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INVITED REVIEW

Biological nitrogen fixation in non-legume plants

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• *Background* Nitrogen is an essential nutrient in plant growth. The ability of a plant to supply all or part of its requirements from biological nitrogen fixation (BNF) thanks to interactions with endosymbiotic, associative and endophytic symbionts, confers a great competitive advantage over non-nitrogen-fixing plants.
• *Scope* Because BNF in legumes is well documented, this review focuses on BNF in non-legume plants. Despite the phylogenic and ecological diversity among diazotrophic bacteria and their hosts, tightly regulated communication is always necessary between the microorganisms and the host plant to achieve a successful interaction. Ongoing research efforts to improve knowledge of the molecular mechanisms underlying these original relationships and some common strategies leading to a successful relationship between the nitrogen-fixing microorganisms and their hosts are presented.
• *Conclusions* Understanding the molecular mechanism of BNF outside the legume–rhizobium symbiosis could have important agronomic implications and enable the use of N-fertilizers to be reduced or even avoided. Indeed, in the short term, improved understanding could lead to more sustainable exploitation of the biodiversity of nitrogen-fixing organisms and, in the longer term, to the transfer of endosymbiotic nitrogen-fixation capacities to major non-legume crops.

Key words: Nitrogen fixation, non-legume, symbiosis, nodulation, actinorhizal plant, *Frankia*, cyanobacteria, *Parasponia*, plant growth-promoting rhizobacteria, PGPR.

INTRODUCTION

Nitrogen is an essential element in plant development and a limiting factor in plant growth. It represents about 2 % of the total plant dry matter that enters the food chain. Nevertheless, plants cannot directly access dinitrogen gas, which makes up about 80 % of the atmosphere. Plants absorb the available nitrogen in the soil through their roots in the form of ammonium and nitrates. The limited bioavailability of nitrogen and the dependence of crop growth on this element have spawned a massive N-based fertilizer industry worldwide (Dobermann, 2007; Westhoff, 2009). About 60 % of synthetic nitrogen fertilizers are presently used for cereals, with irrigated rice production accounting for approx. 10 % of the use. Since >50 % of the fertilizer applied is actually used by plants, the inefficient use of nitrogen contributes to nitrate contamination of soils and ground water, leading to health hazards and compromising agricultural sustainability. Moreover, manufacturing N fertilizer requires six times more energy than that needed to produce either P or K fertilizers (Da Silva *et al.*, 1978).

Only some prokaryotes are able to use atmospheric nitrogen through a process known as biological nitrogen fixation (BNF), which is the conversion of atmospheric N_2 to NH_3, a form that can be used by plants (Lam *et al.*, 1996; Franche *et al.*, 2009). The bacteria responsible for nitrogen fixation are called diazotrophs; they encode nitrogenase, the enzyme complex that catalyses the conversion of N_2 gas to ammonia. The nitrogenase complex is highly conserved in free-living and symbiotic diazotrophs. Various types of associations/interactions occur between diazotrophs and their host plants. The highly specific and most efficient processes for nitrogen fixation involve the formation of root nodules on legumes and non-legumes. The diazotrophic bacteria involved in these endosymbiotic interactions include rhizobia (Gram negative) members of the alpha-subgroup of the phylum proteobacteria that associate with legumes (family Fabaceae) (not included in this review; see Schultze and Kondorosi, 1998; Oldroyd and Downie, 2008; Desbrosses and Stougaard, 2011) and the non-legume *Parasponia* species (family Cannabaceae), and *Frankia* sp. (Gram positive) members of the actinomycete family that associate with a broad spectrum of plants belonging to eight families collectively called actinorhizal plants. In addition, nitrogen-fixing cyanobacteria (mainly *Nostoc* sp.) have also been found to colonize different plant organs, either intracellularly in the family Gunneraceae or extracellularly in liverworts, hornworts, *Azolla* and Cycadaceae. In contrast with these symbioses, some diazotrophs, such as *Azospirillum* spp., *Azoarcus* spp. and *Herbaspirillum*, form associative and/or endophytic relationships with a wide variety of plant roots including those of cereals. In all these associations and symbioses, for the host plants the expected benefit of the interaction is the fixed nitrogen provided by the symbiotic partner, which, in return, receives reduced carbon and possibly all the other nutrients it requires. In addition, the symbiotic or endophytic plant structure colonized by the nitrogen-fixing microorganisms may provide the appropriate conditions for protecting the nitrogenase complex from oxygen exposure.

Rhizosphere associations between nitrogen-fixing microorganisms and plants have been a major driving force in allowing organisms to spread across the biosphere, occupy new niches, and adapt to a variety of environmental stresses. This review presents an overview and recent advances in the understanding of the associations between a wide range of diazotrophs and non-legumes. Discoveries and breakthroughs in legume and non-legume nitrogen fixation provide new insight into ways of manipulating key steps in this process. Finally, new perspectives to engineer nitrogen-fixing ability in non-legume crops based on knowledge of endosymbiotic processes in non-legumes are discussed.

ACTINORHIZAL SYMBIOSES

Actinorhizal plants and their major ecological role

Actinorhizal plants have the ability to develop an endosymbiosis with the nitrogen-fixing soil actinomycete *Frankia*. The establishment of the symbiotic process results in the formation of root nodules in which *Frankia* provides fixed nitrogen to the host plant in exchange for reduced carbon. Actinorhizal plants represent a diverse group of about 220 species belonging to eight plant families distributed in the three orders, Fagales (Betulaceae, Casuarinaceae and Myricaceae), Rosales (Rosaceae, Eleagnaceae and Rhamnaceae) and Cucurbitales (Datiscaceae and Coriariaceae) (Wall, 2000; Pawlowski, 2009; Franche and Bogusz, 2011). All actinorhizal species belong to the Rosid I clade, thus sharing a common ancestor with legumes (Fabaceae), but differing from them in their wide distribution in numerous botanical families. It has been suggested that 100 million years ago (Mya), the common ancestor of Rosid I acquired a unique feature upon which a root nodule symbiosis (RNS) could evolve, and that this evolution occurred several times 50–60 Mya (Doyle, 2011). Three to four independent evolutionary origins have been postulated for actinorhizal symbiosis (Swensen, 1996).

Actinorhizal plants are woody shrubs and trees, except for the genus *Datisca*, which is herbaceous. They are distributed worldwide from cold regions (except Antartica) with, for example, *Alnus* (alder), to warm latitudes with, for example, *Casuarina* (beef wood). Many actinorhizal plants are also capable of forming mycorrhizal associations, and this tripartite symbiosis (host plant–*Frankia*–mycorrhiza) gives them a propensity to grow in marginal and poor soils (Dawson, 2008). Some species are very well adapted to flooded land, arid regions, contaminated soils, extreme pH and high salinity. Due to these properties, some actinorhizal trees are pioneer species that colonize disturbed areas; they play extremely important ecological roles and are intensively used in the revegetation of different landscapes or to prevent desertification. For example, in Africa, Casuarinaceae are planted to stabilize coastal and desert dunes, and for reclamation of salt-affected soils as well as in inter-cropping systems (Diem and Dommergues, 1990). In these arid soils, the species *Casuarina equisetifolia* fixes an average of 15 kg N ha^{-1} $year^{-1}$. But in temperate climates, nitrogen-fixation activity in actinorhizal plants could be similar to the rate of 300 kg ha^{-1} $year^{-1}$ measured in legumes (Wheeler and Miller, 1990). In addition, these perennial plants contribute to the N cycle through litter fall and soil decomposition.

Nitrogen-fixing actinobacteria Frankia

Frankia is a genus of soil actinomycetes in the family Frankiaceae that fix nitrogen, both under symbiotic and free-living aerobic conditions, while most rhizobia do not (Benson and Silvester, 1993). Phylogenetically, the filamentous gram-positive *Frankia* sp. and the unicellular gram-negative paraphyletic rhizobia are quite distant, suggesting that these two major groups of nitrogen-fixing symbionts have acquired mechanisms for nitrogen fixation from different evolutionary origins (Normand *et al.*, 1996). The first successful isolation of *Frankia* was reported relatively recently from *Comptonia peregrina* root nodules (Callaham *et al.*, 1978). At present, over 200 strains of *Frankia* have been isolated from many, although not all, actinorhizal plant species. Phylogenetic analyses revealed that *Frankiae* form a coherent clade within actinobacteria, and that strains generally fall into three major groups or clusters (Normand *et al.*, 1996; Benson and Clawson, 2000; Hahn, 2008). Cluster I consists of strains isolated from plants belonging to the order Fagales; they have the most specific host range and are only able to interact with plants belonging to this clade. Strains belonging to cluster III have a wider host range and can interact with plants belonging to five families within the two distant plant orders Rosales and Fagales. No strain belonging to cluster II has yet been cultured; actinobacteria are thus hypothetical obligate symbionts, even though no genome reduction has been observed in *Frankia* Dg1, the endosymbiotic strain of *Datisca glomerata* (Persson *et al.*, 2011). A fourth group of non-infective or non-effective *Frankia* strains forms a more deeply branching cluster (Kucho *et al.,* 2010). Each group has its own set of characteristics regarding symbiotic properties such as host-plant specificity, physiology and symbiotic relationships (Benson and Clawson, 2000).

Although *Frankia* strains are highly diverse in terms of ecological niches in the soil, current knowledge is focused on its life as an endophyte in root nodules. The difficulties sometimes encountered in growing *Frankia* in culture or in isolating *Frankia* from some plant species such as *Datisca*, reflect this limited knowledge. In pure culture, cultivable *Frankia* form vegetative hyphae that grow slowly, requiring >3–7 d before the colony appears on solid medium (Benson *et al.*, 2011). One striking feature of *Frankia* is its ability to differentiate into two unique structures besides hyphae: (1) spores, which occur in sporangia and contribute to natural dissemination of the actinobacteria (Huss-Danell *et al.*, 1997); and (2) vesicles, which are the sites of nitrogen fixation and differentiate at the tips of hyphae under nitrogen limitation. The vesicles are surrounded by a laminated envelope composed of hopanoid lipids that provide the necessary O_2 protection to prevent nitrogenase inactivation (Harriott *et al.*, 1991; Berry *et al.*, 1993; Dobritsa *et al.*, 2001).

Molecular biology of *Frankia* is limited by the absence of tools for genetic transformation and transposon mutagenesis (Kucho *et al.*, 2009). However, progress in genome sequencing, transcriptome and proteome analyses has provided some important insights into *Frankia*. Genomes of three *Frankia*

strains belonging to different host-compatibility groups were sequenced in 2007, revealing the absence of the canonical *nodABC* genes that code for the lipochito-oligosaccharidic Nod factors in rhizobia (Normand *et al.*, 2007). Only low similarity *nodB* and *nodC* homologues have been detected; these data are in agreement with the absence of functional complementation of rhizobia *nod* mutants with *Frankia* DNA (Cérémonie *et al.*, 1998). Moreover, genes known to be involved in symbiosis such as *nif* (nitrogenase), *hup* (hydogenase uptake) and *suf* (sulfur-iron cofactor synthesis) are scattered throughout the genomes. The absence of symbiotic islands in *Frankia* genomes, in contrast to rhizobia, complicates the identification of symbiotic genes. Nevertheless, with the sequencing of several other *Frankia* genomes in progress, valuable information is expected to become available from further genome comparisons. Preliminary analysis of the uncultured Dg1 symbiont of *Datisca glomerata* reveals that it contains *nodABC*-like genes, thus suggesting the use of Nod factors-like compounds during the infection process (Persson *et al.*, 2011).

Several transcriptome data analyses of free-living and symbiotic *Frankia* have been carried out. Alloisio *et al.* (2010) showed that nodule-induced genes of *Frankia alni* were mostly distributed over several regions with high synteny between three *Frankia* genomes and that, as expected, several genes linked with nitrogen fixation (*nif*, *suf*, *hup2*, *ispg*) were up-regulated. In addition, comparison with rhizobia transcriptome revealed that *Frankia* is metabolically more active in nodules, due to vesicle biosynthesis. Overall, *Frankia* is less dependent on the host plant than rhizobia for symbiotic requirements and, as a result, metabolic activity is higher in symbioses (Alloisio *et al.*, 2010). Interestingly, expression of genes necessary for ammonium assimilation was reduced in symbiosis, suggesting that symbiotic *Frankia* may not perceive N starvation and that *nif* genes are regulated by signals other than those involved in N metabolism (e.g. O_2 level) (Benson *et al.*, 2011). In addition, using high throughput RNA deep sequencing, Bickhart and Benson (2011) demonstrated significant heterogeneity in cell populations of *Frankia* sp. CcI3 growing in different conditions (NH_4^+ added vs. N_2 fixing; young and ageing bacterial culture). Interestingly, the high expression of transposase ORF (open reading frame) observed in 5-d-old and nitrogen-fixing cultures suggests that the stationary phase of growth and nitrogen starvation trigger mechanisms that favour modification of the genome.

