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Oligogalacturonides: Novel Signaling Molecules in Rhizobium-Legume Communications

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Oligogalacturonides are pectic fragments of the plant cell wall, whose signaling role has been described thus far during plant development and plant-pathogen interactions. In the present work, we evaluated the potential involvement of oligogalacturonides in the molecular communications between legumes and rhizobia during the establishment of nitrogen-fixing symbiosis. Oligogalacturonides with a degree of polymerization of 10 to 15 were found to trigger a rapid intracellular production of reactive oxygen species in *Rhizobium leguminosarum* bv. *viciae* 3841. Accumulation of H_2O_2, detected by both 2′,7′-dichlorodihydrofluorescein diacetate–based fluorescence and electron-dense deposits of cerium perhydroxides, was transient and did not affect bacterial cell viability, due to the prompt activation of the *katG* gene encoding a catalase. Calcium measurements carried out in *R. leguminosarum* transformed with the bioluminescent Ca^{2+} reporter aequorin demonstrated the induction of a rapid and remarkable intracellular Ca^{2+} increase in response to oligogalacturonides. When applied jointly with naringenin, oligogalacturonides effectively inhibited flavonoid-induced *nod* gene expression, indicating an antagonistic interplay between oligogalacturonides and inducing flavonoids in the early stages of plant root colonization. The above data suggest a novel role for oligogalacturonides as signaling molecules released in the rhizosphere in the initial rhizobium–legume interaction.

Oligogalacturonides (OG) are short linear molecules of α-1,4-D-galactopyranosyluronic acid residues (from 2 to approximately 20) released upon degradation of homogalacturonan from the plant primary cell wall (Ridley et al. 2001). They may be generated by the action of polygalacturonases and pectate lyases of either plant origin (e.g., during fruit ripening, leaf abscission, pollen tube growth, and pathogen attack) or microbial origin. In addition to plant pathogens, even beneficial microbes such as rhizobia produce pectinolytic enzymes (Angle 1986; Fauvart et al. 2009; Hubbell et al. 1978; Iannetta et al. 1997; Jimenez-Zurdo et al. 1996; Martinez-Molina et al. 1979; Mateos et al. 1992, 2001; Plazinski and Rolfe 1985; Wei et al. 2008). The localized digestion of the plant cell wall at the root hair tip by rhizobial cell-wall-degrading enzymes such as cellulases has been shown to represent a critical early step during plant root colonization by rhizobia (Robledo et al. 2008, 2011). Moreover, it has been recently demonstrated that legumes themselves supply a Nod-factor-inducible pectate lyase, allowing for the penetration of rhizobia in the nascent infection thread (Xie et al. 2012). OG may also be generated from the high molecular weight polysaccharide mucilage released in the rhizosphere by means of the controlled detachment of border cells from the root cap (Hawes et al. 2003; Wen et al. 2007).

OG have long been known to play a signaling role in plants, by activating a Ca^{2+}-mediated signaling pathway leading to the production of reactive oxygen species (ROS) (Lecourieux et al. 2002; Navazio et al. 2002) and activation of defense genes (Denoux et al. 2008; Moscatiello et al. 2006). OG also exert several morphogenetic effects on plants, mainly inhibition of auxin-induced processes (Altamura et al. 1998; Bellincampi et al. 1993, 1996, 2000) and enhancement of cytokinin-induced ones (Falasca et al. 2008). Furthermore, OG have been shown to modulate the pattern of somatic embryogenesis (Baldan et al. 2003).

In the present study, we have considered whether OG may be perceived by *Rhizobium leguminosarum* bv. *viciae* 3841 and carried out a dissection of the early steps of the signal transduction pathway activated by OG in rhizobia. The obtained results provide evidence for a novel signaling role played by these oligosaccharides during plant–rhizobium symbiotic interactions.

RESULTS

OG elicit intracellular ROS accumulation in *R. leguminosarum* bv. *viciae*.

OG with a degree of polymerization (DP) of 10 to 15 were found to evoke a transient accumulation of ROS in *R. leguminosarum* bv. *viciae* 3841 when tested at a concentration range of 10 to 40 µg/ml. Intracellular ROS production that was detected by means of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA)-based fluorescence started after approximately 5 min of bacterial incubation with OG and lasted for approximately 30 min (Fig. 1).

Accumulation of hydrogen peroxide (H_2O_2) was confirmed by experiments of in situ cytochemical localization of H_2O_2, based on the generation of cerium perhydroxides after incubation with $CeCl_3$. Whereas no H_2O_2 accumulation was observed in control rhizobial cells (Fig. 2A), electron-dense deposits of cerium perhydroxides were present in the periplasm (Fig. 2D) or on the surface of the outer membrane (Fig. 2E) at different time intervals from the beginning of OG treatment. The results show that H_2O_2 production is an early rhizobial response to OG: at 5 min after the beginning of the incubation with OG, very dense cerium deposits were visible (Fig. 2B), which became fainter and distributed as patches after 30 min (Fig. 2C). In both control and treated samples of *R. leguminosarum* bv.

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viciae, small electron-dense spherical compartments, commonly known as polyphosphate (volutin) granules and possibly identifiable as acidocalcisomes putatively involved in Ca^{2+} handling (Docampo and Moreno 2011), were often observed (Fig. 2).

Ca^{2+}-based perception of OG by *R. leguminosarum.*

To analyze the potential participation of calcium in the perception of OG by rhizobia, *R. leguminosarum* bv. *viciae* 3841 was transformed with the plasmid pAEQ80, encoding the bioluminescent Ca^{2+} reporter aequorin (Moscatiello et al. 2009). Ca^{2+} measurements carried out in *R. leguminosarum* upon challenge with OG demonstrated the induction in rhizobia of transient elevations in the cytosolic concentration of Ca^{2+} ($[Ca^{2+}]_{cyt}$), whose amplitude was found to be dose dependent, until the saturating concentration of 40 µg/ml (corresponding to 18 µM) was reached (Fig. 3A). Aequorin-expressing *R. leguminosarum* 8401 that lacks its symbiotic plasmid was found to respond to OG with a similar behavior as strain 3841, although with a higher sensitivity in terms of the magnitude of the triggered Ca^{2+} increase (Fig. 3B) and ROS production (data not shown), being the maximal response obtained with a much lower OG dose (5 µg/ml).

