Transcriptomics of Actinorhizal Symbioses Reveals Homologs of the Whole Common Symbiotic Signaling Cascade[1][W]

Valérie Hocher[2], Nicole Alloisio[2], Florence Auguy, Pascale Fournier, Patrick Doumas, Petar Pujic, Hassen Gherbi, Clothilde Queiroux, Corinne Da Silva, Patrick Wincker, Philippe Normand, and Didier Bogusz*

Equipe Rhizogenèse, Institut de Recherche pour le Développement, UMR Diversité Adaptation et Développement des Plantes, 34394 Montpellier cedex 5, France (V.H., F.A., P.D., H.G., D.B.); Université Lyon 1, Université de Lyon, Centre National de la Recherche Scientifique, UMR 5557, Ecologie Microbienne, 69622 cedex Villeurbanne, France (N.A., P.F., P.P., C.Q., P.N.); Commissariat à l'Energie Atomique, Direction des Sciences du Vivant, Institut de Génomique, Genoscope, 91000 Evry, France (C.D.S., P.W.); and INRA, Département de Biologie Végétale, Centre de Recherche de Montpellier, 34060 Montpellier, France (P.D.)

Comparative transcriptomics of two actinorhizal symbiotic plants, *Casuarina glauca* and *Alnus glutinosa*, was used to gain insight into their symbiotic programs triggered following contact with the nitrogen-fixing actinobacterium *Frankia*. Approximately 14,000 unigenes were recovered in roots and 3-week-old nodules of each of the two species. A transcriptomic array was designed to monitor changes in expression levels between roots and nodules, enabling the identification of up- and down-regulated genes as well as root- and nodule-specific genes. The expression levels of several genes emblematic of symbiosis were confirmed by quantitative polymerase chain reaction. As expected, several genes related to carbon and nitrogen exchange, defense against pathogens, or stress resistance were strongly regulated. Furthermore, homolog genes of the common and nodule-specific signaling pathways known in legumes were identified in the two actinorhizal symbiotic plants. The conservation of the host plant signaling pathway is all the more surprising in light of the lack of canonical *nod* genes in the genomes of its bacterial symbiont, *Frankia*. The evolutionary pattern emerging from these studies reinforces the hypothesis of a common genetic ancestor of the Fabid (Eurosid I) nodulating clade with a genetic predisposition for nodulation.

Fixed nitrogen is the factor that most often limits plant growth in ecosystems because it is a major building block of organisms and because it is highly labile, being lost from ecosystems slowly through denitrification and much more brutally in the case of fire, erosion, and glacier retreat, thus upsetting climactic ecosystems. To cope with the rarity of nitrogen and colonize such unsettled biotopes, pioneer plants have developed highly sophisticated systems for housing bacterial diazotrophs in specialized root nodules, called root nodule symbioses (RNS), found in legumes (Fabaceae) and in actinorhizal plants. The latter form a heterologous group comprising members of Fagales, Rosales, and Cucurbitales that develop a root symbiosis with the actinobacterium *Frankia* (Benson and Silvester, 1993). Molecular phylogeny of plant groups that engage in root nodule symbiosis shows that they all belong to a single lineage, the Fabid (Eurosid I) clade, and suggests a common ancestor with a predisposition for nodulation (Soltis et al., 1995). However, marked morphological differences in nodules between actinorhizal and legume plants (Pawlowski and Bisseling, 1996) have led to the suggestion that the ability to enter a symbiosis could have evolved independently several times within the two RNS (Swensen, 1996; Doyle, 1998).

Rhizobial genomes contain common *nod* genes that direct the synthesis of substituted lipochitooligosaccharides called Nod factors that interact with dedicated receptors to trigger the symbiotic program in Fabaceae. *Frankia* has not been genetically transformed despite repeated attempts (Kucho et al., 2010), but the genomes of three *Frankia* strains were recently sequenced (Normand et al., 2007a). *Frankia* genome analysis revealed the absence of canonical *nod* genes. Only a few low-similarity *nodB* and *nodC* homologs were detected, scattered throughout the genome and located far away from other symbiosis-related genes (Normand et al., 2007b). A transcriptomic approach did not enable the

[1] This work was supported by Genoscope and by research grants from the Centre National de la Recherche Scientifique "EC2CO" and from the French Agence Nationale de la Recherche Blanc 7 "Newnod" and "Sesam."