Signals involved in plant–Frankia *recognition and in the plant signalling pathway*

It is assumed that, as observed in rhizobium–legume symbioses, the compatible interaction between *Frankia* and actinorhizal plants that leads to the development of nitrogen-fixing nodules is the result of a fine-tuned exchange of signals between the two partners (Franche and Bogusz, 2011).

On the plant side, although the involvement of flavonoids in symbiosis is poorly understood, several studies indicate that they may play a significant role in the early stage of the interaction. These studies also suggest a role for *Frankia* in chemo-attraction and proliferation (Smolander and Sarsa, 1990), and in the enhancement of nodulation following the addition of seed washes from red alder *Alnus rubra* (Benoit and Berry, 1997). These results were reinforced by Hughes *et al.* (1999), who observed that flavonols (quercetin and kaempferol) contained in *A. glutinosa* (black alder) root exudates enhanced the level of nodulation. In addition, root hair curling, which is the primary event in the symbiotic process, was enhanced by exposure of *Frankia* to *A. glutinosa* root filtrate (Prin and Rougier, 1987; Van Ghelue *et al.*, 1997). More recently, Popovici *et al.* (2010) reported that Myricaceae plants adapt their secondary metabolism in accordance with the compatibility status of *Frankia* bacterial strains, thus suggesting that flavonoids determine the specificity of the microsymbionts. The main plant compounds differentially affected by inoculation with *Frankia* are phenols, flavonoids and hydroxycinnamic acids. Interestingly, Beauchemin *et al.* (2012) demonstrated that *Casuarina* root extracts containing flavonoids altered the physiology, surface properties and plant infectivity of the compatible *Frankia* strain CcI3. In addition, several genes of the isoflavonoid biosynthesis pathway were shown to be up-regulated during early steps of interactions between *Casuarina glauca and Frankia* CcI3 (Auguy *et al.*, 2011). A study is underway to understand the role of flavonoids in the *Casuarina*/*Frankia* symbiosis in more detail by using an RNAi approach to down-regulate the level of chalcone synthase transcripts in *C. glauca* (Rhizogenesis group, Montpellier, France, unpubl. res.).

In legume–rhizobium symbiosis, specific signal molecules secreted by *Rhizobium*, called Nod factors, play a pivotal role in host-symbiont specificity and the induction of all early plant responses including symbiotic gene activation leading to mitotic reactivation of the cortical cells, and formation of pre-infection threads (Oldroyd *et al.*, 2010). As mentioned above, some specificity has been observed in the interaction between *Frankia* strains and their host plants. Studies aimed at purifying and characterizing symbiotic molecules from *Frankia* first relied on a bioassay based on root hair deformation with culture supernatants (Van Ghelue *et al.*, 1997; Bhuvaneswari and Solheim, 2000). Preliminary characterization of *Frankia* root hair deforming factor(s) indicated that their chemical properties differed from those of rhizobium Nod factors (Cérémonie *et al.*, 1999). However, since *N*-acetyl-glucosamine, the backbone of rhizobium Nod factors, has been detected in the *Frankia* root hair-deforming active fraction, it is possible that the two compounds are structurally related (Cérémonie *et al.*, 1999). Recently, a sensitive and reproducible bioassay based on early expression of symbiotic *C. glauca* genes was developed (Rhizogenesis group, Montpellier, France, unpubl. res.). This should help future work dealing with purification and complete characterization of *Frankia* signalling molecules involved in the early dialogue with the root system.

Sugars and phytohormones may also be involved in the molecular dialogue. Interestingly, a study of whole-cell sugar contents showed that a monosaccharide, 2-*O*-methyl-D-mannose, was present in all *Frankia* strains tested. This sugar may thus play a role in interactions and communications between *Frankia* and its hosts (Kucho *et al.*, 2010). From the analysis of *Frankia* supernatants, it was also found that auxins including indole-3-acetic acid (IAA) and analogues are produced by *Frankia* strains (Hammad *et al.*, 2003). Actinobacterial auxin

possibly plays a role in plant cell expansion, cell-wall remodelling, induction of adventitious roots, and in increasing the level of auxin in nodule primordia (Péret *et al.*, 2007; Perrine-Walker *et al.*, 2010).

Infection process and nodule development in actinorhizal plants

Two modes of root infection by *Frankia* have been described that depend on the host plant (Fig. 1) (Wall, 2000; Franche and Bogusz, 2011; Pawlowski and Demchenko, 2012). In the order Fagales, infection proceeds intracellularly via root hairs, whereas in Rosales, actinobacteria enter the root intercellularly. In Datiscaceae and Coriariaceae, the infection process is poorly known due to the difficulty involved in obtaining pure cultures of the symbionts and in studying the early stages of the infection process.

Intracellular infection begins with the deformation of root hairs induced by currently unknown *Frankia* signals. Actinorhizal hyphae are entwined by curled root hairs and only a few of them penetrate at the site of folding. Pronounced deposition of wall material is associated with *Frankia* invasion and hyphae become embedded in a structure analogous to the infection thread found in legume/rhizobia symbioses. Within this structure, *Frankia* filaments are encapsulated within a plant-derived cell-wall matrix consisting of xylans, cellulose and pectins (Berg, 1999). *Frankia* penetration triggers cell divisions in the root cortex subadjacent to the infected root hair, forming a mitotically active zone called the prenodule. The infection threads grow toward the prenodule, penetrate the thin wall of the recently expanded cortical cells, and infect prenodule cells that enlarge and ultimately fix nitrogen. Unlike in legumes, nodule primordia do not arise from these actively dividing cells of the prenodule. The nodule primordium arises from cell divisions induced in pericycle cells located opposite a protoxylem pole and close to a prenodule. In *C. glauca*, the prenodule cells displayed the same differentiation pattern as the one observed in the corresponding *Frankia*-infected cells of the nodule, suggesting that it may be a remaining form of a common nodule ancestor for legumes and actinorhizal plants (Laplaze *et al.*, 2000*a*). In the following stage of nodule ontogenesis, the nodule primordium grows and becomes infected by *Frankia* hyphae progressing from the infected cells of the prenodule. During the infection process, *Frankia* first grows as filamentous hyphae that proliferate in the newly infected host cell, and when the infected cell matures, the tips of the hyphae eventually differentiate vesicles that will fix nitrogen (Newcomb and Wood, 1987).

No root hair deformation is observed in the intercellular root invasion process. *Frankia* hyphae penetrate the middle lamella between adjacent cells of the root epidermis and progress apoplastically between cortical cells, within an electron-dense matrix secreted into the intercellular spaces (Wall and Berry, 2008), which might represent the equivalent of the encapsulation material described previously in infection threads. Cell divisions are induced in the root pericycle opposite a protoxylem pole, leading to the nodule primordium. *Frankia* hyphae infect primordium cells from the apoplast by intense branching of hyphae, concomitant with continuous invagination of the plant plasma membrane.

Mature actinorhizal nodules are multilobed structures, each lobe exhibiting a central vascular bundle surrounded by an endoderm, an expanded cortex and a periderm. Due to activity of the apical meristem, nodule lobes show indeterminate growth and developmental zonation with specific patterns of gene expression (Duhoux *et al.*, 1996; Obertello *et al.*, 2003; Franche and Bogusz, 2011; Pawlowski and Demchenko, 2012). Structural organization varies among actinorhizal root nodules, with some nodules such as those of *C. glauca* exhibiting a so-called nodular root at the apex of each lobe; this root is believed to facilitate the diffusion of gases in and out of the nodule lobe (Callaham and Torrey, 1977; Tjepkema, 1978; Schwintzer and Lancelle, 1983).

Molecular mechanisms underlying actinorhizal infection and nodulation

Whereas no tools are yet available in *Frankia* to perform functional analysis of candidate genes, genetic transformation procedures based on *Agrobacterium tumefaciens* and *A. rhizogenes* are available for some actinorhizal plants, providing a tool for promoter studies and down-regulation of plant genes by RNAi (Franche *et al.*, 1997; Gherbi *et al.*, 2008*a*, *b*; Svistoonoff *et al.*, 2010).

Functional and transcriptome analyses revealed that the common SYM pathway shared by rhizobium–legume and arbuscular mycorrhizal (AM) symbioses also controls nodulation by *Frankia* (Gherbi *et al.*, 2008*a*, *b*; Markmann *et al.*, 2008; Hocher *et al.*, 2011). This pathway includes a receptor-like kinase, nuclear pore proteins and potassium channels required for the induction of calcium oscillations. A putative calcium/calmodulin-dependent protein kinase (CCaMK) is also present and might thus recognize calcium ‘actinorhizal signatures’ (Singh and Parniske, 2012). Interestingly, transcriptome analysis also revealed the presence of genes linked to a ‘NOD’-specific pathway (not shared with AM symbiosis) used by legumes for the nodulation process with rhizobia. These interesting data suggest the possibility of a similar ‘NOD’ pathway between RNS. This overlapping of legume and actinorhizal RNS reinforces the hypothesis of a common genetic ancestor with a genetic predisposition for nodulation in the nitrogen-fixing clade Rosid I (Soltis *et al.*, 1995).

In addition to sequences involved in the *Frankia* signalling pathway, genes *Ag12* and *Cg12*, which encode subtilisin-like serine proteases in *A. glutinosa* and *C. glauca*, respectively, have also been studied (Ribeiro *et al.*, 1995; Laplaze *et al.*, 2000*b*). Subtilases are a superfamily of proteases thought to play a role in different aspects of plant development, including lateral root initiation and responses to pathogens. Using transgenic Casuarinaceae containing *Cg12* promoter–reporter gene fusions, it has been shown that the expression of *Cg12* starts very early during the symbiotic process and is specifically induced in cells infected by *Frankia* (Svistoonoff *et al.*, 2003). When *Cg12*-reporter gene fusions were introduced in the legume *M. truncatula*, a similar pattern of expression was observed during the nodulation process with *Mesorhizobium meliloti* (Svistoonoff *et al.*, 2004). The conservation of the expression profile in *M. truncatula* suggests that a signalling pathway independent of Nod factors, and conserved between the two systems, is activated specifically in cells infected by