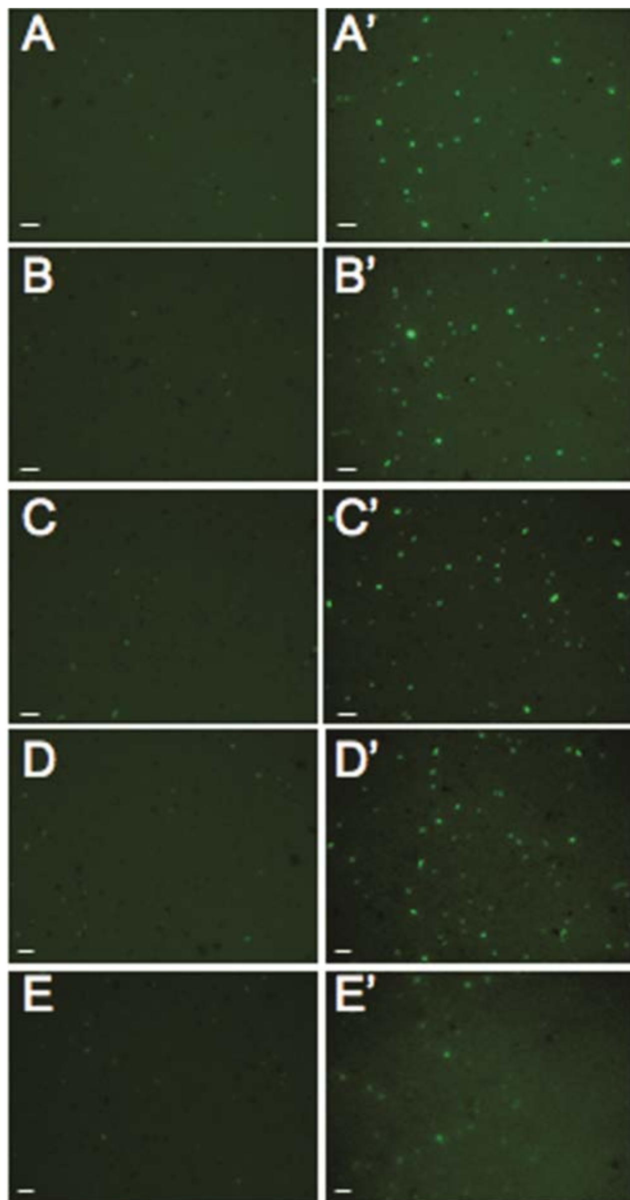

Fig. 1. Effect of oligogalacturonides (OG) on the production of reactive oxygen species (ROS) in *Rhizobium leguminosarum* bv. *viciae* 3841. ROS formation was detected by 2′,7′-dichlorodihydrofluorescein diacetate staining. Fluorescence microscopy images of bacterial cells incubated for different time intervals with **A** to **E,** cell culture medium only (control); **A'** to **E'**, OG at 40 µg/ml (treated samples). **A** and **A',** 5 min; **B** and **B',** 10 min; **C** and **C',** 20 min; **D** and **D',** 30 min; **E** and **E'**, 35 min. Bar: 10 µm.

To check whether rhizobia may perceive the potential oxidative stress caused by OG in a Ca^{2+}-dependent manner, cell cultures of *R. leguminosarum* transformed with aequorin were challenged with increasing concentrations of H_2O_2. Both strains 3841 and 8401 responded to 1 and 10 mM H_2O_2 with remarkable Ca^{2+} changes (Fig. 3A and B, insets), suggesting a potential dual involvement of Ca^{2+} (i.e., in the initial perception of OG and in the subsequent oxidative stress response).

OG activate an ROS scavenging system and counteract naringenin-induced *nod* gene expression.

Reverse-transcription polymerase chain reaction (RT-PCR) analyses of gene expression in *R. leguminosarum* bv. *viciae* 3841 showed no induction by OG (40 µg/ml for 10 min, 30 min, or 1 h) of the genes homologous to the cellulase-encoding gene *celC2* of *R. leguminosarum* bv. *trifolii* (Robledo et al. 2008), and the *picA* and *pgl* loci of *Agrobacterium tumefaciens* (Rong et al. 1994), encoding putative polygalacturonase-like proteins (data not shown). A significant upregulation was detected for the *katG* gene that has been previously shown to encode for the predominant catalase in both *R. etli* (Vargas et al. 2003) and *Bradyrhizobium japonicum* (Panek and O'Brian, 2004). The constitutive transcript level of *katG* was progressively enhanced during the first 30-min treatment of rhizobia with OG at 40 µg/ml (1.6-fold after 10 min and 2.3-fold after 30 min, $P < 0.05$) before decreasing back to basal values after 1 h (Fig. 4).

In keeping with the activation of an efficient ROS scavenging system potentially involved in the dissipation of the OG-induced H_2O_2 production (see previous paragraph), bacterial cell viability was found not to be affected by OG treatment, as demonstrated by staining of rhizobia with SYTO 9 and propidium iodide after 1 h of incubation with the oligosaccharide elicitors (Fig. 5).