[2] These authors contributed equally to the article.

* Corresponding author; e-mail didier.bogusz@ird.fr.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) are: Valérie Hocher (valerie.hocher@ird.fr) and Nicole Alloisio (nicole.alloisio@univ-lyon1.fr).

[W] The online version of this article contains Web-only data.

www.plantphysiol.org/cgi/doi/10.1104/pp.111.174151

detection of a symbiosis island of up-regulated genes (Alloisio et al., 2010). Conversely, *Frankia* is known to synthesize a heat-stable root hair-deforming factor (Ceremonie et al., 1999) as well as phenyl-acetate, an auxin that induces lateral root formation (Hammad et al., 2003; Perrine-Walker et al., 2010).

Recent studies using two model legume species, *Lotus japonicus* and *Medicago truncatula*, elucidated the roles of many genes that are essential for the different steps of nodule development (Oldroyd et al., 2009). A genetic overlap was shown to exist between the legume RNS and the more ancient arbuscular mycorrhizal (AM) symbiosis (Kistner and Parniske, 2002), and the existence of a common pathway for legumes and AM endosymbioses, the "SYM" pathway, was demonstrated (Capoen et al., 2009; Markmann and Parniske, 2009). The need for at least seven legume genes (*SymRK*, *CASTOR*, *POLLUX*, *NUP85*, *NUP133*, *CCaMK*, and *CYCLOPS*) for both bacterial and fungal symbioses led to the hypothesis that preexisting AM genes were recruited during the evolution of root nodule symbiosis (Kistner and Parniske, 2002).

The symbiotic determinants of the actinorhizal plants are still poorly known (Perrine-Walker et al., 2011), aside from the recent demonstration that *SymRK* is a linchpin in *Casuarina* and *Datisca* (Gherbi et al., 2008; Markmann et al., 2008) and plays a role similar to that played in legumes. These results raise two important questions: do all endosymbioses share a unique pathway? Do actinorhizals and legumes share a common nodulation signaling pathway? On the other hand, a number of genes have been shown to be up-regulated in response to interaction with *Frankia* (Pawlowski, 2009). These genes include an enolase (van Ghelue et al., 1996), a subtilisin-like protease (Laplaze et al., 2000), a dicarboxylate transporter (Jeong et al., 2004), and nitrogen assimilation genes (Guan et al., 1996) as well as numerous poorly characterized genes.

The objective of this study was to investigate the genetic bases of symbiotic interactions in actinorhizal plants by focusing on two major actinorhizal species, *Alnus glutinosa* and *Casuarina glauca*. *Alnus* and *Casuarina* react to *Frankia* in a way that is largely similar, with initial deformation and branching of root hairs, division of cortical cells, and the formation of a swelling of the cortex called a prenodule, followed by the emergence from the pericycle of a modified secondary root colonized by *Frankia* that then penetrates cortical cells from an infection thread, where it develops nitrogen-fixing cells. The major difference between the two plants is oxygen level regulation in nodule cortical cells, which is a critical factor in *Frankia*. In *Alnus*, the oxygen level is not regulated, and *Frankia* has to adapt by synthesizing specialized cells called vesicles with thick hopanoid walls that form a diffusion barrier (Berry et al., 1993). When vesicles are absent, as in *Casuarina*, there is a low-oxygen tension in the infected cells (Tjepkema, 1979), presumably due to the lignification of their cell walls (Berg and McDowell, 1988), whereas a symbiotic hemoglobin facilitates the supply of oxygen to the bacterial respiration chain (Gherbi et al., 1997). In addition, nodular roots are prevalent in *Casuarina* and very rare in *Alnus* (Torrey, 1976); these peculiar roots, which emerge from the nodule apex, show a negative geotropism, are free of root hair and of bacterial infection, and are thought to permit the supply of oxygen and nitrogen to nodules under water saturation conditions (Tjepkema, 1978).

To understand the genetic bases of symbiotic interactions in actinorhizal plants, we undertook a study of the ESTs expressed following contact with *Frankia* in *A. glutinosa* and *C. glauca* and used transcriptomic arrays and quantitative PCR to analyze global gene expression. Our data revealed that genes homologous to genes of the entire common legume symbiotic and nodule-specific pathways were present in *A. glutinosa* and *C. glauca*, indicating a possible single origin for legume-rhizobia and actinorhizal symbioses.