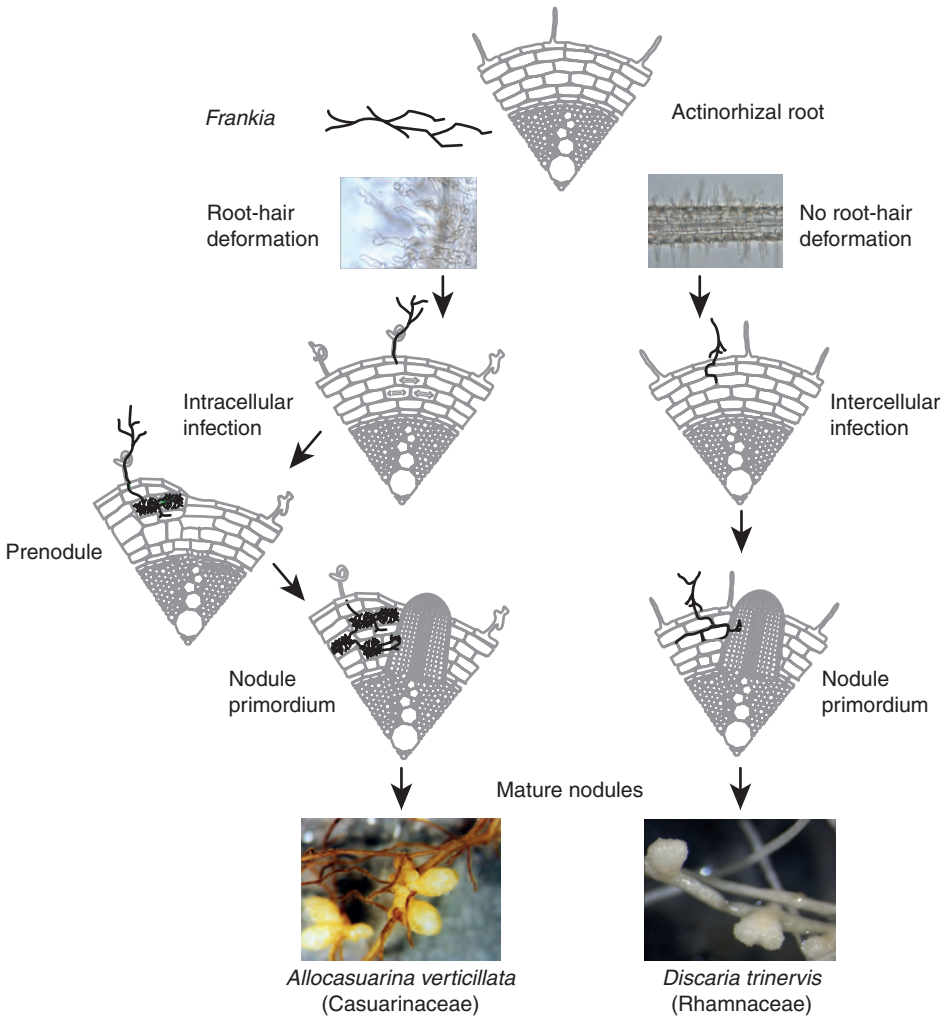

FIG. 1. Schematic representation of actinorhizal root infection by *Frankia*. *Frankia* penetrates via a root hair infection process in host plants from the families Betulaceae, Casuarinaceae and Myricaceae, and intercellularly in Eleagnaceae, Rosaceae and Rhamnaceae. Prenodule formation resulting from mitotic activity in the root cortical cells is observed only during the intracellular infection process. Nodule primordia arise from divisions in root pericycle cells, located opposite a protoxylem pole, and near the site of infection. *Frankia* hyphae progress either from cell to cell in the intracellular mode of infection, or apoplastically in a matrix secreted into the intercellular spaces. *Frankia* hyphae progress towards the nodule primordium where they will penetrate developing cortical cells intracellularly. Mature nodules consist of multiple lobes. Adapted from Franche and Bogusz (2011).

symbiotic bacteria. Intracellular infection mechanisms in legumes and actinorhizal plants were further investigated by introducing the *Enod11* promoter from *M. truncatula* into *C. glauca* (Svistoonoff *et al.*, 2010). In *M. truncatula*, *MtEnod11* gene expression was shown to be correlated with both preinfection and infection events throughout nodulation (Journet *et al.*, 2001). In *C. glauca*, activation of the *ProMtEnod11::gus* reporter was shown to be correlated with *Frankia* infection in root hairs, prenodules and nodules. These results suggest high conservation of regulatory pathways between legumes and actinorhizal plants in cells involved in bacterial infection and accommodation. However, *ProMtEnod11* is not activated in transgenic *Casuarina* prior to infection, during the perception of *Frankia* signal(s), indicating that the pre-infection stage differs between actinorhizal and legume–rhizobium symbioses (Svistoonoff *et al.*, 2010).

Since several plant hormones have been reported to regulate nodulation in legumes (Ding and Oldroyd, 2009), the *C. glauca* auxin influx carrier gene *CgAux1* was characterized to investigate the role of auxin in actinorhizal symbiosis. Using a transcriptional fusion between the promoter region and the β-glucuronidase (*gus/uidA*) reporter gene, *CgAux1* was shown to be expressed during nodule ontogenesis in all *Frankia*-infected cells, including root hairs (Péret *et al.*, 2007). Moreover, accumulation of auxins was reported in *Frankia*-infected cells in actinorhizal nodules of *C. glauca* and this accumulation was shown to be driven by cell-specific expression of auxin transporters and by *Frankia* auxin biosynthesis *in planta* (Perrine-Walker *et al.*, 2010). This localization raises the question of the role of auxin in infected cells. It was suggested that auxin might be involved in the cell wall remodelling processes that occur upon *Frankia* infection resulting from the growth of infection threads (Perrine-Walker *et al.*, 2010). Jasmonic acid belongs to another class of signalling molecules that may be involved in root endosymbioses with AM fungi and rhizobia (reviewed in Hause and

Schaarschmidt, 2009). However, recent results suggest that jasmonic acid does not play a role in nodulation of the model legume *Medicago truncatula* or in the two actinorhizal plant species, *C. glauca* and *D. glomerata* (Zdyb *et al.*, 2011).

Among infection mechanisms leading to RNS, the intracellular infection pathway is probably the most ancestral but also one of the least characterized. To decipher the molecular mechanisms underlying intercellular infection with *Frankia*, and to enable a comparative analysis between intra- and intercellular processes of infection, some tools for functional analysis of candidate genes were recently developed in *Discaria trinervis* (Rhamnaceae), a shrub endemic to Patagonia. Intercellular infection by *Frankia* was first analysed in detail in a time-course experiment in *D. trinervis* (Valverde and Wall, 1999), and an efficient genetic transformation protocol for *D. trinervis* based on *A. rhizogenes* was then set up (Imanishi *et al.*, 2011). First data indicate that *ProMtEnod11* drives expression in the infection zone of *D. trinervis* nodules, thus suggesting conservation of the corresponding regulators among all plants able to enter RNS regardless of the infection mechanism involved. As mentioned previously, *ProCg12* and *ProCgAux1* are two other promoters that drive expression in infected root hairs and prenodules prior to nodule formation in *C. glauca* (Péret *et al.*, 2007; Svistoonoff *et al.*, 2003, 2004). Analyses of these two promoters are currently underway in the intercellularly infected actinorhizal plant *D. trinervis*. The expression patterns that will be observed during the different stages of the root infection and nodule ontogenesis should help to explore signalling mechanisms in symbioses with this ancestral mode of infection, as well as to identify both conserved and divergent regulatory mechanisms (Rhizogenesis laboratory, France and University of Quilmes, Argentina, unpubl. res.).

Unlike legume nodules, actinorhizal nodules are modified lateral roots. Since auxin is a key signal in lateral root initiation, development, emergence and meristem activation (Overvoorde *et al.*, 2010), this raises the question of the role of auxin during actinorhizal nodule development. Interestingly, in studies on *Alnus* root nodules development, Angulo Carmona (1974) suggested that nodules do not form from pre-existing lateral root primordia. In addition, Wheeler *et al.* (1979) showed that the number of lateral root primordia initiated on *Alnus* roots following inoculation by *Frankia* was higher than on uninoculated control roots, indicating that the actinomycete can stimulate lateral root initiation. Further work is thus needed to characterize the role of auxin in symbiotic root development and to understand to what extent the lateral root developmental programme has been hijacked by *Frankia* to form an actinorhizal nodule. A first result was obtained by Sy *et al.* (2007) who showed that the cell-cycle promoter *cdc2aAt* from *Arabidopsis thaliana* has retained its ability to be induced by hormones in transgenic *Allocasuarina verticillata* roots and that, upon *Frankia* infection, this promoter was strongly induced in pericycle cells. These data suggests that, in response to *Frankia*, some pericycle cells in lateral roots can recover mitotic competence due to changes in their hormonal balance.

Recently, microarray expression analysis of transcripts in nodules versus uninfected roots showed that 1500 genes in *A. glutinosa* and 2000 in *C. glauca* are regulated or specifically induced in nodules (Hocher *et al.*, 2011). The majority of regulated genes are involved in transport (e.g. DCAT, dicarboxylate transporter for delivery of photosynthates to the symbiont), metabolism (e.g. GS, glutamine synthetase for the assimilation of the ammonium fixed and its transfer to the plant) and protein synthesis machinery, as expected during a switch from root-specific to nodule-specific gene expression. The analysis also revealed regulated genes involved in cell wall structure, defence (defensins, chitinases) and response to stress (catalase, DnaJ, γ-expansin natriuretic peptide), which is consistent with the results of previous studies (Ribeiro *et al.*, 2011).

O_2 regulation and haemoglobin in actinorhizal nodules

Several reviews summarize many aspects of oxygen metabolism in actinorhizal symbioses (Silvester *et al.*, 1990; Pawlowski, 2008). As mentioned above, in the free-living state, *Frankia* has its own oxygen protection mechanism: it forms specialized thick-walled cells (vesicles) that protect nitrogenase from oxygen (Torrey and Callaham, 1982). Actinorhizal plants have developed different strategies to reduce oxygen levels in nodules, thus reflecting the taxonomic diversity of the hosts and the different structural organization of the actinorhizal nodules. In *Casuarina*, where *Frankia* strains do not form vesicles within nodules, cell-wall lignification observed after *Frankia* infection is believed to provide a barrier against oxygen diffusion (Berg and McDowell, 1988) together with a large amount of symbiotic haemoglobin (class 2) (Fleming *et al.*, 1987; Jacobsen-Lyon *et al.*, 1995; Gherbi *et al.*, 1997). In contrast, in *Alnus* and *Myrica* nodules where *Frankia* do not form vesicles, class 2 symbiotic haemoglobin was not found. However, nonsymbiotic class 1 haemoglobin was detected in *A. glutinosa* (Suharjo and Tjekema, 1995) and *Myrica gale* nodules (Pathirana and Tjepkema, 1995). The corresponding genes were cloned in *A. firma* (Sasakura *et al.*, 2006) and *M. gale* (Heckmann *et al.*, 2006). *Alnus* haemoglobin 1 was strongly induced in actinorhizal nodules by nitric oxide (NO) and cold stress, but not by hypoxia or osmotic stress (Sasakura *et al.*, 2006). As reported by Hill (2012), class 1 nonsymbiotic haemoglobin is involved in reactive oxygen and NO metabolism. A truncated haemoglobin, classified in class 3 (Watts *et al.*, 2001), was also been identified in the actinorhizal plant *D. glomerata* (Pawlowski *et al.*, 2007). This truncated haemoglobin is induced upon plant infection by *Frankia*, leading the authors to suggest a role in NO detoxification. Although reactive oxygen (Tavares *et al.*, 2007) and possibly NO production occur during interaction with *Frankia,* the role of class 1 and 3 haemoglobins in actinorhizal symbioses has not yet been fully elucidated.

NON-LEGUME ROOT ENDOSYMBIOSIS *PARASPONIA-RHIZOBIUM*

General features of the symbiosis

In the order Rosales, in addition to actinorhizal nitrogen-fixing plants, *Parasponia* species (family Cannabaceae,) also display an original nitrogen-fixing root symbiosis (Sytsma *et al.*, 2002). *Parasponia* is the only non-legume host plant known to be nodulated by rhizobia (Trinick, 1973; Akkermans

et al., 1978). The host plants are medium-sized tropical trees (up to 15 m in height), and pioneer species growing on nitrogen-poor and disturbed soils. These trees originated from the Malay Archipelago. Among the five nodulated species identified (Becking, 1992), *P. andersonii*/*Rhizobium* is the most widely studied symbiotic association.

Although different *Rhizobium* species, including some strains isolated from legume nodules, are capable of nodulating *Parasponia* species (Trinick and Galbraith, 1980; Trinick and Hadobas, 1988), they do not represent a specific lineage, thus suggesting the recent emergence of the ability of the host plants to be nodulated by rhizobia (Lafay *et al.*, 2006). More recently, *P. andersonii* was tested for the symbiotic effectiveness of a wide range of *Rhizobium* species and was found to be nodulated by bacteria from four different genera harbouring highly diverse Nod factor biosynthesis genes (Op den Camp *et al.*, 2012). Although *Parasponia* allows such symbiotic promiscuity of rhizobia endosymbionts, the efficiency of the symbiotic nitrogen fixation varies. Microscopy studies of nodules obtained with under-performing rhizobia indicate that the control of symbiotic association is less sophisticated than with legumes (Trinick and Hadobas, 1988; Op den Camp *et al.*, 2012).

Rhizobium *and plant host signalling*

Like in most legume–rhizobium symbioses, the *Parasponia*–rhizobium symbiosis depends on Nod factors. Rhizobial Nod factors are lipochitooligosaccharides that consist of an acylated chitin oligomeric backbone with different functional group substitutions at the terminal or non-terminal residues. These Nod factors are key symbiotic signals and are indispensable in the specific-host rhizobium interaction and at later stages in the infection process and nodule organogenesis (Oldroyd and Downie, 2008). Recently, it was shown that a single gene closely related to lysin-motif (LysM) domain proteins involved in Nod factor perception in legumes, is required for both nodulation and mycorrhization in *P. andersonii* (Op den Camp *et al.*, 2011). It was also shown that the common 'SYM' pathway described for AM, legume–rhizobium and actinorhizal symbioses is activated during *P. andersonii* nodule organogenesis. Together, these data reinforce the hypothesis of a common genetic ancestor of the nodulating clade with a genetic predisposition for nodulation (Soltis *et al.*, 1995). While considerable information is available on the role of particular flavonoids in the rhizobium–legume symbiosis, their role during the different stages of *Parasponia* nodulation is not known. Because it is a Nod-dependent symbiotic interaction, it is likely that the role of flavonoids produced by *Parasponia* is similar to that played in legume nodulation.

