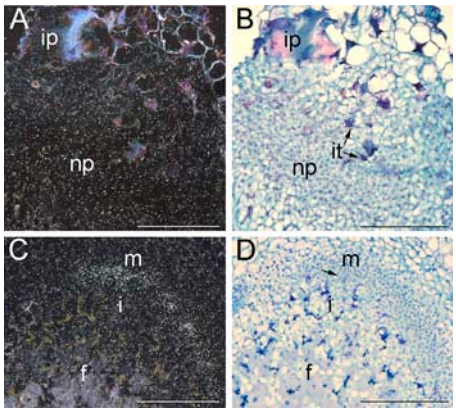

Figure 5. *SrGA20ox1* in situ localization in ORS571-V44/ORS571-X15 complementation nodules. Signals are seen as white or black spots in respectively dark-field or bright-field optics. A and C, Longitudinal sections through a developing complementation nodule, 6 and 14 d after inoculation, respectively. B and D, Bright-field pictures of A and C, respectively. Abbreviations: See legend of Figure 4. Bars = 100 μm.

stem. Wild-type bacteria provoked tissue browning at the base of the root primordia from approximately 3 dpi, while a nonvirulent *hrcR* mutant strain did not elicit any response (Lievens et al., 2004). The *SrGA20ox1* expression was analyzed at different time points after infection with the wild-type and nonvirulent *R. solanacearum* strain. As shown in Figure 6B, *SrGA20ox1* was not induced upon pathogen attack.

Nod Factor-Induced Axillary Root Hair Outgrowth Is Mediated by GAs

Hydroponic *S. rostrata* roots carry bulge-like structures at the LRBs. Upon application of *A. caulinodans* or its purified Nod factors, these bulges grow out into deformed axillary root hairs (Mergaert et al., 1993), a specific effect of Nod factors (Mergaert et al., 1993). To determine whether GAs are also downstream signals of Nod factors, 5.10^{-3} M chlormequat chloride (CCC), an inhibitor of GA synthesis, was added prior to Nod factor, and axillary root hair outgrowth was monitored for 5 d (see "Materials and Methods"). As shown in Figure 7C, no axillary root hairs could be detected. Moreover, GA_3 application (10^{-5} M) provoked outgrowth of two types of root hairs (Fig. 7, D–F): straight ones, similar to those obtained with ethylene and hydrogen peroxide (H_2O_2; D'Haeze et al., 2003), and deformed ones, similar to the Nod factor-induced axillary root hairs but smaller in size (Fig. 7F). Based on this experiment, we conclude that GAs might indeed be downstream signals of Nod factors.

Inhibitors of GA Synthesis Block LRB Nodulation

The restricted, localized expression of a *GA20ox1* gene during nodulation points to a specific role of GAs. The effect of inhibition of GA biosynthesis was tested by exogenous application of three different inhibitors, each one interfering with a distinct stage of the GA biosynthetic pathway (Rademacher, 1991). Various concentrations of daminozide, CCC, or paclobutrazol were applied to determine the optimal quantity that had a clear effect on nodulation without affecting plant health (see "Materials and Methods"). All three inhibitors inhibited the nodulation process. The results obtained with CCC are presented here (Fig. 8).

Addition of 10^{-3} M CCC 2 d prior to bacterial inoculation resulted in a complete loss of nodule formation (Fig. 8A, lane 2). Only one nodule was counted on a total of 10 plants after 6 d. To determine whether CCC blocked nodulation only at the onset of the process or also at later stages, CCC was added 1, 2, 3, and 4 dpi and nodules were counted after 6 d. As shown in Figure 8A (lane 3), CCC addition at 1 dpi still had a clear effect on nodule number (13 compared to 121). When CCC was added 2, 3, or 4 dpi, progressively more nodules were obtained, and at 4 dpi 80% of the wild-type nodule number was reached (Fig. 8A, lanes 4–6).

To confirm the specific effect of CCC on GA synthesis, 2 d before and 1 d after inoculation, GA_3 (10^{-5} M) was added to samples that had been pretreated with CCC 2 d before inoculation, and nodules were counted at day 6. As shown in Figure 8A (compare lanes 7 and 8 with lane 2), a slightly higher nodule number ($P < 0.05$) was obtained, showing that GA_3 could partially complement CCC for the nodulation phenotype. GA_3 (10^{-5} M) added alone 2 d before and 1 d after inoculation had no significant effect on nodule number (Fig. 8A, lanes 9 and 10).