RESULTS

Unigene Data Sets for *A. glutinosa* and *C. glauca* Are Comparable

For *A. glutinosa* and *C. glauca*, sequencing of the cDNA libraries obtained from noninoculated roots (controls), inoculated roots (2 and 7 d post inoculation [dpi]), and nodules (3 weeks post inoculation) gave rise to two sets of high-quality ESTs (32,591 for *A. glutinosa* and 30,525 for *C. glauca*). A detailed description of the EST libraries can be found in Supplemental Table S1. For each species, a complete set of ESTs was analyzed and clustered to generate a set of nonredundant genes. Clustering resulted in 14,301 unigenes (10,424 singletons and 3,877 tentative consensus sequences) for *A. glutinosa* and 14,868 unigenes (11,579 singletons and 3,289 tentative consensus sequences) for *C. glauca*. A similar classification was observed for both species: around 50% of genes had no significant match or known function, while the other 50% were identified by BLAST (Fig. 1A). Moreover, the further classification of the two unigenes sets according to Gene Ontology revealed similar distribution into functional categories for *A. glutinosa* and *C. glauca* (Fig. 1B). For both species, the main categories represented were linked to transport (approximately 10%), metabolism (approximately 15%), and protein synthesis and gene expression (approximately 20%), reflecting high activity levels, possibly linked to nodulation (Journet et al., 2002).

Identification of Genes Differentially Expressed during Actinorhizal Symbioses

Global Analysis of Microarrays

The main objective of this study was to focus on transcriptional changes during actinorhizal symbiosis in two different species. Microarrays were thus used to identify sets of differentially expressed genes for *C.*

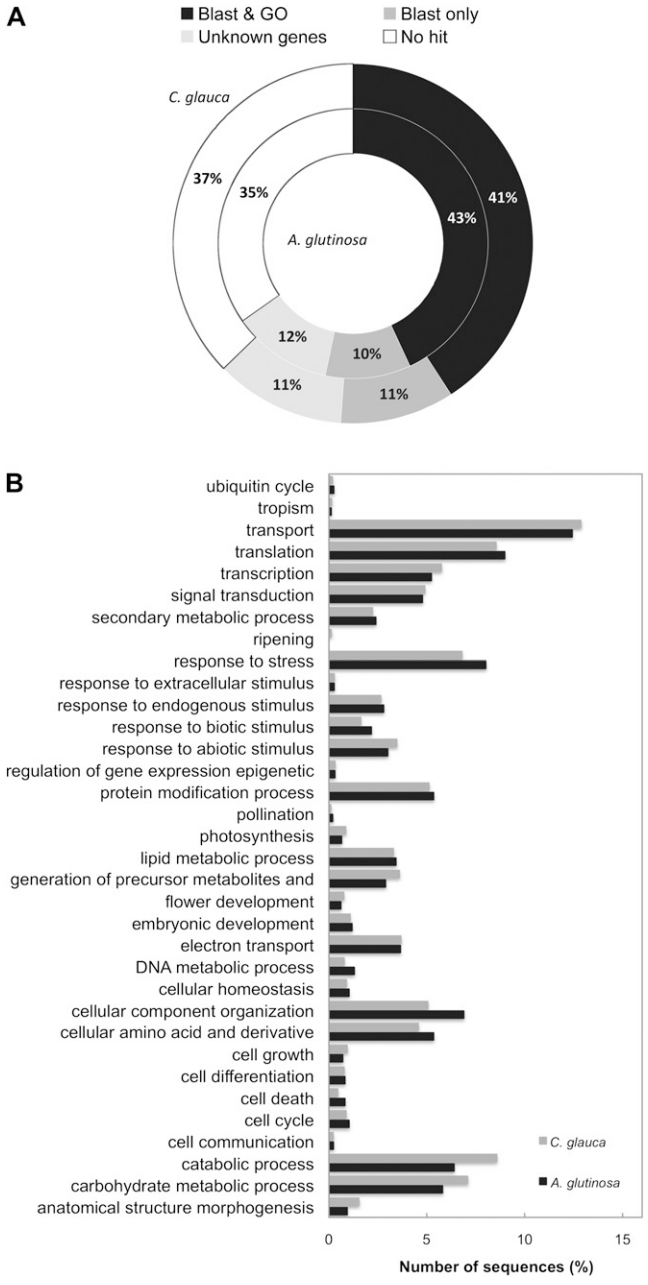

Figure 1. Comparison of unigene classification in *A. glutinosa and C. glauca*. A, Distribution based on E value and top-10 results of BLAST. B, Distribution of annotated unigenes into functional categories according to Gene Ontology (GO) GOSLIM biological process.