Infection process and nodule structure

The infection process that leads to nodule development occurs via the so-called crack entry mode of infection (Sprent and Faria, 1988). Rhizobia enter the root between epidermal cells, this intercellular infection being concomitant with the formation of infection threads of plant origin and with the stimulation of cortical cell division leading to a subsurface swelling zone comparable with the prenodule in actinorhizal plants (Lancelle and Torrey, 1984*a*, *b*; Bender *et al.*, 1987). As the infection threads continue to grow, a few pericycle cells in the immediate vicinity of the prenodule divide giving rise to a nodule-lobe primordium. Progressively, the threads intracellularly invade cells derived from the apical region of the nodule lobe primordium leading to a nodule containing an apical meristem and a central vascular cylinder, which is surrounded by a zone of infected tissue. As in actinorhizal plants, nodule primordium formation does not involve prenodule cells, and the function of prenodules is still not known. The rhizobia remain in threads throughout the symbiotic process and are not released from the threads unlike during bacteroid formation in rhizobium–legume symbioses (Trinick, 1979; Lancelle and Torrey, 1984*b*). In contrast to legumes, where nodule primordia are initiated in the cortex and have a stem-like anatomy with a peripheral vascular bundle, ontogenesis and the final structure of the *Parasponia* nodule lobe is similar to that observed in actinorhizal symbiosis and resembles lateral roots.

Control of oxygen levels in nodules

Symbiotic haemoglobin (Hb) proteins and genes have been isolated from *Parasponia* and characterized (Appleby *et al.*, 1983; Landsmann *et al.*, 1986). *Parasponia andersonii* was shown to possess a single *Hb* gene expressed in both nodules and in non-nodulated roots, suggesting symbiotic and non-symbiotic roles result from a single gene (Landsmann *et al*, 1986, 1988; Bogusz *et al.*, 1988). Its oxygen-binding properties and cellular location in young rhizobia-infected cells are consistent with a role in oxygen transport to rhizobia within root nodules (Wittenberg *et al.*, 1986; Gibson *et al.*, 1989; Trinick *et al.*, 1989). Furthermore, comparative analysis of promoter activity in transgenic *Casuarina* indicated that, in contrast to the lack of conservation of cell-specific expression of *P. andersonii* haemoglobin promoter in transgenic legume nodules (Bogusz *et al.*, 1990), it retains its specific expression in bacteria-infected cells of actinorhizal nodules (Franche *et al.*, 1998). As mentioned above, it is interesting that *P. andersonii* and *C. glauca* nodules have the same origin and structure as lateral roots. This suggests that in Rosales, beyond similar nodule structure and ontogeny, there is a non-legume key symbiotic gene that has identical regulatory mechanisms (Franche *et al.*, 1998).

Along with legume–rhizobium and actinorhizal symbioses, *Parasponia* is a key species for studies of the accommodation of symbiotic bacteria in plant cells. A comparative analysis of these three symbiotic systems should help define strategies for transferring nitrogen-fixing ability to non-legume crops.

CYANOBACTERIAL–PLANT ENDOPHYTIC AND ENDOSYMBIOTIC ASSOCIATIONS

A wide range of cyanobacterial associations

Cyanobacteria are a diverse group of oxygenic photosynthetic prokaryotes that occur in marine, aquatic and terrestrial environments all over the world (Rippka *et al.*, 1979). Some cyanobacteria have the ability to live in association with a wide range of plants from the divisions Bryophyta (liverworts and

hornworts), Pteridophyta (the genus *Azolla*), gymnosperms (family *Cycadaceae*) and angiosperms (family *Gunneraceae*) (Table 1) (for reviews see Meeks, 1998; Adams, 2000; Rai *et al.*, 2000, 2002; Adams *et al.*, 2006; Bergman *et al.*, 2007). A striking difference between cyanobacteria–plant associations, and the non-legumes actinorhiza–*Frankia* and *Parasponia*–*Rhizobium* symbioses where the bacteria are hosted in a root nodule, is that the plant structure colonized by the symbiotic cyanobacteria develops independently of cyanobacterial infection.

Bryophytes are small, non-vascular land plants including liverworts (Hepaticae), horworts (Anthocerotae) and mosses (Musci), a relatively small number of which are able to form epiphytic or endophytic associations with cyanobacteria (Adams, 2002; Meeks, 2003; Adams and Duggan, 2008). Epiphytic associations with mosses are not discussed in this review. Two liverwort species, *Blasia pusilla* and *Cavicularia densa* (Blasiales, Marchantiophyta), and all hornworts (Anthocerophyta) are able to form an endosymbiotic association with cyanobacteria that generally belong to the genus *Nostoc* (Rodgers and Stewart, 1977; Adams and Duggan, 2008). Endosymbiont filaments are hosted in specialized auricles on the ventral surface in Blasiales and in slime cavities within the thallus in Anthicerotophyta such as *Anthoceros* and *Phaeoceros*. During the development of the thallus, new auricles are continuously formed and infected by cyanobacteria.

Cyanobacterial associations with pteridophytes are limited to the genus *Azolla* in the family Azollaceae. *Azolla* is a small floating aquatic fern with a worldwide distribution ranging from tropical to warm temperate regions. It has been exploited for many years as a source of nitrogen for agriculture and is extensively used as a green manure and biofertilizer for rice (Watanabe and Roger, 1984; Ladha *et al.*, 2000; Van Hove and Lejeune, 2002). The nitrogen-fixing cyanobacteria are hosted in a highly specialized cavity located on the dorsal lobe of the leaves (Peters and Mayne, 1974; Zheng *et al.*, 2009). An envelope lines the cavity where cyanobacterial filaments are localized in the periphery within a mucilaginous matrix surrounding a gaseous central region. Morphological analysis of the leaf cavity established that a pore remains open during leaf development, even when the leaf is mature, thus permitting gas exchanges (Veys *et al.*, 1999). Throughout its life cycle, the symbiont remains associated with its host, and is automatically transmitted from generation to generation, including during sexual reproduction (Calvert *et al.*, 1985; Peters and Meeks, 1989). So far, there are no confirmed reports of successful *in vitro* cultivation of the cyanobiont that belongs to the order Nostocales, making *Azolla* symbiosis the only known permanent symbiosis among cyanobacteria–plant associations (Lechno-Yossef and Nierwicki-Bauer, 2002; Pabby *et al.*, 2003). Besides the cyanobiont, it has been shown that minor cyanobacterial and bacterial species coexist in the cavity (Gebhart and Nierzwicki-Bauer, 1991).

Cycads are the only known gymnosperms that have the ability to develop a nitrogen-fixing symbiosis through an intimate association with cyanobacteria. Cycads include approx. 156 species in nine genera that grow naturally in tropical and subtropical regions, and all of them possess a

TABLE 1. *Main features of plant cyanobacterial symbiotic associations*

Plant taxon	Symbiotic host species	Symbiotic	Cyanobiont structure	Proposed time for plant origin
Angiosperm	All known species of *Gunnera*	Stem gland	Intracellular *Nostoc*	80 Mya
Gymnosperm	All known cycads (150 species in 10 genera belonging to 3 families)	Root zone	Intercellular *Nostoc* or *Calothrix*	200–150 Mya
Pteridophyte	All species of the genus *Azolla*	Cavities in each dorsal leaf	Intercellular Nostocales obligatory symbiont	420 Mya for the ferns, 120 Mya for *Azolla* fossils
Bryophyte	Only two of the 330 genera of liverwort; four of the six genera of hornwort	Cavities in the gametophyte	Intercellular *Nostoc*	400–500 Mya

Adapted from Bergman *et al.* (1992*b*) and Bergman *et al.* (2007).

symbiotic cyanobacterium. Cyanobacteria are hosted in specialized coralloid roots that arise from the lateral roots and are formed by the plant before being invaded by the cyanobacteria (Costa and Lindblad, 2002). Filamentous cyanobacteria are located intercellularly in a zone filled with mucilage and comprise a large number of elongated cycad cells that interconnect two adjacent cortical layers in the coralloid roots. These cells may contribute to the transfer of metabolites between the symbionts and the host. In transverse root sections, cyanobacteria are visible as a green ring. *Nostoc* spp. are the most common cyanobionts in Cycadaceae, although *Calothrix* has occasionally been reported (Costa *et al.*, 1999; Thajuddin *et al.*, 2010).

Plants in the genus *Gunnera* are mainly distributed in the southern hemisphere with about 40 species, including small, stoloniferous species such as *Gunnera magellanica* and plants that can reach 3 m in height (e.g. *Gunnera manicata*). Natural populations are restricted to humid areas with heavy rainfall (Bergman *et al.*, 1992*a*; Bergman, 2002). The cyanobiont identified as *Nostoc* enters *Gunnera* plants through specialized glands located on the stem and observed in conditions of nitrogen starvation, even in absence of cyanobacteria (Silvester and McNamara, 1976; Towata, 1985; Bonnett, 1990; Chiu *et al.*, 2005). The glands secrete polysaccharide-rich mucilage that attracts specific symbiotic cyanobacteria (Nilsson *et al.*, 2006), supports their growth on the gland surface, and contributes to their differentiation (Chiu *et al.*, 2005). After entering the gland through existing channels, cyanobacteria induce divisions in the host cells lining the channel and are subsequently taken up into the host cells (Bergman *et al.*, 1992*a*). Cyanobacteria become restricted to certain *Gunnera* cells intermixed with non-infected cells. Once inside the *Gunnera* cells, *Nostoc* filaments grow and divide and fill most of the host cell. As observed with *Frankia* in nodular structures, filaments are always surrounded by the host plasma membrane. After colonization, new glands continue to form on the stem at the base of each leaf and infection continues at the apex of the growing stem. In large *Gunnera* species, the symbiotic tissue is visible as blue-green patches along the rhizomatous stems or along stolons in smaller Gunnera species (Bergman, 2002).

The intimacy of the symbiosis *Gunnera*–*Nostoc* is therefore greater than in other cyanobacterial associations since the cyanobacteria are hosted intracellularly, whereas in bryophytes, *Azolla* and cycads, the cyanobionts occur as endophytes in mucus-filled cavities. With the exception of *Azolla*, the associations are facultative, the two partners can be isolated and cultivated independently, and the symbiosis can be easily reconstituted.

Diversity of cyanobacteria associated with plants

Cyanobionts are filamentous cyanobacteria and generally belong to the genus *Nostoc*, although a few other cyanobacteria such as *Calothrix* and *Chlorogloeopsis* have been reported. Cyanobacteria of the genus *Nostoc* belong to the order Nostocales and to Section IV of cyanobacteria (Rippka *et al.*, 2001). They are all characterized by their ability to differentiate some nitrogen-fixing cells called heterocysts, some resting spores called akinetes, and some motile filaments called hormogonia, which constitute the infective units during the establishment of the symbiotic process and contribute to short distance dispersal in free-living conditions (Campbell and Meeks, 1989; Vagnoli *et al.*, 1992; Adams and Duggan, 2008).

Molecular techniques have shown that, with the exception of the cyanobacteria living in the cavities of the water fern *Azolla*, there is high strain diversity both within and among the different plant hosts (Costa *et al.*, 1999, 2001; Nilsson *et al.*, 2000; Guevara *et al.*, 2002; Rasmussen and Nilson, 2002; Zheng *et al.*, 2002; Papaefthimiou *et al.*, 2008; Rikkinen and Virtanen, 2008). For instance, by studying the 16S rRNA sequence of cyanobionts, a single coralloid root of *Cycas revoluta* was found to harbour up to three cyanobacterial strains and some diversity was also observed in multiple roots from a single plant (Gheringer *et al.*, 2010; Yamada *et al.*, 2012).

The limited genetic variation in cyanobacterial symbionts from *Azolla* was first revealed using RFLP analysis with *nif* gene probes and PCR fingerprinting (Franche and Cohen-Bazire, 1985, 1987; Plazinski *et al.*, 1988; Zheng *et al.*, 1999) and recently confirmed by a phylogenetic tree based on 16S rRNA gene sequences of 35 symbionts extracted directly from leaf cavities of *Azolla* species collected in different geographical regions. *Nostoc azollae* form a homogenous cluster separated from free-living cyanobacterial genera (Papaefthimiou *et al.*, 2008). These data suggest that one cyanobacterial taxon originally infected an ancestor of the *Azolla* species and that the symbiosis ultimately became obligatory for the cyanobacterial partner. Such features suggest long-lasting co-evolution between the partners, potentially extending back as far as 140 million years, corresponding to the oldest fossil records of *Azolla* (Raven, 2002).