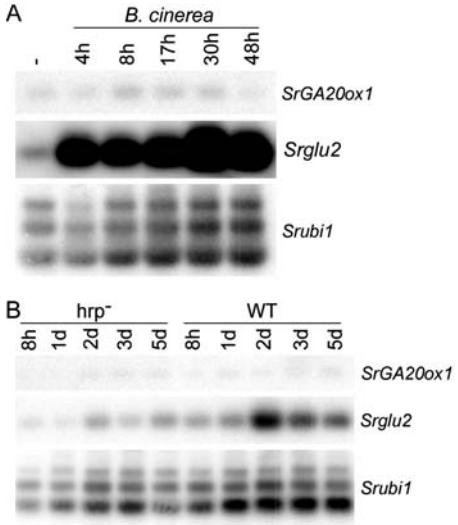

Figure 6. *SrGA20ox1* expression levels upon pathogen attack. A, *SrGA20ox1* expression, measured by RT-PCR, in untreated leaves (−) and 4 (4h), 8 (8h), 17 (17h), 30 (30h), and 48 h (48h) after infection with *B. cinerea*. B, *SrGA20ox1* expression level measured by RT-PCR analysis in adventitious root primordia, infected with a *hrcR* mutant (hrp^-) or wild-type (WT) *R. solanacearum* strain, 8 h (8h) and 1 to 5 d (1d, 2d, 3d, 4d, and 5d) after inoculation. *Srubi1* and *Srglu2* expression levels serve as a constitutive and a positive control (Lievens et al., 2004), respectively.

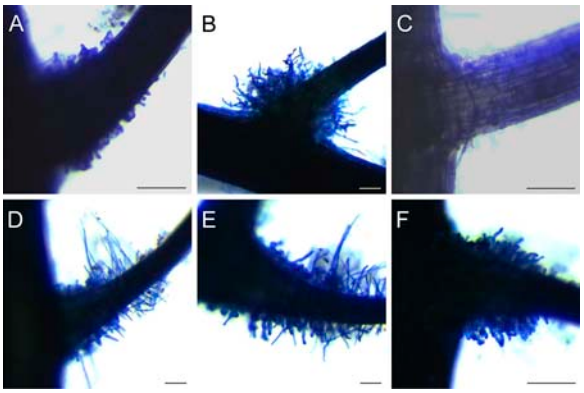

Figure 7. Axillary root hair outgrowth at LRBs of hydroponic roots, analyzed 5 d after treatment and stained with methylene blue for visualization of root hairs. A, Control. B and C, LRBs treated with *A. caulinodans* Nod factors and pretreated with 5.10^{-3} M CCC, respectively. D to F, LRBs treated with 10^{-5} M GA_3. Bars = 500 μm.

To exclude the possibility that the effect of the GA biosynthesis inhibitors on root nodulation resulted from an inhibitory effect on the Nod factor-producing capacities of the azorhizobia, Nod factor production of wild-type *A. caulinodans* under normal growth conditions was compared with the production in the presence of the inhibitors by labeling the molecules in vivo with [^{14}C]acetate. The final concentration for daminozide and CCC was 10^{-2} M, and for paclobutrazol 10^{-4} M, a slightly higher concentration than that applied in the nodulation assays. Extracts from the culture supernatant were analyzed by thin-layer chromatography. No difference could be observed between the patterns obtained for any of the samples, indicating that in the presence of the inhibitors, *A. caulinodans* produces normal Nod factors (data not shown). To test whether the *nod* gene-inducing capacity of *S. rostrata* is compromised by the addition of the inhibitors to the growth medium, *S. rostrata* seedlings were grown overnight on a lawn of an *A. caulinodans* ORS571 strain harboring a *lacZ* fusion in *nod* locus 1, on plates containing the inhibitors at the same concentrations as those in the first control experiment. For all three inhibitors, a blue halo, indicative of β-galactosidase activity, was observed around the seedlings, revealing that *nod* gene activity was induced at levels similar to those in the control plates, to which no inhibitor had been supplemented (data not shown).

These experiments indicate that GA plays a role in the early stages of LRB nodulation. To analyze at which level the nodulation is blocked, the treatments were repeated with *A. caulinodans* (pRG960D-32) expressing *uidA* driven by a *nodA* promoter that enables staining of the bacteria via the GUS assay. Nodules and nodule-like structures were analyzed at 6 dpi. Addition of 5.10^{-3} M CCC 2 d before inoculation completely blocked the rhizobial invasion and no blue staining was observed, indicating the absence of bacteria and IPs (data not shown). When 10^{-3} M CCC was added 2 d before inoculation, no nodules were obtained, but rhizobial colonization was allowed and outer cortical colonization was more pronounced than that of control infection at 1 and 2 dpi (Fig. 8, B, C, and E). When CCC was added at 1 dpi, bumps occurred, in which the bacteria had proliferated partially (compare Fig. 8, F and D). Mostly functional nodules were obtained after CCC had been added 2 dpi (Fig. 8G).