glauca and *A. glutinosa*. An Agilent custom oligonucleotide chip was designed for each species representing 13,909 unigenes for *A. glutinosa* and 14,543 unigenes for *C. glauca*, and gene transcript levels were compared between young nodules (3 weeks old) and uninfected roots. After elimination of residual redundancy, 1,196 (8.5% of the unigenes) and 1,672 (11.5% of the unigenes) genes were found to be significantly regulated in *A. glutinosa* and *C. glauca* nodules, respectively (nodule/root fold change ≥ 2 or ≤ 0.5; $P \leq 0.01$). Moreover, 340 (2.4%) genes in *A. glutinosa* and 339 (2.3%) genes in *C. glauca* were identified as specifically induced or repressed in the nodule. The complete lists of these regulated genes are given in Supplemental Tables S2 and S3. Approximately 50% of differentially expressed genes had a known function, and functional Gene Ontology "biological process" analysis of up- and down-regulated genes indicated that, overall, the response to nodulation was similar in the two species (Fig. 2). The distribution into functional categories was found to be very close to that observed for the unigene sets and thus confirmed high metabolism and transport activity regulation during nodule formation (Journet et al., 2002; Colebatch et al., 2004; El Yahyaoui et al., 2004). Due to the small number of regulated genes, some categories, like "ubiquitin cycle," "cell communication," and "DNA metabolic process," were not represented. It is worth noting that two categories appeared to be particularly underrepresented: the first, "electron transport," was missing even though it represented about 5% of the unigenes (Fig. 1B); the second, the "translation" category, appeared to be underrepresented in regulated genes even though it represented

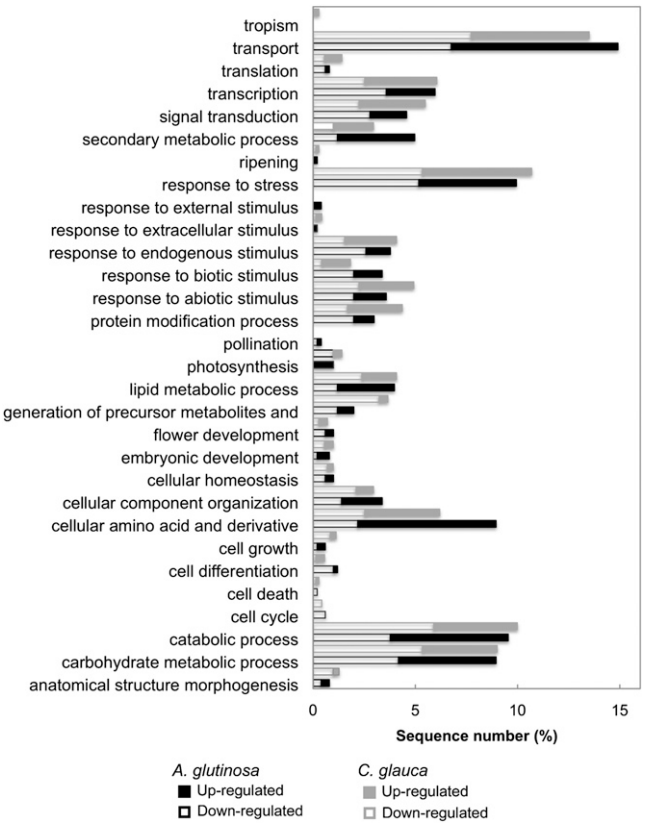

Figure 2. Comparison of *A. glutinosa* and *C. glauca* regulated genes according to Gene Ontology GOSLIM biological process functional categories. The distribution of up- and down-regulated genes is given for each category.