Except for *N. azollae*, plant–cyanobacteria associations can be reconstituted under laboratory conditions in bryophytes, *Gunnera* and, to a lesser extent, in cycads, offering a way to perform studies on the two partners alone and in symbiotic association. It is interesting to note that the cyanobacterial strains that form intracellular symbioses with *Gunnera* remain extracellular when they infect the hornwort *Anthoceros*, and extracellular symbionts that infect *Anthoceros* are able to become intracellular symbionts with *Gunnera*. The possibility for the same *Nostoc* strain such as *N. punctiforme* ATCC 29133 (also referred as PCC 73102) to infect a wide range of hosts, points to a lower degree of specificity in the cyanobacteria–plant associations than that observed in the highly specific *Rhizobium*–legume symbioses or even in actinorhizal symbioses. *N. puctiforme* was first isolated from the roots of the cycad *Macrozamia* sp. in Australia, and has the ability to form a symbiotic association with the angiosperm *Gunnera* (Johansson and Bergman, 1994) and the bryophyte *A. punctatus* (Enderlin and Meeks, 1983).

In addition to taxonomic studies on symbiotic cyanobacteria, data are accumulating on cyanobacterial genomes, with >40 genome sequences of cyanobacterial strains available and many genomes still to come (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) (Hess, 2011). Genome sizes vary from 1·44 Mb to 9·05 Mb, with reported genes ranging from 1241 to 8462. Most genomes are circular and a small number of plasmids can be observed. The facultative symbiont *N. punctiforme* ATCC 29133 has one of the largest genomes:

9·05 Mb and 8462 reported genes (Meeks *et al.*, 2001). For comparison, the free-living non-symbiotic reference strain *Anabaena* PCC 7120 genome is 7.13 Mb and contains 5610 ORFs. The sequence of *N. punctiforme* indicates a genome that is highly plastic and in a state of flux, with numerous insertion sequences and multilocus repeats, as well as encoding transposases and DNA modification enzymes (Meeks *et al.*, 2001; Meeks, 2005*a*, 2009). In contrast to *N. punctiforme*, one obligatory symbiont of *Azolla*, *N. azollae 0708*, has a small genome of 5486 Mb with 5413 genes, among which 1689 are pseudogenes (Ran *et al.*, 2010; Larsson *et al.*, 2011). The number of intact coding sequences is among the lowest in filamentous cyanobacteria sequenced to date.

The role of hormogonia in the infection process

To establish a successful interaction, host plants must attract and internalize the cyanobacteria and then regulate their growth and differentiation. The cyanobacteria must avoid eliciting the plant defence response, and must adapt their metabolism to a new environment. Like in the previously described symbioses with actinorhizal plants and *Parasponia*, these events require sophisticated communication between the plant and the cyanobacteria (Fig. 2) (Gorelova, 2006; Adams and Duggan, 2011).

The conversion of vegetative filaments into motile and short-lived hormogonia is an essential step for the establishment of the symbiotic process and in laboratory conditions, and a cyanobacterial culture rich in hormogonia can increase the efficiency of plant infection (Uheda and Silvester, 2001). Hormogonia are short gliding filaments that lack heterocysts, with cells that are smaller than the cells in vegetative filaments (Duggan *et al.*, 2007). The reduced cell size results from cell divisions that are not accompanied by an increase in cell biomass and a significant synthesis of DNA. Different environmental stimuli and/or plant factors released during nitrogen starvation can stimulate the induction of hormogonia (Meeks and Elhai, 2002; Adams *et al.*, 2006). Hormogonia are in a transient, non-growth state, and they maintain their gliding activity for 48–72 h before reverting back to vegetative growth. During the interaction with a host plant, hormogonia revert to filaments with nitrogen-fixing heterocysts after entering the host.

Under low nitrogen conditions, certain plants exude one or several hormogonia-inducing factors (HIF), thereby dramatically increasing the frequency at which nearby *Nostoc* spp. filaments convert to hormogonia (Campbell and Meeks, 1989). As reviewed by Adams and Duggan (2008), HIF have been found in the hornwort *A. punctatus* (Meeks, 2003), in *Blasia*, cycads and the angiosperm *Gunnera* (Rasmussen *et al.*, 1994; Bergman *et al.*, 1996; Knight and Adams, 1996; Cohen and Meeks, 1997; Campbell *et al.*, 1998; Ow *et al.*, 1999). In *Gunnera*, the viscous mucilage secreted by the stem contains an HIF and extracts from other tissues or seeds have no effect on the cyanobacteria. In both *Gunnera* and *A. punctatus,* the putative signal has been identified as a heat labile compound, possibly a protein of <12 kDa (Campbell and Meeks, 1989; Rasmussen *et al.*, 1994; Meeks, 2003). Cycad root extracts also promoted hormogonium formation in competent *Nostoc*. Surprisingly, HIF is not restricted to symbiotic plant extracts since hormogonium formation has also been detected in artificial associations between cyanobacteria and non-host plants such as wheat (Gantar *et al.*, 1991, 1993; Gusev *et al.*, 2002). The biochemical composition of the HIF(s) has not yet been determined.

Although our knowledge on the molecular events involved in the differentiation of hormogonia is still limited, several genes affecting this process have been identified. In contrast to *Frankia* where no genetic tools are available to create mutations, procedures for genetic analysis and transposon mutagenesis have been developed in the large host range *Nostoc* ATCC 29133, thus providing a valuable tool for investigating the function of putative symbiotic genes (Cohen *et al.*, 1994). A mutation in *NtcA,* which encodes a transcription factor essential for nitrogen metabolism, resulted in a reduction in the frequency of hormogonia induced by the HIF and the resulting hormogonia did not infect *Anthoceros* (Wong and Meeks, 2002). Mutations in *sigH* and *trpN* which, respectively, encode an alternative group 2 sigma factor and a tetratricopeptide repeat protein, were also associated with increased symbiotic competence (Campbell *et al.*, 1998). In 2008, Chapman *et al.* isolated two different mutants of *N. punctiforme* resulting from the insertion of a transposon in the *cyaC* gene encoding an adenylate cyclase which catalyses the formation of the intracellular messenger cyclic AMP. Following cocultivation experiments with the symbiotic partner *B. pusilla*, both mutant strains were found to form hormogonia about 12 h earlier than in the wild-type strain, and displayed reduced symbiotic competence. Recently knowledge on hormogonia differentiation has progressed thanks to transcriptome analyses by Campbell *et al.* (2007, 2008). Their data revealed that, although hormogonia do not grow, they are characterized by a high dynamic transcriptional state, with 1827 genes differentially transcribed in *N. punctiforme* 24 h after their induction. A plant extract of *A. punctatus* containing the still unknown HIF was found to down-regulate 345 genes and up-regulate 689 genes in 30 min (Campbell *et al.*, 2008).

Hormogonium production is not sufficient for the establishment of the symbiosis, since some non-infective *Nostoc* strains are also capable of forming motile hormogonia in the presence of the angiosperm *Gunnera* spp. (Johansson and Bergman, 1994; Rasmussen *et al.*, 1994). Rapid migration of hormogonia inside the symbiotic structure is a critical factor for the successful establishment of nitrogen-fixing associations (Nilsson *et al.*, 2005). This step in the process involves a combination of motility and chemoattraction. The cell surface of hormogonia of symbiotically competent *Nostoc* is covered with pili that confer a form of surface motility called gliding. In contrast, immotile vegetative trichomes of mature *Nostoc* filaments are devoid of these specialized filaments (Duggan *et al.*, 2007). Following the analysis of insertion mutants in *N. punctiforme* ATCC 29133, the inactivation of two ORF coding for the *pilT* and *pilD* coding for pilus-like structures was seen to alter the level of surface piliation and to reduce the symbiotic competency of *N. punctiforme* in *Blasia* (Duggan *et al.*, 2007).

Chemoattraction of cyanobacteria was first shown with the host plant *Blasia* (Knight and Adams, 1996). Although hormogonium is a prerequisite for symbiosis, not all hormogonia-forming cyanobacteria are capable of infecting plants

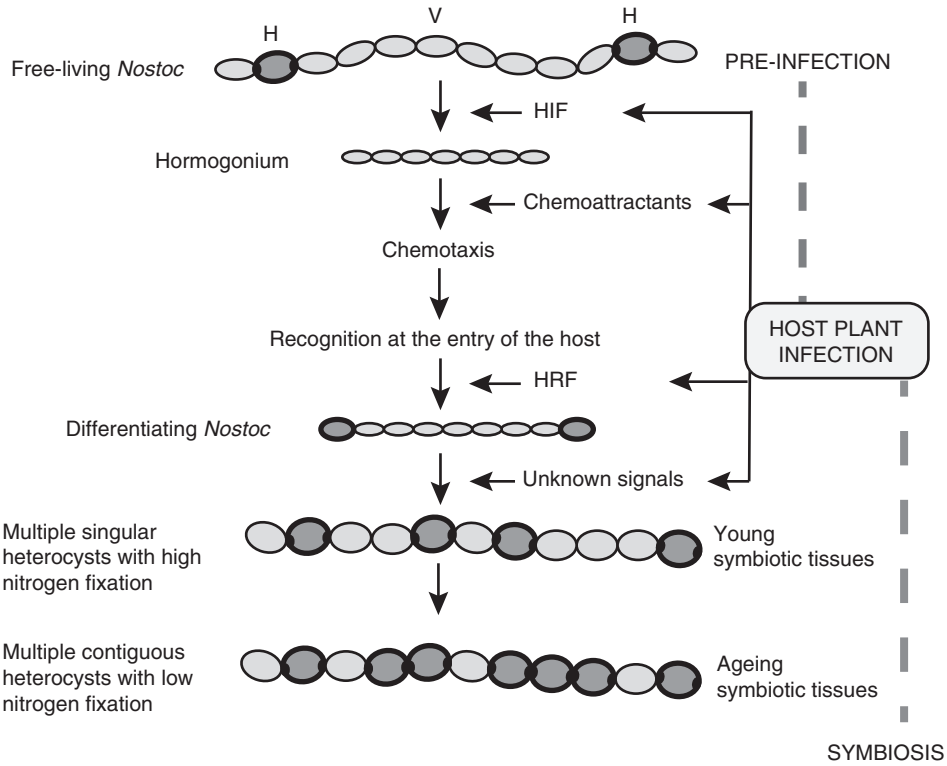

FIG. 2. Schematic representation of the infection process in cyanobacteria–plant symbioses. In nitrogen-free medium, *Nostoc* sp. filaments consist of vegetative cells (V) and regularly spaced heterocysts (H) that fix nitrogen. A hormogonium inducing factor (HIF) produced by the host under nitrogen starvation conditions leads to differentiation of motile small-celled hormogonial structures. Following the exchange of appropriate recognition signals, hormogonia penetrate the host symbiotic cavities and revert to vegetative filaments with a large number of heterocysts. The repression of hormogonia is linked to a hormogonia repressing factor (HRF). In ageing symbiotic tissues, multiple contiguous heterocysts are observed that exhibit low nitrogen-fixation activity. Adapted from Rai *et al.* (2000) and Meeks (2005*b*).

(Rasmussen *et al.*, 1994) and chemoattraction is an important factor in the initiation of the association. Whereas factors stimulating the formation of hormogonia are not highly specific, the induction of chemotaxis could be a more specific event. In *Blasia*, a chemoattractant compound was characterized as a low molecular-weight (<1 kDa) compound, stable at temperatures up to 95 °C, and almost completely inactivated by acetic acid (Watts *et al.*, 1999). Some sugars such as arabinose, glucose and galactose can also attract hormogonia (Nilsson *et al.*, 2006). Although chemoattractants are responsible for some specificity in the interaction, they can also be secreted by non-host plants. Chemotactic attraction has been detected in wheat (Gantar *et al.*, 1993; Gusev *et al.*, 2002; Nilsson *et al.*, 2002), in *Trifolium rupens*, and to a lesser extent in *Arabidopsis thaliana* and *Oryza sativa* (Nilsson *et al.*, 2006).