Sections through a developing nodule treated with 10^{-3} M CCC 2 d prior to infection showed large IPs in which the plant cell structures were still visible (Fig. 8H). Neighboring cells were often stained pink by toluidine blue and contained pinkish granular threads, indicating that the nuclei had disintegrated and that the cells were dying (rectangles in Fig. 8, H and I). Often outer cortical cells with initiated ITs were blue because of the presence of bacteria in the cytoplasm (triangles in Fig. 8, H and I) and showed signs of cell death (pinkish granular threads; Fig. 8I). No cell division and no sign of primordium formation were observed (Fig. 8, H and I). In contrast, nodule primordia were seen in sections through LRBs that were treated with CCC at 1 dpi (Fig. 8J), comparable to those of control infection at 1 dpi (Fig. 8M). Many more IPs than in the wild type were observed and new IPs were still being made (Fig. 8K), a process that does not occur at 6 dpi in control samples. The infection had proceeded further than in samples where CCC was added 2 d before inoculation and ITs were broader (Fig. 8L) than those of the wild type. No transition from globular primordium to a zonated structure took place and no meristem was observed (Fig. 8L).

GAs Block the RHC Invasion

To analyze the effect of GA on the RHC invasion, *S. rostrata* plants were grown in Leonard jars, the experimental system that allows aerated root growth and nodulation via RHC in zone I (Goormachtig et al., 2004a). Four days prior to inoculation 10^{-3} M CCC was added, and nodules were counted at 14 dpi. No effect on the nodule number was observed (Fig. 8N). On the other hand, no nodules were formed when 10^{-5} M GA_3 was applied 4 d prior to inoculation. Microscopic analysis revealed root hair deformation, but no ITs within the root hairs (data not shown).

DISCUSSION

SrGA20ox1 Codes for an Active GA20ox and Is Induced upon Adventitious and LRB Nodulation

Differential display has been used to search for genes that are involved in adventitious root nodulation on *S. rostrata* stems (Lievens et al., 2001). One of the tags was homologous to genes coding for different GA20ox. The deduced amino acid sequence of the full-length cDNA clone *SrGA20ox1* was approximately 50% identical to GA20ox sequences from other species, in agreement with the commonly observed degree of amino acid sequence identity of 50% to 60% between GA20ox of unrelated species (Hedden, 1999). The