Treatment of rhizobia with OG (40 µg/ml for 1 h) did not induce the expression of *nodC*, an *N*-acetylglucosaminyltransferase-encoding gene centrally involved in Nod-factor biosynthesis. When OG were administered to *R. leguminosarum* 3841 together with the flavonoid naringenin, the transcriptional activation of *nodC* induced by naringenin (10 µM) was found to be effectively inhibited (Fig. 6A). A similar, although partial, antagonistic effect played by OG on the inducing activity of naringenin was obtained by using *R. leguminosarum* 8401 (pSym$^-$), containing the pIJ1477 plasmid (*nodC-lacZ* fusion), and the pIJ1518 plasmid, carrying the gene encoding for the transcriptional activator NodD, essential for the expression of the common nodulation (*nod*) genes (Fig. 6B), in Miller assays. In parallel experiments, it was verified that the treatment with OG did not have any effect on the bacterial culture growth for up to 8 h of incubation (data not shown). Considering that our exposure of exponentially growing cells to OG lasted for only 1 h, such control ruled out the possibility that the lower expression level observed could be due to a general effect on cell growth rate.

In contrast, the constitutive expression of nodD was not altered by treatment with OG, administered either singly or in combination with naringenin, in both strain 3841 (Fig. 6A) and 8401 containing the pIJ1478 plasmid (*nodD-lacZ* fusion) (Fig. 6C). Likewise, no effect on *nodD* expression was observed in backgrounds also containing the full functional copy of the gene because the treatment of strain 8401 pIJ1478

(*nodD-lacZ*) pIJ1518 (*nodD*) with either OG or OG + naringenin did not alter the negative autoregulation of the *nodD* gene (data not shown).

An OG preparation with a DP of 1 to 5, as well as the final product of OG degradation (i.e., the galacturonic acid monomer), could neither trigger $[Ca^{2+}]_{cyt}$ changes nor affect naringenin induction of *nod* genes (Supplementary Fig. S1), indicating that the DP size of the OG fractions is likely to play a crucial role in their bioactivity.

In agreement with the in vitro data about *nod* gene expression, *Vicia sativa* subsp. *nigra* roots treated with culture filtrates from *R. leguminosarum* bv. *viciae* 3841 cells, previously incubated overnight with OG of DP 10 to 15 (40 µg/ml) and naringenin (10 µM), were found to lack the root hair deformations that were observed with culture filtrates from naringenin-induced rhizobia, suggesting the effective absence of Nod factors in supernatants of cultures in which naringenin was supplied along with OG (Supplementary Fig. S2).

DISCUSSION

Research on OG in the last two decades has been mainly focused on the effects of these plant cell-wall-derived oligosaccharides on plant growth and development and elicitation of plant defenses during plant–pathogen interactions. Different groups have demonstrated that OG activate transient cytosolic Ca^{2+} changes and an oxidative burst in plant cells (Lecourieux et al. 2002; Navazio et al. 2002). More recently, cDNA microarray analyses have uncovered the transcriptional activation by OG of genes involved in multiple defense signaling pathways: in particular, the mitogen-activated protein kinase gene family, genes involved in plant cell wall modification, jasmonic acid/ethylene–associated processes, and several transcription factor-encoding genes (Denoux et al. 2008; Moscatiello et al. 2006). Recently, a role of the plant wall-associated kinase 1 (WAK1) as a receptor for OG has also been demonstrated (Brutus et al. 2010). It has to be noted that, from the plant point of view, OG should be considered as host-associated molecular patterns rather than pathogen-associated molecular pattern, because they do not derive from the pathogen but from the host plant cell (Galletti et al. 2009).

OG may be generated not only during the interactions of plants with pathogens but also with beneficial microbes such as rhizobia, by the localized action of pectinolytic enzymes of either microbial or plant origin (Ljunggren and Fahraeus 1959, 1961; Xie et al. 2012) during root infection by rhizobia. Nev-