Cyanobacteria in symbiotic status

Symbiosis causes modifications in both partners. For the host, symbiosis leads to an increase in size of the symbiotic structures and organs inhabited by the cyanobiont caused by local plant cell proliferation, the accumulation of mucus, and the formation of specialized plant cells penetrating cyanobacterial colonies that contribute to the exchange of metabolites. For the cyanobiont, after entering the host, hormogonia revert to non-motile vegetative filaments, the differentiation of nitrogen-fixing heterocysts is observed at an unusually high frequency, and vegetative cells show altered morphology, i.e. are enlarged and irregular in shape comparedwith their free-living counterparts (Meeks and Elhai, 2002).

The repression of hormogonia is linked to the release by the host of an unidentified hormogonia-repressing factor. This factor induces genes such as *hrmA* belonging to the hormogonium-regulating locus *hrmRIUA*, which plays a central role in the repression of hormogonia formation and exhibits high similarity with sugar uronate metabolism operons of bacteria (Meeks, 2003). *hrmA* is induced by an aqueous extract of *Anthoceros* tissue, leading to the suggestion that a factor in the extract prevents hormogonium formation (Cohen and Meeks, 1997). In addition to the flavonoids, naringenine and, to a lesser degree, neohesperidine and prunine, can induce the expression of *hrmA* (Cohen and Yamasaki, 2000). *hrmA* was also induced by aqueous extracts of fronds of *Azolla pinnata* and *A. filiculoides*, and this induction was correlated with the amount of deoxyanthocyanin contained in the extract, even though pure deoxyanthocyanin had only a weak effect on the induction process (Cohen *et al.*, 2002). Recently, quantitative analyses of soluble sugars in the mucilage of *Gunnera* revealed that low levels of soluble sugars help attract the cyanobiont *N. punctiforme*, but are not involved in the formation of hormogonia. Conversely, high levels of soluble sugars in plant cells help prevent further

development of hormogonia once *Nostoc* is inside the plant cells (Khamar *et al.*, 2010).

The development of heterocysts after plant infection is essential for a functional nitrogen-fixing symbiosis. Whereas in a free-living *Nostoc* strain, the average heterocyst frequency in a nitrogen-free medium is 5–10 %, heterocysts appear at an average frequency of 35 % in plant associations, and a gradient in the number of heterocysts is observed (Meeks and Elhai, 2002). In the glands of *Gunnera*, 5–10 % of heterocysts were observed in the young glands and up to 75 % in older glands (Bergman *et al.*, 1992*a*; Zhang *et al.*, 2006). At the molecular level, differentiation from a vegetative cell to a heterocyst is a complex process that is regulated by several important genes (Buikema and Haselkorn, 1991; Adams and Duggan, 1999; Haselkorn, 2007). The expression of four cyanobacterial genes connected to signalling (*ntcA* and *glnB* (P_{II})), heterocyst differentiation (*hetR*) and dinitrogen fixation (*nifH*) was monitored in cyanobacteria from two *Gunnera* species at eight different stages of development from newly infected tissues at the apex to more mature tissues (Wang *et al.*, 2004). The *hetR* gene was highly expressed and correlated positively with an increase in heterocyst frequency and with *ntcA* expression; *nifH* expression was already high in the apical part of Gunnera and *glnB* expression decreased from the apex along the stem. Analysis of *Nostoc* mutants revealed that the mutant in *ntcA* that encodes a global nitrogen regulator (Marcozzi *et al.*, 2009) has lost the ability to infect the hornwort *Anthoceros* (Wong and Meeks, 2002).

When the proteome of *Nostoc* sp. freshly isolated from stem glands of *G. manicata* was compared with the proteome obtained in the same strain grown in a free-living state, several proteins were shown to be affected in the symbiotic process, with 23 proteins being up-regulated and ten down-regulated (Ekman *et al.*, 2006, 2008). When the cyanobacteria enter the host, the normally photoautotrophic genus *Nostoc* is exposed to specific conditions and has to adapt to intracellular conditions, darkness and a microoxic environment. Despite all these physiological changes, data obtained by Ekman *et al.* (2006) revealed that most of the proteins present in symbiosis are also present in the free-living state, indicating that most cellular functions remain unmodified in the host. However, some adaptations to the symbiotic state were observed. These included up-regulation of the genes involved in nitrogen fixation such as *nifH* coding for dinitrogenase reductase, whose level was about four times higher in the symbiotic state than in the free-living culture. Because nitrogen fixation is a highly energy-demanding process, enzymes such as ATP synthase were up-regulated in the cyanobiont. Conversely, enzymes involved in the Calvin cycle such as phosphoribulokinase were down-regulated, together with *Vipp1*, a protein needed for thylakoid biosynthesis. Several proteins differentially expressed in the cyanobiont were involved in exopolysaccharide synthesis, along with surface, and membrane-associated proteins, suggesting adaptation of the surface for the establishment of the symbiosis and the exchange of nutrients. A third class of modifications was observed in the composition of phycobiliproteins, with allophycocyanin and phycocyanin being up-regulated in symbiosis, while phycoerythrin was down-regulated. These differential levels of expression are likely to be linked to the dark interior of the gland tissues.

ASSOCIATIVE AND ENDOPHYTIC NITROGEN FIXATION IN RICE, MAIZE AND WHEAT

Associative and endophytic nitrogen-fixing bacteria

Members of the Poaceae family do not naturally form symbiotic nitrogen-fixing associations. However, it has been shown that they can derive a substantial part of their nitrogen from BNF. Although the amount of fixed nitrogen is not as large as that measured in legumes nodulated by rhizobia, in actinorhizal plants or in cyanobacterial associations, increases in yields have been reported in the field (Dobbelaere *et al.*, 2003; Vessey, 2003; Verma *et al.*, 2010; Bhattacharyya and Jha, 2012).

Bacteria that colonize the rhizosphere are called rhizobacteria, and rhizobacteria with beneficial effects on plant development are referred to as plant-growth-promoting rhizobacteria (PGPR) (Kloepper and Beauchamp, 1992). Some of these PGPR are diazotrophic bacteria and have the ability to develop root associations with different plants including grasses. When they are found in close association with roots, they are usually designated ‘associative’ nitrogen-fixing bacteria (Elmerich, 2007). ‘Endophytic’ nitrogen-fixing bacteria have been defined as bacteria detected inside surface-sterilized plants or extracted from inside plants, having no visible harmful effects on the plants, fixing nitrogen, and proved by microscopic evidence to be located inside the plant (Hallmann *et al.*, 1997; Reinhold-Hurek and Hurek, 1998). However, the frontier between associative and endophytic plant colonization is not always clear, since associative bacteria can also be observed in plant tissues, although they are less abundant than strains originally classified as endophytic (Elmerich, 2007). In contrast to endosymbioses, no differentiated structures in the roots are induced by these bacteria and, although endophytic bacteria invade plant tissues, they cannot be regarded as endosymbionts that reside intracellularly in living plant cells. Endophytic diazotrophs may have an advantage over root-surface associative diazotrophs, as they colonize the interior of plant roots and can establish themselves in niches that provide more appropriate conditions for effective nitrogen fixation and subsequent transfer of the fixed nitrogen to the host plant (Reinhold-Hurek and Hurek, 1998, 2011).

Diazotrophic rhizobacteria have been identified in several genera of alpha- and beta-proteobacteria including *Acetobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Enterobacter*, *Herbasprillum*, *Glucenobacter* and *Pseudomonas* (reviewed in Baldani *et al.*, 1986; Döbereiner *et al.*, 1993; Vessey, 2003; Schmid and Hartmann, 2007; Cocking, 2009; Richardson *et al.*, 2009). Among these, *Azoarcus* spp., *Herbaspirillum seropedicae* and *Glucenobacter* are recognized as endophytes. They differ from other rhizobacteria such as *Azospirillum and Azotobacter*, in that they are tightly associated with plants and do not survive well in soil (Reinhold-Hurek and Hurek, 1998). Some of the main bacteria that can live in association with maize, rice and wheat and contribute to improved plant growth are presented in Table 2.

Plant colonization process

The plant–bacterial interaction takes place in the rhizosphere where PGPR are stimulated by plant root exudates and attracted

by root mucilage (reviewed in Vanbleu and Vanderleyden, 2007; Raaijmakers *et al.*, 2009; Compant *et al.*, 2010). The composition of root exudates depends on the type of soil, the availability of nutrients, the plant genotype and growth stage, and environmental biotic and abiotic stresses. In addition, there are differences in root exudation patterns along the root system that result in differences in the composition of the associated communities of bacteria. Some studies have shown that, as in the endosymbiotic process between legumes and rhizobia (Zhang *et al.*, 2009) and actinorhizal plants and *Frankia* (Abdel-Lateif *et al.*, 2012), flavonoids seem to be important plant signals for interaction with the bacteria. Some flavonoids were found to stimulate the colonization of wheat by *Azospirillum brasilense* and to be responsible for an almost 100 % increase in the number of lateral root cracks colonized in *Arabidospsis* by *Herbaspirillum seropedicae* (Webster *et al.,* 1998). In addition, the flavanone naringenin was found to regulate genes of *H. seropedicae* predicted to be involved in the colonization process (Tadra-Sfeir *et al.*, 2011).

Root colonization involves migration towards the plant roots, adsorption and anchoring onto the root system, as well as microbial proliferation and the formation of microcolony/biofilm structures at the surface of roots (Reinholdt *et al.*, 1986; Zhu *et al.*, 2002; Alexandre and Zhulin, 2007; Vanbleu and Vanderleyden, 2007; Compant *et al.*, 2010; Reinholdt-Hurek and Hurek, 2011). Diazotrophic PGPR probably employ an array of distinct mechanisms, either alone or in combination, to colonize successfully the plant roots and compete with other soil microorganisms. Among the mechanisms concerned, chemotaxis resulting from the presence of flagella will allow the bacteria to get into contact with roots, together with type IV pili and twitching motility. Twitching motility is based on a mechanism which includes pilus extrusion, surface attachment of the pilus tip, and pilus retraction to convey the bacterial cell to the point of attachment (Böhm *et al.*, 2007). In *Azoarcus*, type IV pili were shown to be involved in adherence to plant surfaces and the *pilA*, *pilB* and *pilT* genes were essential for root-surface colonization and for infection of plant tissues in rice (Dörr *et al.*, 1998; Krause *et al.*, 2006; Böhm *et al.*, 2007).

As observed in different plant–bacteria associations (Downie, 2010; Gough and Cullimore, 2011), surface polysaccharides such as exopolysaccharides and lipopolysaccharides (LPS) are involved in the colonization of roots. In a *Tn5* mutant of *A. brasilense* affected in the biosynthesis of dTDP-rhamnose, LPS composition was modified and resulted in impaired attachment of the mutant to maize roots and reduced root colonization (Jofré *et al.*, 2004). Additional studies recently undertaken on *H. serepedicae* by Balsanelli *et al.* (2010) confirmed these data. Two knock-out mutants were obtained in the genes *rfbB* (dTDP-D-glucose 3,5-epimerase) and *rfbC* (dTDP-4keto-L-rhamnose reductase) involved in the biosynthetic pathway of rhamnose. The ability of the knock-out mutants to attach to the surface of the maize root was 100-fold lower than that of the wild type, and the number of bacteria colonizing the internal plant tissues was also 100-fold lower. In addition to LPS, a major outer membrane protein from *A. brasilense* strain Cd was purified and shown, by *in vitro* adhesion assays, to bind to roots of wheat, corn and sorghum seedlings (Burdman *et al.*, 2001). In addition to its

TABLE 2. *Association of cereals and nitrogen-fixing PGPR*

Cereals	Diazotroph inoculant	Benefits % increase	References
Rice	*Azoarcus*	16 (total dry weight)†	Reinhold-Hurek and Hurek, 1997
			Engelhard *et al.*, 2000
	Burkholderia	68 (shoot biomass)† 19 (seed biomass)†	Baldani *et al.*, 2000
	B. vietnamiensis	13–22 (yield)*	Trân Van *et al.*, 2000
	Gluconacetobacter diazotrophicus	30 (total dry weight)†	Muthukumarasamy *et al.*, 2005
	Herbaspirillum seropedicae	37.6 (plant dry weight)†,	James *et al.*, 2002
	Serratia marcescens	23 (total dry weight)†	Gyaneshwar *et al.*, 2001
Maize	*Burkholderia* sp.	5.9–6.3 (yield)*	Estrada *et al.*, 2005
	Azospirillum brasilense	13–25 (yield)†	Riggs *et al.*, 2001
		33 (grain yield)*	Dobbelaere *et al.*, 2001
	Azotobacter		Pandey *et al.*, 1998
	H. seropedicae	19.5 (yield)*	Riggs *et al.*, 2001
	Pseudomonas sp.	11.7 (total biomass)†	Shaharoona *et al.*, 2006
Wheat	*H. seropedicae*	49–82 (total biomass)†	Riggs *et al.*, 2001
	Azospirillum sp.		Boddey *et al.*, 1986
	Azotobacter sp.		Mrkovacki and Milic, 2001

*, †, Experiments in fields (*) or in controlled conditions (†).
Adapted from Bhattacharjee *et al.* (2008) and Bhattacharyya and Jha (2012).

involvement in root adsorption, this protein acted on cell aggregation of *Azospirillum*. Bacterial cell aggregation was also modified in mutants of *A. brasilense* Sp7 affected in the chemotaxis Che1 pathway (Edwards *et al.*, 2011). Moreover, an outer membrane lectin that specifically recognizes and binds to the extracellular exopolysaccharides from *Azospirillum* is thought to play a role in cell-to-cell adhesion (Mora *et al.*, 2008).