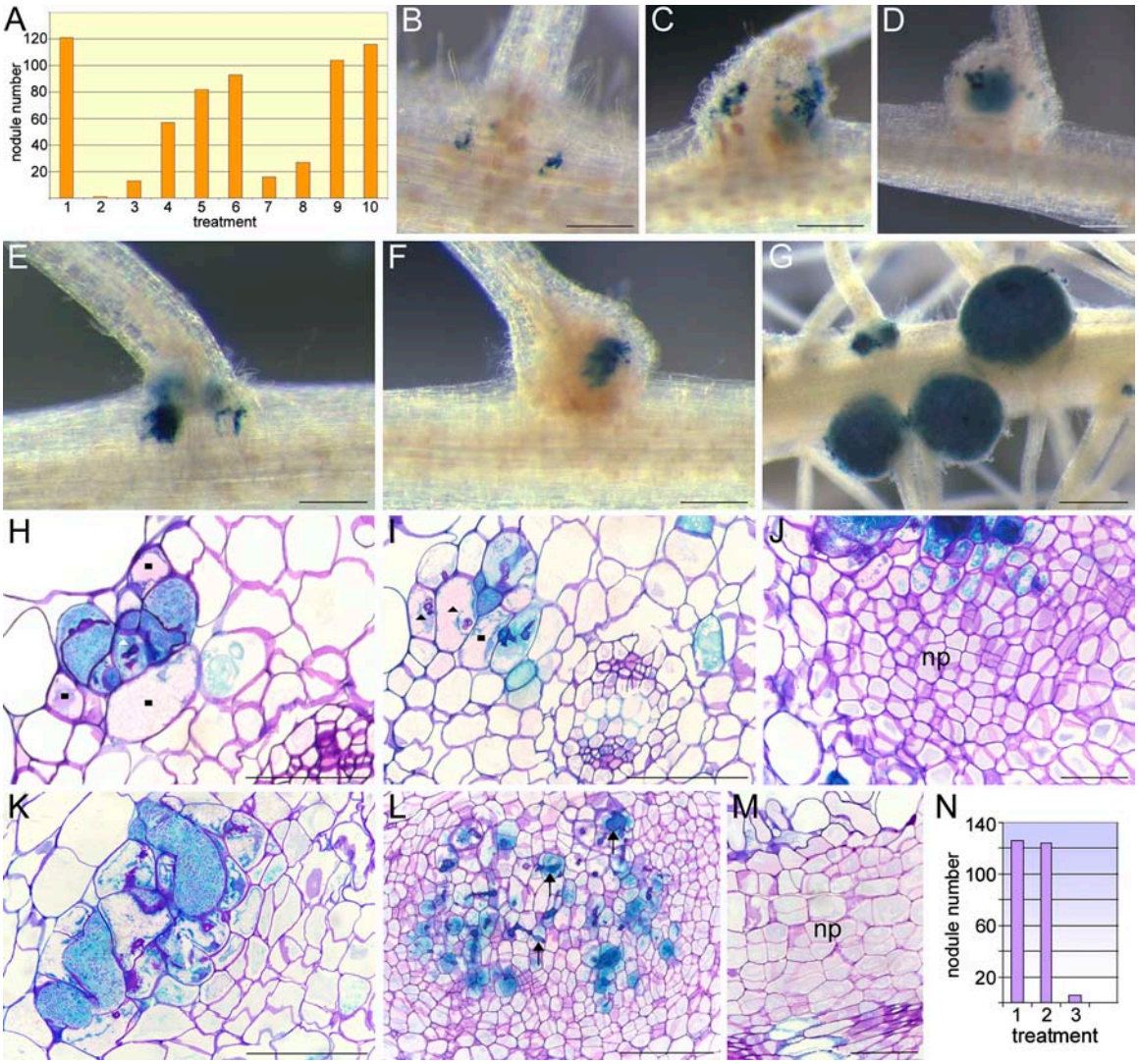

Figure 8. Involvement of GAs during *S. rostrata* nodulation. A, Graphic representation of the effect of CCC and GA_3 on nodule number during LRB nodulation. The number of nodules counted on 10 plants is given. Lane 1, Control roots. Lanes 2, 3, 4, 5, and 6, Addition of 10^{-3} M CCC 2 d before inoculation, at 1 dpi, 2 dpi, 3 dpi, and 4 dpi, respectively. Lane 7, Addition of 10^{-3} M CCC and 10^{-5} M GA_3 2 d before inoculation. Lane 8, Addition of 10^{-3} M CCC 2 d before inoculation and 10^{-5} M GA_3 at 1 dpi. Lane 9, Addition of 10^{-5} M GA_3 2 d before inoculation. Lane 10, Addition of 10^{-5} M GA_3 at 1 dpi. B to D, GUS-stained LRBs infected with *A. caulinodans* (pRG960D-32), at 1 dpi, 2 dpi, and 3 dpi. E to G, GUS-stained LRBs infected with *A. caulinodans* (pRG960D-32) after addition of 10^{-3} M CCC 2 d prior to inoculation, 1 dpi, and 2 dpi, respectively. H and I, Toluidine blue-stained sections through E. Rectangles and triangles indicate dying cells and ITs containing cells with blue precipitate within the cytoplasm, respectively. J to L, Toluidine blue-stained sections through F. Arrows in L indicate irregular ITs. M, Toluidine blue-stained section through B. N, Graphic representation of the effect of CCC and GA_3 on nodule number during RHC nodulation. Lane 1, Control roots. Lanes 2 and 3, Addition of 10^{-3} M CCC and 10^{-5} M GA_3 4 d before inoculation, respectively. Abbreviations: See legend of Figure 4. Bars = 1 cm (B to G); 100 μm (H to M).

identity of the protein was confirmed by an in vitro assay. Soluble extracts from *E. coli* producing the full-length SrGA20ox1 protein displayed GA20ox activity, converting the substrate GA_{12} mainly to GA_9.

As for other plants, also in *S. rostrata*, a small family of *GA20ox* genes is present. To reduce the possibility that more than one gene would be visualized during the expression analysis, primers and probes were used that cover the 3′ untranslated region of the gene because probes of this region only recognized one band by DNA gel-blot analysis.

RT-PCR analysis showed that *SrGA20ox1* transcript accumulation starts between 4 and 8 h after contact with the microsymbiont, whereas mitotic activity in the mid cortex or bacterial invasion of the outer cortex can be perceived only approximately 1 dpi (Goormachtig et al., 1997). In *S. rostrata*, this is one of the earliest induced genes yet identified (Lievens et al., 2001). *SrGA20ox1* transcripts were also detected in shoot apices, leaves and to a lower extent in roots and flowers, indicating that the expression is not restricted to nodulation. Until now, only a few genes have been isolated

that are uniquely expressed during nodulation, demonstrating recruitment of similar functions for different developmental processes.

Two Distinct Patterns of *SrGA20ox1* Expression during LRB Nodule Development

GA20ox are multifunctional enzymes that catalyze oxidation on carbon-20 of C_{20}-GA precursors, some of the final steps in GA biosynthesis. After GA20ox-mediated synthesis of C_{19}-GAs, they are converted into bioactive GAs by the action of GA3ox (Hedden, 1999). Considerable evidence indicates that the stages catalyzed by GA20ox are important regulatory checkpoints. Experiments with GA response mutants demonstrated a feedback regulation of GA biosynthesis, involved in adjusting levels of bioactive GAs (Hedden and Kamiya, 1997) that act at the transcriptional level on *GA20ox* and *GA3ox* (Chiang et al., 1995; Phillips et al., 1995; Xu et al., 1995; Martin et al., 1996). Also, for *SrGA20ox1*, preliminary data showed that the basal expression observed in roots disappeared upon GA treatment (W. Capoen and M. Holsters, unpublished data). In many plant species, GA levels control growth through GA20ox activity that is a major determinant of GA production. For example, in bolting Arabidopsis plants, higher GA levels in the rapidly elongating shoots were accompanied by increased *GA20ox* gene expression (Xu et al., 1995). A similar observation was reported from spinach (*Spinacia oleracea*; Wu et al., 1996). In addition, transgenic Arabidopsis plants overexpressing *GA20ox* had elevated levels of bioactive GAs and were taller than control plants, indicating that GA20ox might be a rate-limiting step in the biosynthesis of GAs (Coles et al., 1999). Comparable results were obtained for potato (*Solanum tuberosum*) plants overexpressing *GA20ox* (Carrera et al., 2000). The specific expression patterns of *SrGA20ox1* suggest roles for GA in LRB nodulation. *SrGA20ox1* transcripts could not be detected in uninoculated root primordia, but throughout early nodule development were located transiently in cells associated with the invading bacteria, in cells adjacent to the epidermal fissure, IPs, or ITs. This induction faded out as the invasion front moved on. When a zonated developing nodule was established, transcripts accumulated in a number of cell layers at the transition between meristematic and infection zone. When the meristematic activity of the LRB nodules disappeared, *SrGA20ox1* transcripts did not accumulate anymore. In conclusion, on the basis of *SrGA20ox1* expression, GAs might be produced during intercellular invasion at LRBs and in differentiating cells derived from the nodule meristem.