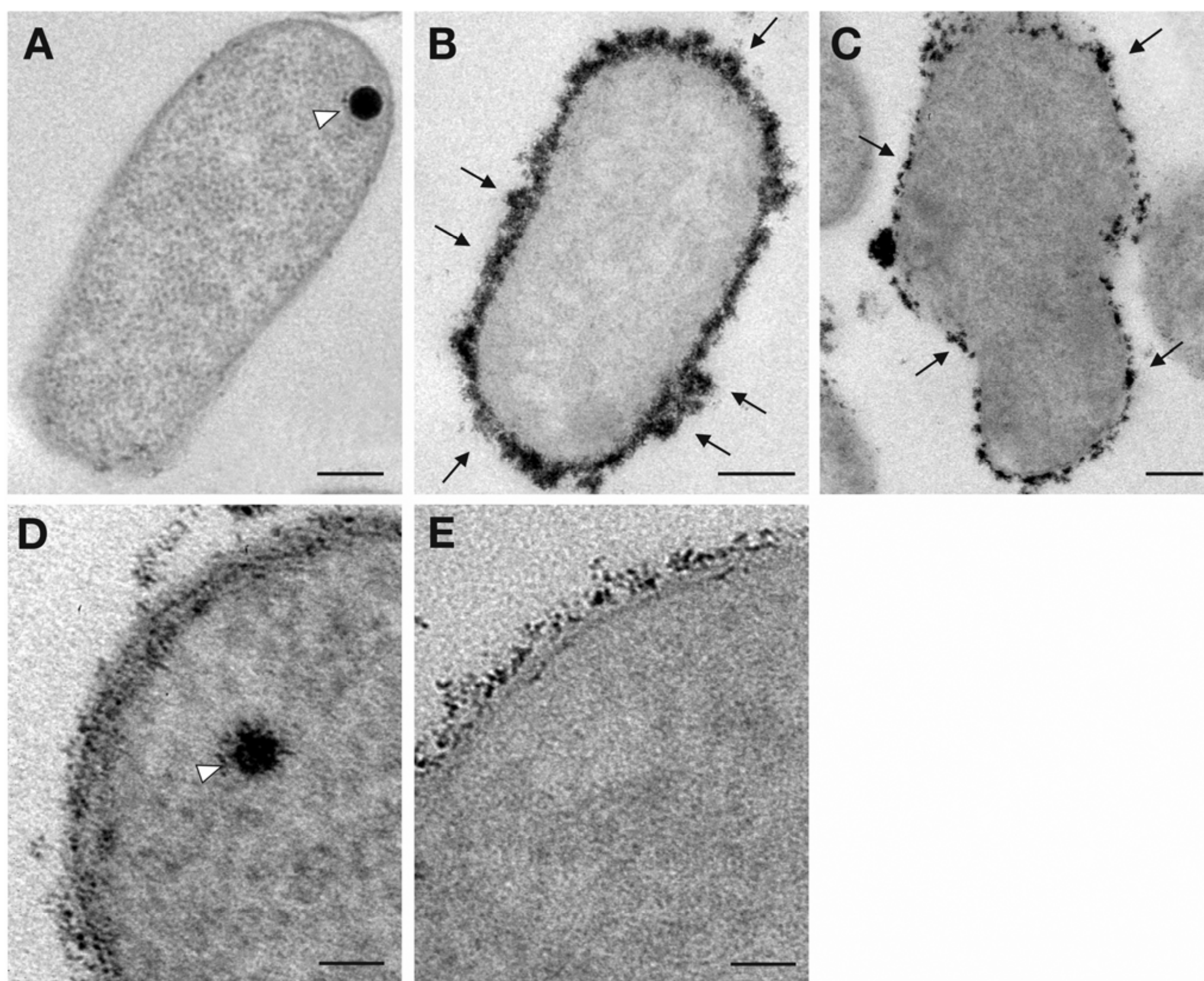

Fig. 2. Localization of H_2O_2 accumulation in *Rhizobium leguminosarum* bv. *viciae* 3841 in response to oligogalacturonides (OG). Electron micrographs of **A,** control rhizobia and **B** and **C,** bacteria treated with OG at 40 µg/ml for **B,** 5 min and **C,** 30 min. At the end of treatment, samples were incubated with $CeCl_3$ and processed for transmission electron microscopy (TEM). **D** and **E,** Higher-magnification TEM images of OG-treated rhizobia showing cerium perhydroxide precipitates in the periplasm or on the surface of the outer membrane. Arrows, electron-dense deposits of cerium perhydroxides. Arrowhead, acidocalcisome (polyphosphate/volutin) granules. Bar: A to C, 200 nm; D and E, 50 nm.

ertheless, the potential role exerted by OG during beneficial plant–microbe interactions has not yet been evaluated.

In this work, OG with a DP of 10 to 15 were found to trigger an oxidative stress response in *R. leguminosarum* bv. *viciae* 3841, given by a transient accumulation of ROS and the subsequent prompt activation of a catalase-based scavenging system. Interestingly, the occurrence of an oxidative burst at the site of attempted invasion seems to be a common trait in both pathogenic and symbiotic interactions (Nanda et al. 2010; Torres 2010). Indeed, ROS production by the plant host has been highlighted by several groups during the early stages of legume-rhizobium interactions (Bueno et al. 2001; Cardenas et al. 2008; D'Haeze et al. 2003; Lohar et al. 2007; Peleg-Grossman et al. 2007; Ramu et al. 2002; Santos et al. 2001). Reviews on the production of ROS by legumes in the context of nodulation have been compiled (Glyan'ko and Vasil'eva 2010) and the mechanisms of their scavenging have been listed (Matamoros et al. 2003). Salzwedel and Dazzo (1993) demonstrated rhizobial species-dependent transient induction and localization of legume host root peroxidase at the site of incipient microsymbiont penetration and its role during successful primary host infection. Moreover, a *Sinorhizobium meliloti* mutant overexpressing the housekeeping catalase KatB exhibited a delayed nodulation phenotype, suggesting that H_2O_2 is required for optimal progression of *Medicago sativa* infection (Jamet et al. 2007). ROS, far from being just toxic byproducts of aerobic metabolism, are increasingly being appreciated as central players in signaling networks from bacteria to eukaryotic cells (Mittler et al. 2011). The role of redox signals in establishing and maintaining symbiosis between rhizobia and legumes has been reviewed by Chang and associates (2009).

Our results, as regards OG-induced H_2O_2, have been obtained in vitro on rhizobia growing in TY medium; nevertheless, also under these ex planta conditions, rhizobia are known to be fully able to be induced by plant flavonoids and to produce, in return, their active Nod factors that can trigger nodulation. Because OG-producing pectate lyase has been demonstrated to act right at the onset of nodulation (Xie et al. 2012), it is possible to speculate that H_2O_2 may be produced during the early phases of rhizobium–legume interactions, as a result of the activation of not only the ROS-producing system of the plant host but also the symbiotic microorganism.

ROS are known to be formed in bacteria when O_2 oxidizes redox enzymes involved in electron transfer to other substrates (Imlay 2003). Possible sources of endogenous H_2O_2 have recently been investigated in *Escherichia coli* (Korshunov and Imlay 2010).

To cope with OG-induced oxidative stress, *R. leguminosarum* cells were found to rapidly upregulate the catalase-encoding gene *katG*. The prompt induction of oxidative stress-related genes is a common mechanism of primary and secondary oxidative stress response in bacteria (Mols and Abee 2011).