In endophytes, undifferentiated tissues above the root tips and the points of emergence of lateral roots are the sites for primary colonization and entry into the plant (Reinhold-Hurek and Hurek, 1998). It has been suggested that cellulolytic and pectinolytic enzymes contribute to the infection process by degrading plant cell walls, thus providing a means to pass through the endoderm and to continue colonization inside the plant (Reinholdt-Hurek *et al.*, 1993; Kovtunovych *et al.*, 1999; Adriano-Anaya *et al.*, 2005). This hypothesis was confirmed by some studies on the role of an endoglucanase gene referred as *eglA* in *Azoarcus* BH72 (Reinhold-Hurek *et al.*, 2006). Whereas in rice plants inoculated with the control *Azoarcus*, bacteria were observed inside root epidermis cells 3 weeks after inoculation, the number of colonized cells was considerably decreased with an *eglA* mutant and the level of *nifH* mRNA was reduced in rice plants. The mutation also had an impact on the spreading into the shoot, thus leading to the conclusion that the cellulolytic degradation of plant cell walls plays an important role in plant colonization by *Azoarcus* BH72 and may contribute to systemic infection. However, since genes encoding cell wall-degrading enzymes have not been found in all endophytic PGPR, some of them may passively enter the root system, using disrupted endodermal cell layers resulting from the emergence of developing lateral roots.

After penetration, some endophytes may then colonize nutrient-rich intercellular spaces of the root cortex, move towards the xylem, and spread into stems and leaves (Olivares *et al.*, 1996). The interaction can develop rapidly with some endophytes such as *H. seropedicae*, which was observed in cortical cell layers of maize 12 h after inoculation and in xylem after 24 h (Monteiro *et al.*, 2008). Endophytic bacteria are found in roots, stems, leaves and seeds; nevertheless, in most plants, roots have the higher number of endophytes than above-ground tissues. Up to 10^8 colony-forming units per gram of root fresh weight have been reported (Barraquio *et al.*, 1997), whereas bacterial densities in leaves may reach 10^3–10^4 colony-forming units of fresh weight with active colonizers (reviewed in Compant *et al.*, 2010). However, it should be noted that recent metagenomic studies revealed a wide range of endophytes that are adapted to proliferate and spread in field-grown plants, although some have not yet been cultured or are difficult to isolate as pure cultures (Ikeda *et al.*, 2010; Sessitsch *et al.*, 2012).

Factors involved in plant growth promotion

Most of the PGPR isolates significantly increase plant height, root length and dry-matter production in agricultural crops like maize, wheat and rice. This plant growth promotion is the result of many different factors that can act directly or indirectly (reviewed in Dobbelaere *et al.*, 2003; Rosenblueth and Martinez-Romero, 2006; Saharan and Nehra, 2011).

Since mutants of *A. brasilense* and *Azoarcus* sp. deficient in nitrogenase activity were shown to retain their ability to promote plant growth, these data initially raised the question of the relative contribution of nitrogen fixation to increasing plant growth in grasses (Hurek *et al.*, 1994; Pedrosa and Elmerich, 2007). The contribution of associative and endophytic nitrogen fixation has now been clearly established, even though it appears to be highly variable, depending on the bacterial strain, the plant genotype and growth stage, and environmental conditions (Table 2). Evidence for nitrogen fixation was demonstrated by a number of techniques including acetylene reduction assays, ^{15}N dilution studies, immunogold labelling with antibodies against the iron protein of nitrogenase, expression of transcriptional fusion between *nifH* and reporter genes, and qRT-PCR on transcripts encoding the nitrogenase complex. These techniques revealed nitrogenase activity in several endophytic bacteria of grasses including *G. diazotrophicus* (Sevilla *et al.*, 2001), *Azoarcus* sp. strain BH72 in rice (Hurek *et al.*, 2002), *Herbasprillum* sp. in rice (Elbeltagy *et al.*, 2001; James *et al.*, 2002; Roncato-Maccari *et al.*, 2003) and *Klebsiella* sp. in maize and wheat (Chelius and Triplett, 2000; Iniguez *et al.*, 2004). Nevertheless, in some associations such as *Klebsiella* sp. and rice, or *Azospirillum* and maize, the addition of a supplementary carbon source such as sodium malate was necessary to observe significant nitrogenase activity, suggesting a shortage of suitable carbon sources during the nitrogen-fixation process (Egener *et al.*, 1999; Wouters *et al.*, 2000; Saikia and Jain, 2007).

In PGPR and, in particular, the well-known *Azospirillum*, the production of phytohormones rather than nitrogen fixation is considered to be a major factor for plant growth promotion (Fulchieri *et al.*, 1993; Dobbelaere *et al.*, 2001; Baca and Elmerich, 2007; Spaepen *et al.*, 2007, 2008). The production of the auxin IAA together with cytokinin has been reported in numerous rhizobacterial strains. These two hormones play a central role in regulating plant development, including processes that determine root architecture, such as root pole establishment during early embryogenesis, root meristem maintenance, root gravitropism and lateral root organogenesis (Kramer and Bennett, 2006). Phytohormones produced by bacteria thus enhance root branching and root elongation, which in turn favour the uptake of soil water and minerals and has a positive effect on plant growth (Steenhoudt and Vanderleyden, 2000). However, the effect of exogenous IAA on the root system can vary from growth stimulation to inhibition and is usually a function of the amount of IAA that is available to the plant and the sensitivity of the plant tissues to changes in IAA concentration (Keyeo *et al.*, 2011). Recent transcriptome analyses in *A. brasilense* revealed that interfering with IAA biosynthesis led to broad transcriptional changes in the bacteria, suggesting that IAA is an important signalling molecule involved in the plant–PGPR communication process (Van Puyvelde *et al.*, 2011). Besides auxin and cytokinin, the synthesis of gibberellin and, to a lesser extent ethylene, can also be observed. Gibberellin produced by *Azospirillum* was found to play an important role in the early stages of plant growth in Graminae by enhancing shoot and root growth and increasing root-hair density.

Although ethylene is essential for normal growth and development in plants, and is required for the induction of systemic resistance and in defence pathways in plants, at high concentrations it can be harmful and can reduce plant performance. PGPR produce the enzyme 1-aminocyclopropane-1-carboxylate deaminase, whose activity can divert 1-aminocyclopropane-1-carboxylate from the ethylene biosynthesis pathway (Blaha *et al.*, 2006; Desbrosses *et al.*, 2009). Rhizobacteria may thus reduce the accumulation of ethylene and re-establish a healthy root system. Production of NO due to the activity of nitrate reductase has also been observed in PGPR such as *A. brasilense* Sp245 (Pothier *et al.*, 2007, 2008). This NO may have some positive effects on root development since it is a key signal molecule that controls root growth, stimulates seed germination and is involved in plant defence responses against pathogens (Fernández-Marcos *et al.*, 2012).

Other direct effects of PGPR include the production of siderophores, vitamins and the solubilization of phosphorous. Iron is essential for plant growth and, in microorganisms, it acts as a global regulator of many cellular, metabolic and biosynthetic processes and, in bacteria, is a key element for nitrogen-fixation activity. Since in nature, iron is not readily available, microorganisms produce a wide range of small high-affinity chelating molecules called siderophores for its acquisition (Saha *et al.*, 2012). Microbial siderophores may stimulate plant growth directly by increasing the availability of iron in the soil surrounding the roots or by inhibiting pathogen growth. Concerning vitamins, the production of thiamine, biotin, riboflavin and niacin has been documented in some strains of *Azosprillum* and *Azotobacter* (reviewed in Richardson *et al.*, 2009). This exogenous supply may also stimulate root development. Phosphorous is one of the most essential nutrients for plants, but the majority of soil P is present in insoluble forms, while the plants can only absorb it in the soluble forms HPO_4^{2-} and $H_2PO_4^-$. Phosphate-solubilizing bacteria such as *Azospirillum* and *Burkholderia* convert insoluble phosphorous into soluble form through acidification, secretion of organic acids or protons and chelation, thereby helping to improve phosphate nutrition in the associated plants (Sturz and Nowak, 2000; Richardson *et al.*, 2009).

For some PGPR, positive effects on plant growth are indirect and result from mechanisms involving antagonism toward phytopathogens and the induction of systemic resistance pathways in the plant (Verhagen *et al.*, 2004; Bally and Elmerich, 2007; Raaijmakers *et al.*, 2009). These beneficial bacteria can help suppress a broad spectrum of viral, bacterial and fungal pathogens (reviewed in Sahan and Nehra, 2011). For instance, the inoculation of rice plants with the *Azospirillum* strain sp. B510 enhanced disease resistance to virulent rice blast fungus and to the bacterial pathogen *Xanthomonas oryzae* (Yasuda *et al.*, 2009). The biocontrol of phytopathogens in the root zone can also be achieved through the production of antifungal or antibacterial agents, siderophore production or through competition for colonization sites and nutrients (Rienhold-Hurek and Hurek, 2011). Interestingly PGPR can also confer tolerance to a number of abiotic stresses and can stimulate plant growth even in areas affected by drought (Alvarez *et al.*, 1996; Creus *et al.*, 1996), salt (Creus *et al.*, 1997; Jofré *et al.*, 1998; Bacilio *et al.*, 2004), and in soils polluted by heavy metals (Belimov and Dietz, 2000).

As the above-mentioned factors act in combination, a prerequisite for using these bacteria as nitrogen biofertilizers is to find the most appropriate combination of diazotrophic PGPR strain–plant cultivars to achieve a significant increase in plant biomass in the field (Table 1) (Roesh *et al.*, 2006; Bhattacharjee *et al.*, 2008). Some *Azospirillum*, *Glucenobacter* and *Acetobacter* inoculants are available for a variety of crops in Europe and Africa and from 5 % to 30 % increases in yields have already been reported (Vessey, 2003; Karthikeyan *et al.*, 2007; Bhattacharyya and Jha, 2012). In rice and maize, associative nitrogen fixation can supply 20–25 % of total nitrogen requirements and, in wheat, inoculation with *A. brasilense* significantly increased the yields of foliage, grain and branching of root hairs (reviewed in Okon and Labandera-Gonzalez, 1994; Saikia and Jain, 2007; Mano and Morisaki, 2008; do Vale Barreto Figueiredo *et al.*, 2010; Montanez *et al.*, 2012). However, the impact of associative nitrogen fixation and plant-growth promotion is usually more marked in soils with poor fertility.

Molecular mechanisms underlying the association with cereals

So far, our knowledge of bacterial endophytes has come from the study of model PGPR, via genomic and functional analysis of candidate genes. As reviewed by Reinhold-Hurek and Hurek (2011), several genomes of endophytes are now available, including *Azoarcus* sp. BH72 (Krause *et al.*, 2006), *Klesiella pneumoniae* 342 (Fouts *et al.*, 2008), *Pseudomonas stutzeri* A1501 (Yan *et al.*, 2008), *Gluconacetobacter diazotrophicus* Pal5 (Bertalan *et al.*, 2009), *Azospirillum* sp. B510 (Kaneko *et al.*, 2010) and *Herbaspirillum seropedicae* SmR1 (Pedrosa *et al.*, 2011). The exploration of these genomes revealed a number of characteristics that are important for rhizosphere competence. Gene-encoding products with relevant functions linked to plant–microbe interactions were identified, such as nitrogen fixation, production of hormones and degradation of ethylene intermediate, iron transport, flagella, pili and quorum sensing that modulate functions related to rhizosphere competence and adaptation.