8% to 10% of the unigenes. For each category, Figure 2 shows the distribution between up- and down-regulated genes. The percentages of up- and down-regulated genes in each category were very similar in the two actinorhizal symbiotic species.

The Top-60 Differentially Regulated Genes

A comparison of the top-60 differentially or specifically regulated genes confirmed the high similarity between the two plant species for the control of nodulation. Around 50% of the top-20 up-regulated genes (Table I) had no homolog, and the other 50% were mostly linked to defense reactions, transport, and cell wall modification. Concerning down-regulated genes (Table II), genes encoding late embryogenesis-abundant protein-related, peroxidase precursor, chitinase, nitrogen transporter, and cytochrome 450 were found in both species. Interestingly, *Ag12* (for *A. glutinosa12*) and *Cg12* (for *C. glauca12*) were among the most strongly induced genes in nodules (Table III). These two homologs encode a subtilase, considered a nodulin linked to symbiosis (Ribeiro et al., 1995; Laplaze et al., 2000; Svistoonoff et al., 2003). Two other actinorhizal nodulins were also found in both species among the top-20 induced genes: a dicarboxylate transporter (*Dcat1*) that could supply the intracellular bacteria with carbon, and a γ-expansin natriuretic peptide belonging to a novel class of peptide signal molecules (plant natriuretic peptides) involved in biotic and abiotic stress response.

Actinorhizal Nodulins

The microarray expression levels were compared with previously published RNA gel-blot analyses of 11 *A. glutinosa* nodulins (five nodule specific and six nodule enhanced) and one pathogenesis-related protein (nodule down-regulated). Results and references are presented in Table IV. Excellent agreement was found between the microarray and RNA gel-blot data, except for the three less expressed nodulins (Gln synthetase, enolase, and Ag13 protein), which did not show significant up-regulation in nodules. This discrepancy could be due to the different nodule ages, 3 weeks for the microarrays and 5 to 13 weeks for

Table I. *List of the 20 most up-regulated genes during nodulation in A. glutinosa and C. glauca*

The fold change nodule (NOD) versus noninoculated root (NIR) was validated by a Student's test ($P < 0.01$). NA, Not annotated.

A. glutinosa			*C. glauca*		
Clone Name	Fold Change NOD/NIR	Product Description	Clone Name	Fold Change NOD/NIR	Product Description
AGCL10Contig3[a]	1,539.56	Actinorhizal nodulin GHRP	CGCL2388Contig1	3,256.26	Nucleoid DNA-binding protein cnd41
AGCL23Contig1[b]	1,123.35	γ-Expansin natriuretic peptide[c]	CGCL139Contig1	2,724.01	NA
AG-N01f_001_E15	481.46	Receptor protein kinase perk1-like	CGCL244Contig1	2,689.17	NA
AG-N01f_021_C14	400.94	Integral membrane family protein	CGCL683Contig1	2,136.16	Cys-rich protein
AG-N01f_032_M24	305.73	PMR5 (powdery mildew resistant 5)	CG-N02f_001_B22	1,166.02	Purine transmembrane transporter
AG-R01f_030_G24	288.39	NtPRp27-like protein	CGCL1417Contig1	1,027.49	Multidrug/pheromone exporter, MDR family, ABC transporter family
AG-N01f_011_P13	277.20	NA	CG-N02f_022_D11	763.95	NA
AG-N01f_002_I06	263.57	NA	CG-N02f_017_B16	616.81	Cellulase-containing protein
AGCL454Contig1	240.45	NA	CGCL1398Contig1	448.81	NA
AG-N01f_017_D15	173.71	β-Tubulin 14	CGCL167Contig1	372.36	NA
AGCL3713Contig1	155.02	Defensin amp1 protein	CG-N02f_031_K14	301.55	Flavanone 3β-hydroxylase
AG-N01f_032_B10	135.71	Anthocyanin-*O*-methyltransferase	CG-N02f_029_M21	292.47	AAA-type ATPase family protein
AGCL563Contig1	127.02	NA	CGCL2384Contig1	221.47	NA
AGCL1540Contig1	99.80	NA	CGCL291Contig1	216.13	NA
AG-N01f_041_J16	93.01	NA	CG-N02f_007_K17	200.54	NA
AGCL726Contig1	85.59	NA	CGCL2872Contig1	168.62	Bifunctional nuclease
AG-N01f_016_D15	82.23	NA	CG-N02f_033_F11	166.22	NA
AGCL2340Contig1	81.09	Cellulose synthase catalytic subunit	CG-N02f_002_L04	125.82	Disease resistance-responsive family protein
AG-R01f_024_I03	65.66	NA	CG-N02f_028_L08	124.61	Peroxidase 1
AG-N01f_026_N11	62.42	NA	CG-N02f_005_A03	114.77	Invertase pectin methyl-esterase inhibitor family protein