Transformation of *R. leguminosarum* 3841 and 8401 with a plasmid encoding for the Ca^{2+}-sensitive photoprotein aequorin allowed for the elucidation of the signaling pathway evoked by OG that was found to involve a potential twofold participation of calcium as intracellular messenger both upstream and downstream of the oxidative burst. The recombinant aequorin technique has been firmly demonstrated to be one of the most suitable methods to perform Ca^{2+} measurements in bacteria (Barrán-Berdón et al. 2011; Campbell et al. 2007). The Ca^{2+}-based signaling mechanism underlying OG perception by *R. leguminosarum* bv. *viciae* extends the range of environmental stimuli that evoke transient Ca^{2+} changes in rhizobia (Moscatiello et al. 2009, 2010) and confirms the versatility and

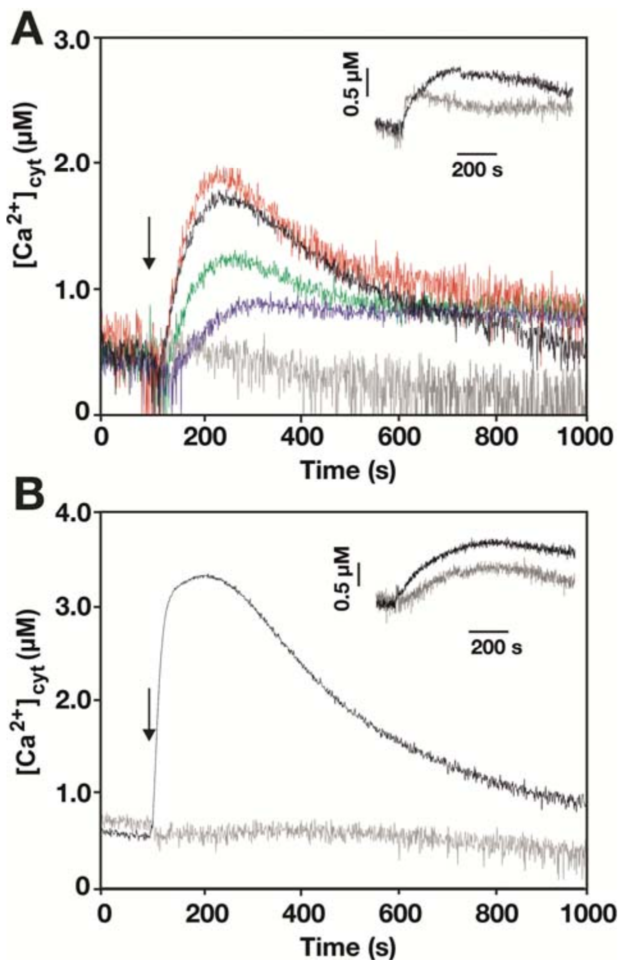

Fig. 3. Monitoring of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in aequorin-expressing *Rhizobium leguminosarum* in response to oligogalacturonides (OG). OG were administered to **A,** *R. leguminosarum* 3841 (blue trace, 10 µg/ml; green trace, 25 µg/ml; black trace, 40 µg/ml; red trace, 50 µg/ml); and **B,** *R. leguminosarum* 8401 (black trace, 5 µg/ml). Rhizobia were treated with buffer for Ca^{2+} measurements as control (gray trace). The arrow indicates the time of injection (100 s). In the insets, the effect of oxidative stress on $[Ca^{2+}]_{cyt}$ in *R. leguminosarum* is shown. Different doses of H_2O_2 (gray trace, 1 mM; black trace, 10 mM) were applied to aequorin-expressing *R. leguminosarum* **A,** inset, 3841 and **B,** inset, 8401. Ca^{2+} traces are representative of three independent experiments which gave very similar results.

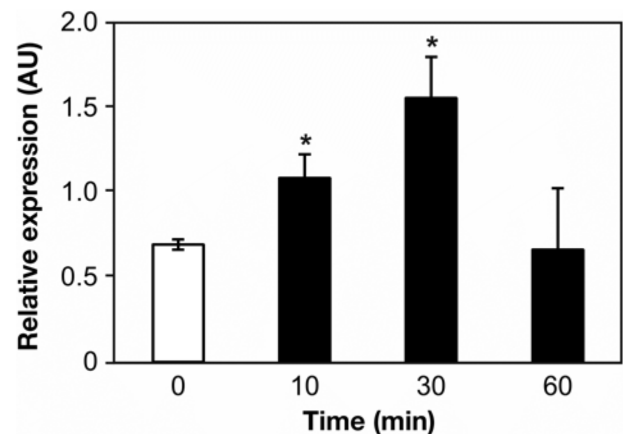

Fig. 4. Reverse-transcriptase polymerase chain reaction analysis of *katG* gene expression in *Rhizobium leguminosarum* bv. *viciae* 3841. Rhizobia were treated with oligogalacturonides (OG) (40 µg/ml) for different time intervals. Relative transcript abundance was normalized against 16S rRNA. Data are the means ±standard error of three independent experiments; * indicates statistically significant at $P < 0.05$.

potential universality of Ca^{2+} as a cellular regulator not only in eukaryotes (Cai and Clapham 2012; Clapham 2007; Dodd et al. 2010) but also in bacteria (Dominguez 2004; Stael et al. 2012). It is still unknown whether the extracellular medium represents the main source for Ca^{2+} fluxes in bacteria, or whether intracellular Ca^{2+} stores such as the so-called acidocalcisomes (Docampo and Moreno 2011) may also be involved in Ca^{2+} handling. Indeed, it has been shown that Ca^{2+} may be mobilized from the periplasmic space in *E. coli* (Jones et al. 2002) and from still unidentified intracellular Ca^{2+} stores in cyanobacteria (Torrecilla et al. 2004).

Our data shed light on a previously uninvestigated involvement of OG in the molecular communications underlying plant-rhizobium symbiotic interactions (Murray 2011; Oldroyd et al. 2011). On the basis of our results, we propose a new signaling role for these long-standing molecular signals: they are perceived and transduced not only by plants but also by rhizobia, with a similar DP size and concentration range required for their biological activity. It has to be noted that OG seem to be the only informational chemicals known to date to be sensed by both partners of the nitrogen-fixing symbiosis. Interestingly, in both the plant host and the microsymbiont, OG activate a Ca^{2+}-mediated signaling pathway and a transient oxidative burst.