GAs Act Downstream of Nod Factors during LRB Nodulation

Up-regulation of *SrGA20ox1* transcripts upon azorhizobial invasion depends on Nod factor-producing bacteria. *SrGA20ox1* transcript accumulation is induced when pure Nod factors are applied to hydroponic roots, suggesting that GAs might be direct or indirect downstream signals of Nod factors.

A. caulinodans provokes different Nod factor-related effects on *S. rostrata* roots (Mergaert et al., 1993), of which the outgrowth and deformation of axillary root hairs at LRBs has been used to analyze the role of H_2O_2 and ethylene in Nod factor signaling (D'Haeze et al., 2003). Inhibitors of GA biosynthesis inhibit the Nod factor-dependent root hair outgrowth, and GAs can partially mimic the Nod factor effect. Coinoculation between ORS571-V44, which does not produce Nod factors, and ORS571-X15, which has defective surface polysaccharides and is restricted to superficially located IPs, and subsequent analysis of the resulting complementation nodules addressed the question whether *SrGA20ox1* expression is triggered by locally produced Nod factors or from a distance. ORS571-V44 can invade the plant when Nod factors are supplemented from outside to initiate the nodule formation. The Nod factors, provided by ORS571-X15, are not expected to travel within the plant tissue because they attach to cell walls (Goedhart et al., 2000). Because no *SrGA20ox1* gene expression could be detected around IPs and ITs that contained ORS571-V44 bacteria, Nod factors are locally responsible for the invasion-related *SrGA20ox1* expression pattern. Moreover, GAs were able to enhance the expression of another infection-related and Nod factor-depending tag coding for a peroxidase, *Srprx1* (J. Den Herder, S. Goormachtig, and M. Holsters, unpublished data).

What could be the function of GAs during IP and IT formation? By using pharmacological approaches, we showed that GAs are needed to initiate intercellular invasion. Addition of inhibitors of GA synthesis 2 d prior to bacterial inoculation completely blocked nodulation, an effect that could be partially complemented by exogenously added GAs. No GUS staining that marks the presence of bacteria was observed when the roots were pretreated with 5.10^{-3} M CCC. The bacterial invasion during LRB nodulation is initiated by IP formation, which involves local cell death (D'Haeze et al., 2003). In the aleurone of cereals, GA accelerates cell death after reserve remobilization by sensitizing the cells to H_2O_2 (Bethke et al., 1999; Fath et al., 2001). By analogy, GAs produced at the onset of LRB invasion might make the surrounding outer cortical cells more prone to death. Interestingly, the *GA20ox1* gene was not switched on after infection of *S. rostrata* leaves with *B. cinerea* and after infection of adventitious root primordia with *R. solanacearum*, two pathogens that induce cell death and necrosis (Lievens et al., 2004).

Addition of CCC at a slightly lower concentration (10^{-3} M) inhibited nodule formation, but bacterial colonization was allowed and even enhanced. Many more IPs were formed compared to wild-type. ITs were broad and had an irregular form, indicating that GAs might be an important signal to control IT structure. Both IT formation and pollen tube growth have common features (Szczyglowski and Amyot,

2003; Rodríguez-Llorente et al., 2004). It is interesting that GAs are needed for pollen tube elongation in Arabidopsis (Singh et al., 2002).

The enhanced formation of IPs and the release of bacteria within cells containing ITs observed at 10^{-3} M CCC contrasted with the inhibition of IP formation at slightly higher concentrations (5.10^{-3} M). Possibly, 10^{-3} M CCC might enable enough GAs to be produced to make the tissue sensitive to cell death. Enhanced cell death and IP formation within this sensitive tissue may be a consequence of the inhibition of IT growth and proper bacterial invasion. Indeed, enhanced primary colonization has been observed during invasion by mutant bacteria that are impaired in triggering IT growth (Mathis et al., 2005). The plant feedback mechanisms that control bacterial invasion might simply not be switched on when invasion is impaired. Thus, these apparently opposite effects reflect that GA levels have to be strictly controlled and that different processes require different doses of GA.