[a]Same sequence as for reported actinorhizal nodulin GHRP (Table IV). [b]Same sequence as for *A. glutinosa* γ-expansin natriuretic peptide cDNA (GU062393). [c]EST with the same product description as an EST listed in Table III but showing a different sequence, revealing the likely presence of different genes and/or alleles.

Table II. *List of the 20 most down-regulated genes during nodulation in A. glutinosa and C. glauca*

The fold change nodule (NOD) versus noninoculated root (NIR) was validated by a Student's test ($P < 0.01$). NA, Not annotated.

A. glutinosa			*C. glauca*		
Clone Name	Fold Change NOD/NIR	Product Description	Clone Name	Fold Change NOD/NIR	Product Description
AG-N01f_019_F11	0.06	NA	CG-R02f_047_E06	0.01	Chitinase (class II)
AG-R01f_031_P04	0.06	Cytochrome P450	CG-R02f_034_F01	0.01	Cytochrome P450
AG-R01f_019_E22	0.06	Sugar transporter	CG-R02f_021_J04	0.01	NA
AG-R01f_016_D22	0.06	CLE5 (clavata 3 ESR-related 5) receptor binding	CG-R02f_021_J02	0.01	Stigma-specific stig1 family protein
AGCL1170Contig1	0.06	TPA_inf: aquaporin TIP4;1	CG-R02f_011_F09	0.01	Pollen ole e 1 allergen and extensin family protein[a]
AG-R01f_011_O10	0.06	β-1,3-Glucanase	CG-R02f_044_A07	0.01	Pectin-esterase family protein
AG-R01f_034_A02	0.06	NA	CGCL1068Contig1	0.01	Nitrate reductase
AG-J07f_004_O01	0.05	Ammonium transporter	CG-R02f_032_M15	0.01	Short-chain alcohol dehydrogenase
AGCL3378Contig1	0.05	Cinnamate 4-hydroxylase	CG-R02f_024_C08	0.01	Carboxypeptidase D
AG-J07f_002_P01	0.05	NA	CG-R02f_048_H06	0.01	High-affinity nitrate transporter
AG-R01f_010_L01	0.04	Glc sorbosone dehydrogenase-like protein	CGCL47Contig1	0.00	SEPALLATA3-like MADS box
AG-N01f_039_B03	0.04	Peroxidase precursor	CG-R02f_011_B02	0.00	Nitrate transporter
AGCL103Contig1	0.04	Suspensor-specific protein	CG-R02f_001_N20	0.00	Pollen-specific kinase partner protein
AG-J07f_001_A06	0.03	NA	CG-R01f_002_A09	0.00	Peroxidase precursor[b]
AGCL2795Contig1	0.03	Xyloglucan-specific fungal endoglucanase inhibitor	CG-R02f_008_G05	0.00	Secoisolariciresinol dehydrogenase
AGCL1854Contig1	0.02	Putative chitinase	CGCL968Contig1	0.00	Late embryogenesis-abundant protein-related (Lea)
AGCL656Contig1	0.01	Short-chain alcohol dehydrogenase	CG-R02f_025_K08	0.00	Peroxidase precursor[b]
AGCL2384Contig1	0.00	NA	CG-R02f_009_P04	0.00	NA
AG-R01f_021_P13	0.00	Type II proteinase inhibitor family protein	CG-R02f_044_N23	0.00	TPA: class III peroxidase 102 precursor
AG-R01f_037_A23	0.00	Late embryogenesis-abundant protein-related (Lea)	CG-R02f_020_D19	0.00	Pollen ole e 1 allergen and extensin family protein[a]

[a,b]ESTs followed by the same letter indicate ESTs having the same product description but different sequences, showing thus the likely presence of different genes and/or alleles.

published gel-blot analyses. *C. glauca* homologs of these actinorhizal nodulins were identified in the unigene database, and interestingly, for most, a similar regulation pattern was observed in the microarray data (Table IV). As only a few mechanisms have been described for actinorhizal symbiosis, this observation is of paramount importance, as, to our knowledge for the first time, similar global expression was found in two actinorhizal species.