Other oligosaccharide elicitors (i.e., oligoguluronates and oligomannuronates) have recently been shown to trigger transient $[Ca^{2+}]_{cyt}$ increases in both gram-negative and gram-positive bacteria such as *E. coli* and *Bacillus subtilis*. The observed increased intracellular Ca^{2+} levels have been hypothesized to contribute to the enhancement of secondary metabolite levels in microbes (Murphy et al. 2011). It remains to be ascertained whether elicitation with OG may lead to the accumulation of some secondary metabolites in rhizobia.

Interestingly, we found that OG effectively blocked the activation of naringenin-inducible *nod* genes, lending themselves as novel potential anti-inducers in *R. leguminosarum*, in addition to the well-known noninducing flavonoids in this species such as genistein and daidzein. The level of transcription inhibition observed by RT-PCR in strain 3841 was total, whereas that assayed at post-translational level by Miller assay in 8401 ($pSym^{-}$) containing pIJ1477 and pIJ1518 was partial. This can be explained by the fact that the latter construct bears the *nod* genes in plasmids whose copy number is higher than that of the pSym in wild-type 3841. Moreover, the occurrence of basal levels of expressed proteins constitutes a common trait in bacterial phenotypic assays when compared with the stringency of transcriptional regulation events.

The lack of any detectable effect by OG on nodD expression (in both 3841 and 8401 pIJ1478) suggests that the action is played on genuine flavonoid-inducible genes, because the constitutively expressed *nodD* is not affected. Moreover, because NodD is also known to negatively regulate the expression of its own gene, the fact that, in a *nodD*$^{+}$ background (8401

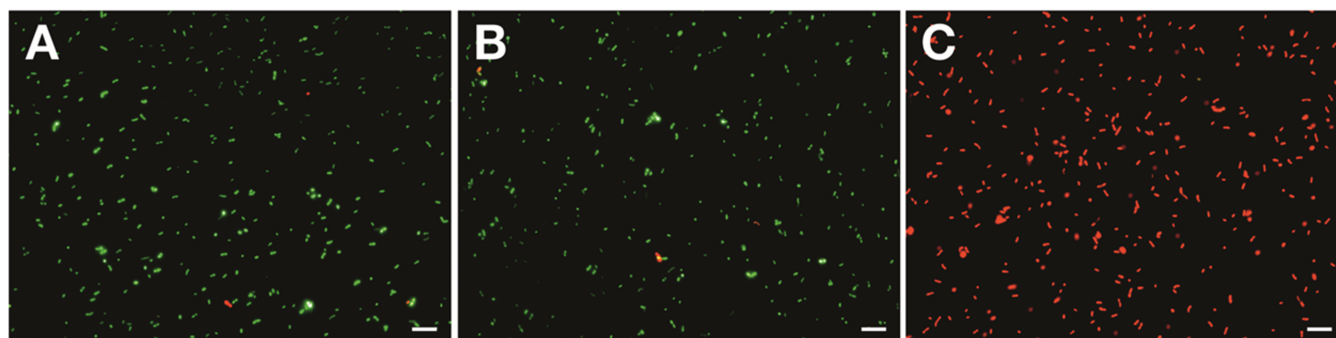

Fig. 5. Viability of *Rhizobium leguminosarum* bv. *viciae* 3841 after treatment with oligogalacturonides (OG). Bacterial cell viability was monitored with the LIVE/DEAD *Bac*Light Bacterial Viability kit, consisting in a mixture of the nucleic acid stains SYTO 9 and propidium iodide. Mid-exponential-phase bacterial cells were treated for 1 h in **A,** control conditions; **B,** OG at 40 µg/ml; or **C,** 70% isopropyl alcohol. Merged images of SYTO 9 and propidium iodide fluorescence are shown. Bar, 10 µm.