GAs Are Essential for Induction of Cortical Cell Division and Differentiation of the Nodule Primordium

The pharmacological studies showed that GAs are needed for nodule primordium formation in the cortex and the establishment of a nodule meristem. Supplementation of 10^{-3} M CCC allowed extensive colonization, but no cell division was initiated in the cortex. When the inhibitor was added 1 d after invasion, after a nodule primordium had been formed, the ITs could spread in the preformed primordium, but further development was arrested. By inhibiting GA synthesis after formation of an indeterminate meristem, nodule development was no longer affected and fewer, but functional, nodules were initiated, reflecting the imperfect synchronization of nodulation at all the LRB sites.

It is well known that GAs control various plant developmental processes by promoting cell division or cell elongation (Stuart et al., 1977; Toyomasu et al., 1998; van den Heuvel et al., 2001). Recently, in cucumber (*Cucumis sativa*) and tomato (*Lycopersicon estivum*) GAs have been shown to be needed to induce the wound meristem during tissue reunion in the hypocotyls (Asahina et al., 2002). Interestingly, induction of the nodule primordium and of a wound meristem has been proposed to have much in common (Brewin, 2004). The need for GAs for the nodule primordium formation and differentiation might support this hypothesis.

GAs Are Required for Differentiation of the Nodule Meristem Descendants

SrGA20ox1 transcripts accumulated in a narrow zone of nodule meristem descendants and this pattern was still observed in coinoculation nodules that were invaded by non-Nod factor-producing bacteria. Differentiating cells derived from the meristem undergo enlargement, a process that may be regulated by GAs (Huttly and Phillips, 1995; Jacobs, 1997; Kende and Zeevaart, 1997; Dolan and Davies, 2004). Expression of *GA20ox* genes at the periphery of the meristem has been used to propose a role for GA in the shoot apical meristem and leaf development (Barley and Waites, 2002; Vogler and Kuhlemeier, 2003). Proteins of the KNOTTED (KNOX) homeodomain produced within the shoot apical meristem repress *GA20ox* expression to maintain the meristem. Disappearance of KNOX proteins, concomitantly with the appearance of GA20ox, may be associated with differentiation of meristematic cells for the formation of leaf primordia, for instance (Sakamoto et al., 2001; Barley and Waites, 2002). Interestingly, a KNOX homolog from barrel medic is produced in the meristem of indeterminate nodules (Koltai et al., 2001). Thus, this KNOX protein could inhibit GA production to keep a meristem identity, while the presence of GA20ox would trigger differentiation into central tissue cells.

It has been a long-standing question whether a nodule is a modified root, stem, or an organ *sui generis* (Hirsch and LaRue, 1997). Several experiments support the hypothesis that lateral root programs have been recruited for nodule formation (Ferguson and Mathesius, 2003). However, also shoot-linked expression patterns for nodulins have been reported, and the organization of the nodule vascular bundles is similar to that in a shoot. Here, we show that the meristem organization during nodule formation might have features in common with shoot apical meristems.

GA Inhibits RHC Nodulation

The most widespread mode of invasion is via curling of zone I root hairs and this invasion process occurs on *S. rostrata* roots when they are grown aeroponically (Goormachtig et al., 2004a, 2004b). The main difference between the two modes of invasion is that in LRB nodulation the epidermis, where several control checkpoints are established, is circumvented (Goormachtig et al., 2004b). After the invasion of the root hair during RHC, or IP formation during LRB, the process for IT formation and initiation of cell division within the cortex is the same (Goormachtig et al., 2004b). In contrast to LRB, application of GA_3 to roots grown in Leonard jars inhibited nodulation. Microscopic analysis revealed Nod factor-dependent root hair deformations, but curling of the root hairs was hampered, which is a requirement for IT initiation. Thus, excessive amounts of GAs inhibit the epidermal response during RHC. A similar observation has been made for ethylene (Goormachtig et al., 2004a).

Recently, addition of GA_3 at low concentration (10^{-9} M) in pea has been shown to enhance nodule formation, whereas at 10^{-6} M or higher the process was inhibited. Our data might fit with these results because inhibition is observed when 10^{-5} M GA_3 is added. It would be interesting to know at which stage, depending on the concentration range, GA_3 interferes with the pea nodulation: either at primordium formation or infection, or both (Ferguson et al., 2005).

Nodulation in aerated soils not only gives rise to the RHC process, but also to indeterminate nodules (Fernández-López et al., 1998). We were not able to detect *SrGA20ox1* transcripts by in situ hybridization during the RHC invasion because of problems with tissue fragility of aerated roots (data not shown), but we detected the transcripts in the nodule meristem descendants, where it presumably plays the same role in cell differentiation.