Validation of Microarray Results

Results of real-time quantitative (qRT)-PCR analysis of 23 *A. glutinosa* and 29 *C. glauca* genes revealed similar regulation patterns to those observed by microarray (Supplemental Table S4).

Homologs of Genes Involved in the Nod Factor Signal Transduction Pathway of Legumes Were Identified in *A. glutinosa* and *C. glauca*

BLAST analysis of our two unigene sets revealed that most legume symbiotic genes have homologs in the two actinorhizal plants. Twelve homologs (*Lys-6*, *SYMREM1*, *DMI2/SymRK*, *DMI3/CCaMK*, *RPG*, *Hap2-1*, *NSP1*, *ERF1*, *Cyp2*, *Cyp4*, *HMGR*, and *RALFL1*) were identified in *A. glutinosa*, and 16 homologs (*Lys-6*, *SYMREM1*, *CASTOR*, *Nup133*, *DMI3/CCaMK*, *IPD3/CYCLOPS*, *Hap2-1*, *ERN1*, *CPP-L56*, *NIN*, *LIN/CERBERUS*, *HK1*, *Cyp2*, *Cyp4*, *HMGR*, and *RALFL1*) were identified in *C. glauca* (Fig. 3). Interestingly, these homologs covered the entire symbiotic pathway from the signal perception to the nodulation process via the bacterial and fungal common pathway, as described (Kistner and Parniske, 2002; Markmann and Parniske, 2009; Madsen et al., 2010). Eight of them (*Lys-6*, *SYMREM1*, *DMI2/SymRK*, *DMI3/CCaMK*, *Hap2-1*, *HMGR*, *Cyp2*, and *Cyp4*) were identified in both species. For others, it is likely that their transcripts were not detected and have not been sequenced in one of the two actinorhizal species. For instance, this was the case of *SymRK*, whose symbiotic role has already been demonstrated in *C. glauca* (Gherbi et al., 2008) but was not present among *C. glauca* unigenes. Interestingly, expression analysis of these symbiosis homologs in *A. glutinosa* and *C. glauca* revealed that the accumulation

Table III. *List of the 20 most specifically induced genes during nodulation in A. glutinosa and C. glauca*

The fold change nodule (NOD) versus noninoculated root (NIR) was validated by a Student's test ($P < 0.01$). NA, Not annotated.

A. glutinosa			*C. glauca*		
Clone Name	Fold Change NOD/NIR	Product Description	Clone Name	Fold Change NOD/NIR	Product Description
AG-N01f_008_E02	22,688.37	Germin-like protein	gi_4691230_emb_AJ012164	7,848.98	Cg12, subtilase
AGCL324Contig1	10,465.36	NA	CG-R02f_025_H02	7,026.29	γ-Expansin natriuretic peptide
AGCL473Contig1	9,898.67	γ-Expansin natriuretic peptide[a]	CG-N02f_009_P18	6,703.78	NA
AG-N01f_036_K05	8,311.45	NA	CGCL126Contig1	5,735.54	Aquaporin, MIP family, PIP subfamily[d]
gi_757521_emb_X85975.1	7,823.16	Ag12, subtilisin-like protease[b]	CGCL488Contig1	5,627.12	τ-Glutathione *S*-transferase
AGCL2186Contig1	6,071.43	Dicarboxylate transporter (*AgDCTA1*)[c]	CG-N02f_007_N11	5,617.62	Aquaporin, MIP family, PIP subfamily[d]
AGCL24Contig1	5,891.36	NA	CG-N02f_012_A22	5,515.40	Subtilase
AG-N01f_015_L13	5,707.03	NA	CGCL1727Contig1	5,061.24	Cytochrome P450
AG-N01f_005_I04	4,584.00	NA	CG-N02f_037_M03	4,582.71	NA
AG-N01f_011_F15	4,538.73	Ag12, subtilisin-like protease[b]	CG-N02f_031_I07	4,520.42	Protein kinase family protein
AGCL240Contig1	3,761.25	NA	CG-N02f_028_D21	4,276.36	Male sterility 2-like protein
AGCL10Contig1	3,537.90	NA	CGCL3025Contig1	4,217.26	NA
AGCL806Contig1	3,390.73	NA	CGCL58Contig1	4,083.10	NA
AG-N01f_027_M10	2,971.80	Dicarboxylate transporter (*AgDCTA1*)[c]	CGCL672Contig1	3,984.77	NA
AGCL581Contig1	2,941.04	NA	CG-N02f_013_C02	3,898.23	Dicarboxylate transporter (*AgDCTA1*)
AG-N01f_032_L02	2,678.19	Plastocyanin-like domain-containing protein	CGCL491Contig1	3,762.19	Integral membrane family protein
AGCL716Contig1	2,592.40	Integral membrane protein	CGCL484Contig1	3,652.93	Receptor protein kinase related
AGCL507Contig1	2,470.97	γ-Expansin natriuretic peptide[a]	CG-N02f_029_C14	3,203.59	NA
AG-N01f_043_P03	2,354.12	pdr3_tobac, pleiotropic drug resistance protein 3	CG-N01f_010_G05	3,053.58	NA
AG-N01f_026_P02	2,109.03	Ag12, subtilisin-like protease[b]	CGCL125Contig1	3,008.36	NA