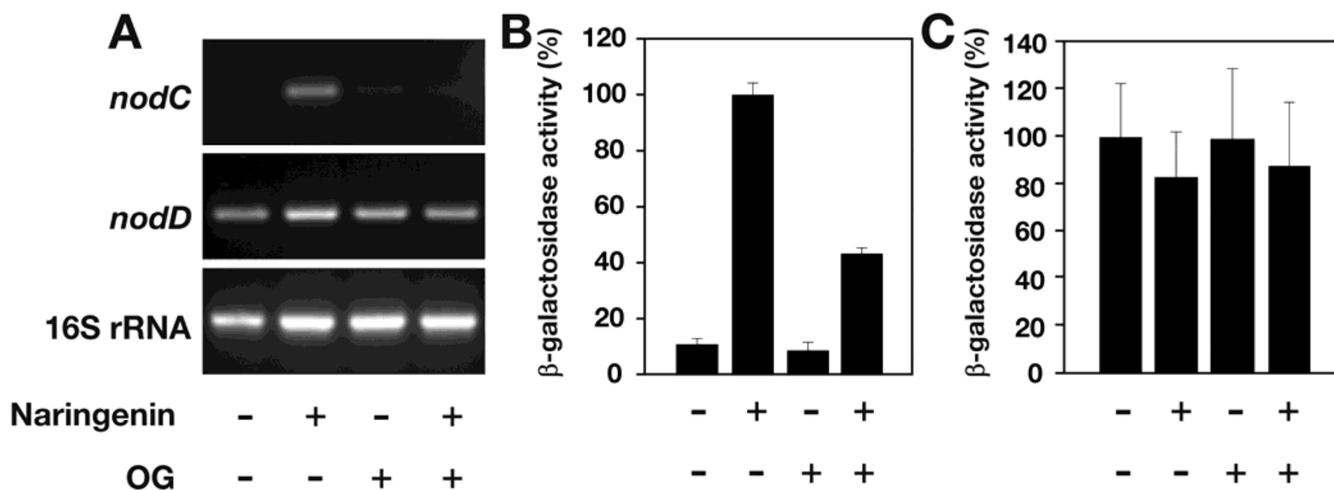

Fig. 6. Analysis of *nodC* and *nodD* gene expression in *Rhizobium leguminosarum*. Induction of nodulation genes was analyzed by **A,** reverse-transcriptase polymerase chain reaction in *R. leguminosarum* 3841 and **B,** β-galactosidase activity in *R. leguminosarum* 8401 containing plasmids pIJ1477 (*nodC-lacZ* fusion) and pIJ1518 (*nodD*) or **C,** pIJ1478 (*nodD-lacZ* fusion). Rhizobia were incubated for **A,** 1 h or **B** and **C,** 4 h with culture medium only, OG (**A,** at 40 µg/ml or **B** and **C,** at 5 µg/ml), naringenin (10 µM), or the two treatments combined, as indicated. In A, transcription levels of 16S rRNA were used as standards.

pIJ1478 pIJ1518), *nodD* transcription is not enhanced either suggests that the OG effect is not exerted via an interaction with the NodD protein or that, if this occurs, it does not impair its autoregulatory domain.

The finding that even the 8401 (pSym$^-$) mutant strain of *R. leguminosarum* (pSym$^-$) responds to OG with a transient Ca^{2+} change indicates that the OG receptor-encoding gene is not on the symbiotic plasmid. The molecular identity of such a receptor remains unknown, because database searches in the sequenced genome of *R. leguminosarum* 3841 (Young et al. 2006) did not reveal any genes homologous to plant WAK (data not shown), one isoform of which has recently been identified as encoding the OG receptor in plants (Brutus et al. 2010).

Hypothesizing on the possible reasons for a regulatory role of OG on nodulation genes, the naringenin-counteracting action played by OG may fulfill a modulatory role on *nod* gene expression along plant root invasion. During the infection thread progression throughout the root cortex, a localized degradation of the plant cell wall is repeated at each cell junction (Oldroyd et al. 2011), thereby providing multiple potential sites for OG generation. At each of these events, a tune-down of the *nod* genes might ensure a fine modulation of the Nod factor signal concentrations to the benefit of a minimal level of impact in the use of the specific entry tools, which is deemed appropriate for the compatible invasion of the homologous host legume.

To further dissect the complex Ca^{2+}-mediated signaling cascades triggered by flavonoids (Moscatiello et al. 2010) and OG (this article) and their potential interplay, future investigations should be addressed to unraveling components of the Ca^{2+} signature decoding machinery in bacteria, in particular Ca^{2+} sensors and their targets (Michiels et al. 2002; Rigden et al. 2003, 2011; Zhou et al. 2006).

In this work, we have shown a previously underestimated role of OG as host-derived signals that may be sensed by rhizobia in a Ca^{2+}-dependent manner and activate in the microsymbiont a transient production of H_2O_2. It is becoming increasingly evident that common themes underlie the molecular dialogue of plants with pathogenic and mutualistic microorganisms, which often makes use of similar molecular components and signaling processes (Gough and Cullimore 2011; Oldroyd and Robatzek 2011). A transient state of alert in which the two interacting partners have to reciprocally recognize themselves as friends and not foes may be essential for an optimal establishment of the symbiotic association. It is interesting to remark that, in pathogens, the invader is the one whose activity would generate OG and trigger plant defense responses whereas, in symbionts, their Nod factors would induce the plant to make its own pectate lyase (Xie et al. 2012) that could bring about OG which, in turn, would tune down the Nod factor production.

MATERIALS AND METHODS

Chemicals.

OG with a DP of 10 to 15 were produced and isolated as described by Moscatiello and associates (2006). OG with a DP of 1 to 5 were provided by D. Bellincampi (Rome). Coelenterazine was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Molecular biology reagents were purchased from Promega Corp. (Madison, WI, U.S.A.), Qiagen (Hilden, Germany), and Clontech (Mountain View, CA, U.S.A.). The flavonoid naringenin and all other reagents were obtained from Sigma-Aldrich (St. Louis).

Bacterial strains, plasmids, and culture conditions.

R. leguminosarum bv. *viciae* 3841 was provided by P. Young (York, U.K.). *R. leguminosarum* 8401 that lacks the Sym plasmid and carries the pIJ1477 plasmid (*nodC-lacZ* fusion) and pIJ1518 (cloned *nodD*), pIJ1478 (*nodD-lacZ* fusion), or pAEQ80 (cloned aequorin) (Moscatiello et al. 2010) were also used. Strains were grown in TY medium, containing the appropriate antibiotics (streptomycin at 500 µg/ml for strain 3841, tetracycline at 2 µg/ml for strain 8401 pIJ1477 and 8401 pIJ1478, and kanamycin at 50 µg/ml for strains containing pIJ1518 or pAEQ80 plasmids) at 28°C under shaking at 170°C.

Plant material.

Seeds of *V. sativa* subsp. *nigra* (Vergerio Mangimi s.r.l., Padova, Italy) were surface sterilized by immersion in H_2SO_4 (4 min) followed by five washes in H_2O (30 min), 3% NaClO (4 min), and five washes in H_2O (30 min), and allowed to germinate for 3 days on 0.7% water agar at 24°C in the dark. Seedlings were transferred on 0.1% Jensen medium solidified with 1% agar, and 20 µl of culture filtrates (through 0.20-µm sterile filters) from *R. leguminosarum* bv. *viciae* 3841 cell suspensions that had previously been subjected to different overnight treatments were applied onto the roots. A sterile glass coverslip (12 mm in diameter) was placed on the root over the applied drop. Root hair observations were carried out after 12 h with a Leica DMI4000 B inverted microscope.