CCC application did not inhibit the nodulation process, although for LRB nodulation GAs are needed for IT growth and primordium formation and differentiation, processes that are common to both nodulation types. The simplest reason might be the consequence of the inaccessibility of the roots to pharmalogical compounds in the experimental set up for RHC nodulation. On the other hand, the requirement for GA might depend on the physiology of the root tissue in which invasion occurs. Different physiological environments are present during both infection ways (hydroponic versus aerated) and nodules are formed at different locations within the root (LRB versus zone I root hairs above the tip). As a result, for nodule formation, different concentrations of GAs might be needed to elicit the same processes.

Our work demonstrates that GAs have a function during LRB nodulation, which is recruited for nodule formation under hydroponic conditions (Goormachtig et al., 2004a). GAs are downstream signals of Nod factors for intercellular invasion and for formation of IPs and ITs at LRBs, and are essential for nodule primordium formation and differentiation. Like ethylene, GAs play an important role in adaptations to water logging. Upon submergence shoot elongation of deepwater rice (*Oryza sativa*) depends on GAs, as does hyponastic growth of leaves of marsh dock (*Rumex palustris*; Vriezen et al., 2003; Cox et al., 2004). Therefore, it is not surprising that GAs are needed during LRB nodulation.

MATERIALS AND METHODS

Biological Material

Sesbania rostrata Brem seeds were surface sterilized, grown, and inoculated as described (Goormachtig et al., 1995; Fernández-López et al., 1998). For the inhibitor assays, plants were grown in 70-mL tubes containing sterile nitrogen-free Norris medium (pH 7.0; Vincent, 1970) and sealed from the exterior by an aluminum foil cap surrounding the hypocotyl. For the Nod factor experiment, 20 roots were grown together in a 1-L pot for each stage. The bacterial strains ORS571, ORS571-X15 (Goethals et al., 1994), and ORS571-V44 (Van den Eede et al., 1987) of *Azorhizobium caulinodans* were cultivated as previously described (Goormachtig et al., 1995). Purified Nod factors were obtained as described by Mergaert et al. (1997) and added at a final concentration of 10^{-8} M (5.10^{-9} M of each fraction pI and pII; see Mergaert et al., 1997). *Ralstonia solanacearum* and *Botrytis cinerea* infections were performed as described (Lievens et al., 2004).

Isolation of the Full-Length *SrGA20ox1* Clone

The sequence of cDNA clone *Srdd16* was used as a source of primer sequences for the isolation of the corresponding full-length sequence by using the Marathon cDNA Amplification Kit (Clontech). cDNA was synthesized from RNA extracted from root primordia harvested 2 dpi with *A. caulinodans* ORS571. Several rounds of nested PCR amplification with the anchor primers AP1 and AP2 provided by the manufacturer in combination with gene-specific antisense primers sh8 (5′ GCAGCAGGAGCAGATATAACAGAAGC 3′), sh7 (5′ GTGGTTTGGAGGATAGCAACCACTTGG 3′), and sh23 (5′ CAGGCTCTGAGTTATTGTCATGGAAGGGG 3′) were necessary to obtain the full-length sequence, which was designated *SrGA20ox1*. A fragment of this sequence that corresponded with the open reading frame, initiating with the start codon and ending between the stop codon and the polyadenylation sequence, was amplified from the same cDNA template with Vent polymerase (New England Biolabs) and sense primer sh29 (5′ ATGGATTCAGGTTTGTGCTTAGTGTCTG 3′) and antisense primer sh30 (5′ GCAGCAGGAGCAGATATAACAGAAGC 3′), cloned in the pGEM-T vector (Promega), and designated pGEMTc8.2fl1.

DNA Gel-Blot Analysis

DNA gel-blot analysis was performed as described by Lievens et al. (2004). Labeled probes were generated from either the full-length cDNA insert of pGEMTc8.2fl1 or the differential display clone *Srdd16*.

Heterologous Expression in *Escherichia coli* and GA20ox Activity Assay

The insert from pGEMTc8.2fl1 was PCR amplified using primers containing a *Bam*HI restriction site and inserted into the *Bam*HI site of pET-3a (Novagen). Heterologous expression of the construct was carried out essentially as described by MacMillan et al. (1997). A 50-mL culture of *E. coli* containing the recombinant plasmid was induced at an OD_{600} of 0.36 with isopropyl-β-D-thiogalactoside (1 mM final concentration) and then grown at 30°C for 5 h. Cells were pelleted for 5 min at 4,000*g* and resuspended in 1 mL of 100 mM Tris-HCl (pH 7.5 at 25°C) containing 4 mM dithiothreitol and lysozyme (2 mg). After incubation at room temperature for 15 min, 25 μg DNAse was added, followed by an incubation at room temperature for another 30 min. After centrifugation at 15,000*g* for 5 min, 90 μL of the supernatant was incubated overnight at 30°C with [^{14}C]GA_{12} (167 Bq, 30 pmol) and cofactors in a total volume of 100 μL. The samples were processed and the reaction products were separated by HPLC according to MacMillan et al. (1997). The products were identified by gas chromatography-mass spectrometry (MacMillan et al., 1997).