The growing number of available genomes of rhizobacteria makes it possible to use comparative analyses to improve our understanding of the common or specific features of plant diazotrophic endophytes. For example, comparison of the genome of *G. diazotrophicus* Pal5 and that of *Azoarcus* sp. BH72 showed that these two bacteria have evolved different strategies to colonize plants (Bertalan *et al.*, 2009). *Gluconacetobacter diazotrophicus* is capable of growing on a wide variety of carbon sources and has a larger number of transport systems, whereas *Azoarcus* has complex signalling mechanisms to communicate with its host plant. Comparisons can also be extended to closely related beneficial and pathogenic endophytes. In 1997, Olivares *et al.* reported a genomic comparison of the endophyte *H. seropedicae* SmR1 and the phytopathogen *Herbaspirillum rubrisubalbicans* M1, which causes mottled stripe disease in sugarcane (Olivares *et al.*, 1997). Suppressive subtractive hybridization, together with direct comparison of genome sequences, revealed some structural differences in LPS and adhesins between the two strains, suggesting that molecular determinants of bacterial

cell surface structure may be responsible for their contrasted phenotypic behaviour as an endophyte or phytopathogen (Monteiro *et al.*, 2012). These comparisons are expected to improve our understanding of contrasted phenotypes such as endophytic associations and pathogenic life styles.

On the plant side, global approaches based on PGPR have targeted plant transcriptomics (Cartieaux *et al.*, 2003; Verhagen *et al.*, 2004), proteomics (Miché *et al.*, 2006; Cheng *et al.*, 2010) and metabolomic approaches (Walker *et al.*, 2011). First results of 'omics' approaches were reported with the model plant *A. thaliana*, followed by studies in rice (*Oryza sativa*), the model plant for monocot species, and more recently in maize. Transcriptomic studies initially helped reveal that, when *A. thaliana* was colonized by a PGPR, an accumulation of transcripts involved in the responses to pathogens and abiotic stresses was observed (Cartieaux *et al.*, 2003), thus conferring resistance to subsequent infection by pathogenic bacteria. More recently, proteome analysis of *O. sativa* was used to examine the extent to which a defence response might be involved in the interaction with an *Azoarcus* sp. with different rice cultivars (Miché *et al.*, 2006). Root proteomes of two rice cultivars with differing degrees of compatibility with the endophytic bacteria, were compared in response to jasmonate and to inoculation with the *Azoarcus* strain BHNG3.1. Jasmonate is a key signalling phytohormone in numerous plant responses to stresses such as pathogen attacks, and plays an important role in rice defence mechanisms (Rakwal *et al.*, 1999). Data suggested that plant defence involving jasmonate plays a role in restricting colonization when the host–bacterium interaction is less compatible (Miché *et al.*, 2006). Consequently, to obtain optimal plant–bacteria interactions, in the future it will be important to determine which factors suppress the defence response in a compatible variety.

EXTENDING NITROGEN-FIXING ABILITY TO NON-LEGUME CROPS: WHAT CAN BE LEARNED FOR NON-LEGUME SYMBIOSES?

In recent years, new insights into rhizobium–legume, rhizobium–*Parasponia*, actinorhizal and AM symbioses led to renewed interest in the possibility of transfering nitrogen-fixing ability to non-legume crops (Charpentier and Oldroyd, 2010; Beatty and Good, 2011; Geurts *et al.*, 2012). It has been known for several years that several components (SYMs) of the legume symbiotic signalling pathway acting downstream from Nod factor receptors play a role in both nodulation and in the more ancient AM symbiosis. The demonstration that the common SYM gene *SymRK* is also required for actinorhizal nodulation (Gherbi *et al.*, 2008*a*, *b*; Markmann *et al.*, 2008) raised the question as to what extent the nodulation signalling pathway is conserved in legumes and non-legume actinorhizal plants. In addition, Hocher *et al.* (2011) highlighted the fact that, beyond SymRK, the whole array of compounds of the Nod factor signal transduction pathway is shared between RNS in legumes and actinorhizal plants. The fact that a series of well-characterized symbiotic genes in legumes exhibit similar expression patterns in actinorhizals lends credibility to a common 'SYM' pathway for endosymbioses and, for the first time, points to the possibility of a similar 'NOD' pathway between RNS. This overlapping of legume and actinorhizal RNS reinforces the hypothesis of a common genetic ancestor of the nodulating clade with a genetic predisposition for nodulation (Soltis *et al.*, 1995).

AM symbioses appeared some 400 Mya (Remy *et al.*, 1994), while nitrogen-fixing RNS evolved approx. 60–70 Mya (Doyle, 1998), supporting the hypothesis that the symbiotic signalling mechanisms involved in rhizobium–legume associations derived in the course of evolution from pre-existing mycorrhizal signalling components. The majority of land plants, including cereals, can form an AM association with fungi belonging to the phylum Glomeromycota. Most of the genes closely related to those involved in the signalling pathways leading to nodulation or AM symbiosis (i.e. *SymRK/DMI2*, *CCaMK/DMI3*, Cytokinin receptor, *NIN*) have been identified in the rice genome. Moreover, it has been shown that rice *CCaMK* is able to restore AM symbiosis in a *Medicago dmi3* mutant and a rice *ccamk* mutant fails to develop AM (Chen *et al.*, 2007). The rice *CCaMK* is also able to restore nodulation in the *dmi3 Medicago* mutant, although nodules mostly did not contain rhizobia or bacteria were not released from infection threads (Godfroy *et al.*, 2006). These data show that elements of the rice AM genetic programme can trigger the appropriate downstream signalling pathway, thus paving the way for strategies to engineer nitrogen-fixing symbiosis in cereals by redirecting the evolutionary conserved common symbiotic pathway.

In addition to the use of knowledge accumulated on model legumes, we highlight the fact that non-legume actinorhizal and *Parasponia* symbioses could be more suitable models to obtain nitrogen-fixing cereals. Several steps including recognition, infection, intracellular accommodation of nitrogen-fixing endosymbionts, and nodule organogenesis are necessary to establish highly efficient nitrogen-fixing cereals. Because of the absence of the common *nodABC* genes in the genomes from clusters I and III strains (Normand *et al.*, 2007), the fact that *Frankia* strains can interact with non-legumes belonging to eight angiosperm families raises the question of whether *Frankia* is not a better choice than Rhizobia to infect cereals. In 75 % of legume genera, rhizobia enter the root hairs via an intracellular infection, as observed in the two model legumes *M. truncatula* and *L. japonicus*. In contrast, in 75 % of actinorhizal genera and in *Parasponia*, endosymbionts form nitrogen-fixing nodules via intercellular invasion (Sprent, 2007; Wall, 2000). In evolutionary terms, intracellular infection via root hairs would be the most recent and sophisticated mechanism and intercellular infection probably the most primitive mode of root colonization (Madsen *et al.*, 2010). Since much less is known about intercellular infection than about root hair infection, Imanishi *et al.* (2011) recently developed an efficient genetic transformation protocol for *Discaria trinervis*, an actinorhizal plant belonging to the Rosales order, in order to understand this ancestral and simpler infection mode. The study of *Parasponia–Rhizobium* and actinorhizal symbioses are thus of great interest in the context of engineering non-legume crops to allow infection by rhizobia or *Frankia*.

As previously mentioned, actinorhizal and *Parasponia*–rhizobia symbioses exhibit the same origin and structure as lateral roots. Interestingly, in *Parasponia* and in actinorhizal plants in the order Fagales, infection by endosymbionts led to cell

division in the cortex, resulting in a small external protuberance called the prenodule (Angulo Carmona, 1974; Callaham and Torrey, 1977). Although nodule primordium formation does not involve prenodule cells, Laplaze *et al.* (2000*a*) showed that, in the actinorhizal plant *C. glauca*, the prenodule represents a very simple symbiotic organ where both *Frankia* and plant cells differentiate into their symbiotic condition. Before considering the engineering of a fully developed symbiotic nodule in non-legume crop, an intermediate step similar to the prenodule could be considered. Since *Parasponia* and actinorhizal nodules are modified lateral roots, we can expect a common subset of phytohormones and genes in the control of their development. Recently, Péret *et al.* (2007) and Perrine-Walker *et al.* (2010) showed that auxin accumulated in *Frankia*-infected cells, suggesting a role for auxin in the establishment of actinorhizal symbiosis. Further work is needed to explain how *Frankia* and rhizobia trigger the lateral root development programme of the host root system. This could be a clue on how to initiate the formation of a nodule primordium in a non-legume crop.

For some unknown reasons, angiosperms appear better adapted for developing intracellular symbiosis, since the angiosperm *Gunnera* is the only host plant in which intracellular symbiosis with cyanobacteria is observed. Whereas the need for a pre-existing gland restricts the possible transfer of this colonization process outside Gunneraceae, understanding the physiological conditions and molecular mechanisms underlying the intracellular penetration of *Nostoc* sp. in the angiosperm could provide valuable information, for example in the context of a comparative analysis of intracellular colonization in root and nodule cortical cells by *Frankia* and rhizobia. The advantage of using cyanobacteria to create new symbioses with agricultural plants is the broad host range of some strains, such as those belonging to the genus *Nostoc*, which can infect different plant organs. Furthermore, nitrogen fixation is accomplished in heterocyst cells that naturally protect the nitrogenase complex from inactivation by oxygen. The associative competence of symbiotic *Nostoc* strains isolated from *Gunnera* and *Anthoceros* was studied in rice roots by Nilsson *et al.* (2002, 2005). The association obtained was tight and the cyanobacteria could not be removed by washing or by sonication. When associated with rice roots, the *Nostoc* strains increased their nitrogen fixation and their presence appeared to improve root and shoot growth, and increased the weight of the rice grains (Nilsson *et al.*, 2002).

In contrast to extensive molecular knowledge of rhizobium–legumes interactions, there are still only limited data available on the molecular aspects and signalling in the interactions leading to associative and/or endophytic interactions. Although the interactions with PGPR appear to be less complex than endosymbioses, they require the exchange of appropriate signals between the two partners to achieve successful colonization and phytostimulation, and for the bacteria to escape plant defence mechanisms. An intensive search for plant and bacterial signals, the receptors involved, cellular mediators and target genes in both partners is a primary goal to improve our understanding of these non-legume symbioses. This will provide additional knowledge leading to a broad view of the plant and microbial genes that could be manipulated to engineer new nitrogen-fixing plants.

CONCLUSIONS

A number of non-legume plants have evolved multiple strategies in association with diazotrophs to deal with N deficiency. The most sophisticated associations are root nodule endosymbioses between *Frankia* and actinorhizal plants, rhizobium and *Parasponia* sp., and cyanobacteria that associate with *Gunnera* sp. in cells of the specialized stem gland. In recent years, a major breakthrough has been the demonstration of a common genetic basis for plant root endosymbioses with AM fungi, rhizobia and *Frankia* bacteria in both legumes and non-legumes. This finding strengthens the hypothesis of a single origin for all nitrogen-fixing root nodule endosymbioses, and that RNS could have been partially recruited from the more ancient AM.

Compared with legumes, important questions remain to be answered including whether *Frankia* signals are structurally similar to Nod and Myc-factors, whether signals from actinorhizal plant and plant-hosting cyanobacteria are flavonoids – as in legumes and probably in *Parasponia* – and whether the conservation of the signalling pathway in *Parasponia* and actinorhizal plants goes beyond the common legume/rhizobium AM pathway. Progress in the knowledge of the basic mechanisms underlying symbiotic and endophytic associations in non-legumes has been generally slow, mainly due to the difficulties encountered in designing tools for the identification of candidate genes and their functional analysis. The value of comparative genomic approaches to help identifying, in addition to *nif* genes, common conserved gene functions specific to endosymbiotic and/or endophytic bacteria has been demonstrated. On the plant side, tools for 'omics' approaches and high-throughput sequencing technologies to finely explore transcriptomes are expected to provide new opportunities to decipher the molecular mechanisms underlying successful associations with diazotrophs.

The creation of artificial symbioses or associations between nitrogen-fixing microorganisms and plants of great agricultural importance is a primary goal in agriculture to reduce the demand for chemical nitrogen fertilizers. Since much of the basic work and major breakthroughs have been done on model legumes, strategies to expand the genetic capacity to fix nitrogen in symbiosis are currently based on that knowledge (Charpentier and Oldroyd, 2010; Beatty and Good, 2011). Recent advances in the understanding of endosymbiotic and endophytic nitrogen fixation with non-legume plants may represent original and alternative new avenues for engineering non-legume nitrogen-fixing crops.

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