Transformation of *R. leguminosarum.*

The expression vector pAEQ80 carrying the apoaequorin cDNA under the control of the strong isopropyl β-D-thiogalactopyranoside (IPTG)-inducible synthetic promoter P_{syn} and conferring resistance to kanamycin (Moscatiello et al. 2009) was introduced into the *R. leguminosarum* bv. *viciae* 3841 using a freeze-thaw method (Vincze and Bowra 2006).

Detection of ROS.

Intracellular ROS production was detected as described by Maxwell and associates (1999). Exponentially growing cultures of *R. leguminosarum* (optical density at 600 nm [OD_{600}] of approximately 0.25) were loaded with 10 µM H_2DCFDA (Invitrogen) for 30 min. This nonpolar compound passively diffuses into cells where it is converted by endogenous esterases in 2′,7′-dichlorodihydrofluorescein, a nonfluorescent derivative that is rapidly oxidized to the highly fluorescent 2′,7′-dichlorofluorescein by intracellular peroxides. Excess dye was removed by extensive washing with fresh culture medium. Bacteria were treated with OG or cell culture medium only (control) and observed under a Leica DM5000 B fluorescence microscope, with excitation at 450 to 490 nm and emission at 500 to 550 nm. Images were acquired with a Leica DFC 425 C digital camera using the Leica Application Suite software.

Cytochemical localization of H_2O_2.

Cytochemical localization of H_2O_2 based on the generation of cerium perhydroxides was carried out as described by Bestwick and associates (1997). Briefly, mid-exponential-phase *R. leguminosarum* cells were treated with OG (40 µg/ml) for different time intervals or with cell culture medium only (control). After centrifugation, the bacterial pellet was incubated for 1 h in 5 mM $CeCl_3$ and 50 mM 3-(N-morpholino)propanesulfonic acid, pH 7.2. The resedimented rhizobia were immediately fixed for 1 h in 1.25% (vol/vol) glutaraldehyde/1.25% (vol/vol) paraformaldehyde in 50 mM sodium cacodylate (CAB) buffer, pH 7.2. After two washes for 10 min in CAB buffer, cells were postfixed for 45 min in 1% (vol/vol) osmium tetroxide in CAB and washed as above. Dehydration was performed in a graded ethanol series. Samples were then transferred into propylene oxide and progressively embedded in Epon. Thin sections were obtained on a Reichert-Ultracut microtome, mounted on uncoated copper

grids, and observed using a Tecnai 12-BT transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 120 kV equipped with a Tietz camera.

Ca^{2+} measurement assays with recombinant aequorin.

Aequorin expression was induced by inoculating a loopful of pAEQ80-containing *R. leguminosarum* strains into 30 ml of TY medium supplemented with the appropriate antibiotics and 1 mM IPTG overnight, until an $OD_{600} = 0.25$ (corresponding to early exponential phase) was reached. Bacterial suspensions were washed and resuspended in buffer A (25 mM Hepes, 125 mM NaCl, and 1 mM $MgCL_2$, pH 7.5) and incubated with 5 µM coelenterazine for 90 min, as described by Campbell and associates (2007). Aequorin-based Ca^{2+} measurements were carried out in the presence of 6 mM $CaCl_2$ (the same concentration as in TY medium) in a purpose-built luminometer (Electron Tubes Limited, Uxbridge, U.K.) as previously described (Moscatiello et al. 2010).

Semiquantitative RT-PCR analysis.

Extraction of RNA and RT-PCR analysis of gene expression were carried out as previously described (Moscatiello et al. 2009). Briefly, cells were grown to an OD_{600} of approximately 0.25 and subjected to the different treatments. After adding RNAprotect Bacteria Reagent (Qiagen), cells (5×10^8) were lysed with lysozyme (Sigma) at 0.5 mg/ml for 5 min. RNA was isolated with RNeasy mini kit (Qiagen) according to the manufacturer's instructions, treated with DNase I (Promega Corp.), and quantified. cDNA was synthesized from 5 µg of RNA using Random Decamers (Ambion) and SMARTScribe Reverse Transcriptase (Clontech) and diluted 1:5. First-strand cDNA (5 µl) was used as a template for subsequent PCR analyses with Advantage 2 Polymerase mix (Clontech). The oligonucleotide primers used in this study (Supplementary Table S1) were designed against *nodC*, *katG*, and the gene sequences homologous to *celC2* of *R. leguminosarum* bv. *trifolii* (Robledo et al. 2008), and *picA* and *pgl* of *A. tumefaciens* in the sequenced genome of *R. leguminosarum* bv. *viciae* 3841 (Young et al. 2006). To amplify the 16S ribosomal RNA (rRNA) gene, Y1 and Y2 primers were used (Young et al. 1991). RT-PCR experiments were conducted in duplicate on three independent experiments. The statistical significance of differences between means was evaluated by the Student's *t* test.

β-Galactosidase assay.

R. leguminosarum 8401 pIJ1477 pIJ1518, containing both a *nodC-lacZ* gene fusion and a cloned *nodD*, and 8401 pIJ1478, containing a *nodD-lacZ* gene fusion, were grown for 4 h in control conditions or with the specified compounds. The β-galactosidase activity assay was carried out as described by Miller (1972).

Bacterial cell viability assay.

Bacterial cell viability was monitored by the LIVE/DEAD *Bac*Light bacterial viability kit (Invitrogen). This fluorescence-based assay utilizes a mixture of the nucleic acid stains SYTO 9 and propidium iodide to distinguish live and dead bacteria. The excitation and emission maxima are 480 and 500 nm, respectively, for SYTO 9 and 490 and 635 nm, respectively, for propidium iodide. Live bacteria fluoresce green, whereas dead bacteria fluoresce red. As a positive killing control, rhizobia were treated with 70% isopropyl alcohol (100% dead bacteria).

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