RNA Analysis

RNA was prepared according to the protocol of Goormachtig et al. (1995) and the RT-PCR analysis was carried out as described (Corich et al., 1998). A 3′-sequence fragment of *SrGA20ox1* was amplified with sl32 (5′-AGAGCCGACGAAGATACCCT-3′) and sl106 (5′-GCCGTACAAAGTAGAATTAGGTTAAG-3′) as sense and antisense primer, respectively. As a constitutive control, a ubiquitin cDNA fragment was amplified (Corich et al., 1998). Twenty PCR cycles were performed, and PCR products were detected radioactively with probes generated from the cDNA fragment *Srdd16* and *Srubi1* (Corich et al., 1998) by means of the T7 QuickPrime kit (GE-Biosciences). The membranes were analyzed with a PhosphorImager (GE-Biosciences).

GA Inhibitor Experiments

Daminozide (Sigma-Aldrich) and CCC (Sigma-Aldrich) were prepared as aqueous solutions. Paclobutrazol (Duchefa) was stored as a stock solution of 5.10^{-2} M in methanol and diluted to lower concentrations in water. All chemicals were filter sterilized before addition to the liquid root medium. Pure methanol added to control plants at concentrations comparable to those present in the medium of roots treated with paclobutrazol did not affect nodulation. Media levels in the plant growth tubes were kept maximal by regular refilling, upon which the inhibitor concentrations were adjusted by adding the appropriate volumes of fresh stock solution.

To establish the optimal concentration for GA_3 and CCC, a range of concentrations was examined and the general fitness of the plant and the effect on axillary root outgrowth and nodulation were analyzed. For the antagonist, CCC, 10^{-2} M, 5.10^{-3} M, 10^{-3} M, 10^{-4} M, and 10^{-6} M were tested. After 2 weeks, only 10^{-2} M had a clearly negative effect on the health of plants compared to

that of nontreated plants, including smaller stems, browning of leaves, and a general low fitness. Plants treated with 5.10^{-3} M and 10^{-3} M, but not with 10^{-4} M and 10^{-5} M, had clearly stunted shoots and dark-green leaves, typical effects of GA inhibition. For the root hair essay, 5.10^{-3} M CCC was chosen because this concentration gave an effect on more than 95% of the plants whereas for 10^{-3} M this percentage was a bit lower. For the interference on nodulation, the effects of 5.10^{-3} M and 10^{-3} M are described in the results. Addition of 10^{-4} M of CCC had no effect on nodule number just as it had no typical GA antagonistic effect.

For GA_3, concentrations of 10^{-4} M, 10^{-5} M, and 10^{-6} M were tested. After 2 weeks, neither concentration affected the general plant health as scored above. The typical GA effects, such as the elongated stems and light-green leaves, were observed. For the axillary root hair experiment, the 10^{-5} M was chosen because it was the lowest concentration that affected more than 95% of the plants. A similar effect was obtained with 10^{-6} M, but the efficiency was lower. Each experiment was done at least in triplicate. Because of the heterogeneity of the seeds of *S. rostrata*, the nodule number has a degree of variation, and, therefore, the sum of the nodules counted on 10 plants is given for each treatment.

Control experiments in which Nod factors were labeled in vivo with 2-[^{14}C]acetate in the presence of GA inhibitor were carried out according to Mergaert et al. (1993). *A. caulinodans* ORS571 was grown overnight in the presence of the appropriate amount of GA inhibitor. The cultures were centrifuged and the pellet was resuspended in 1 mL water. Both pellet and culture supernatant samples were processed as described and analyzed by reversed-phase thin-layer chromatography. The results were visualized on a PhosphorImager (GE-Biosciences).

The *nod* gene induction was assayed as described by Goethals et al. (1989). Two-day-old *S. rostrata* seedlings were incubated in the dark at 37°C for 12 h on plates containing 80 μg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and the appropriate amount of GA inhibitor and covered by a lawn of *A. caulinodans* ORS571 (pRG290-12::T20) in λ top agar supplemented with the GA inhibitor as well.

Microscopic Analyses

In situ hybridization was performed on 10-μm sections of paraffin-embedded tissue as described by Goormachtig et al. (1997). The plasmid pBlueSKSrdd16 was digested with *Bam*HI and *Pst*I to obtain templates to produce a ^{35}S-labeled antisense and sense probe with T7 and T3 RNA polymerase (GE-Biosciences), respectively. Hybridizations with the sense probe did not result in above-background signals (data not shown). Histochemical analysis and staining for GUS were performed according to Fernández-López et al. (1998) and D'Haeze et al. (2003).

Sequence Analysis

DNA was sequenced with universal SP6 and T7 primers. Sequence data were assembled and analyzed with the GCG Wisconsin Package (Accelrys). Percentage of identity and similarity between sequences was determined with the Gap program and alignments were produced with the PileUp program (GCG Wisonsin Package).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ090959.

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