[a]EST with the same product description, as an EST listed in Table I but showing a different sequence, revealing the likely presence of different genes and/or alleles. [b,c,d]ESTs followed by the same letter indicate ESTs having the same product description but different sequences, showing thus the likely presence of different genes and/or alleles.

of transcripts in nodules versus uninfected roots was not only comparable between the two actinorhizal species but also similar to those found in legumes (Fig. 3). A detailed description of the homologs, BLAST results, and references are given in Supplemental Tables S5 and S6.

DISCUSSION

Land plants have evolved from rootless sea-dwelling ancestors about 400 million years ago through major evolutionary reorganization involving the development of roots and the ability to cope with a completely different array of surrounding pathogenic and saprotrophic microbes, prominent among which are fungi (Simon et al., 1993). Present-day land plant families are almost all able to establish root symbioses with AM fungi, which require dedicated kinases in the plant and a lipochitooligosaccharide effector in the fungus (Maillet et al., 2011). This evolutionary quantum leap was presumably accomplished by duplicating and rewiring preexisting sensing kinases with downstream regulators and defense proteins, since the detection of GlcNAc oligomers made sense in a biotope awash with fungal pathogens that contained chitin in their cell walls (Miya et al., 2007). With our approach, the aim was first to determine whether the symbiosis of *Frankia* with *Casuarina* and *Alnus* uses the common SYM pathway described for AM fungi and legume-rhizobium symbioses and second if a "NOD"-specific pathway was only shared by RNS.

Table IV. *Comparison of microarray expression with previously reported expression analyses of 11 actinorhizal nodulins and one pathogenesis-related protein of A. glutinosa*

Homologs found in the *C. glauca* database are indicated. NOD/NIR, The fold change nodule versus noninoculated root.

A. glutinosa Nodulins					*C. glauca* Homologs			
Name	Accession No.	Fold Change NOD/NIR	Student's *t*	References of RNA Gel-Blot Analyses in Nodules and Roots of *A. glutinosa*	Clone Name	E Value	Fold Change NOD/NIR	Student's *t*
Dicarboxylate transporter (*AgDcat1*)	AJ488290.1	12,186.0[a]	0.010	Specific in nodules (6–8 weeks old; Jeong et al., 2004)	CG-N02f_009_D12	3.00E-79	850.9[a]	0.004
Subtilisin-like protease (*Ag12*)	X85975.1	7,823.2[a]	0.006	Specific in nodules (5–13 weeks old; Ribeiro et al., 1995)	AJ012164	0.0	7,849.0[a]	0.003
Actinorhizal nodulin AgNOD-GHRP (*AgNt84*)	U69156.1	2,223.9	0.005	Specific in nodules (8–12 weeks old; Pawlowski et al., 1997)	CG-N02f_022_H01	4.00E-28	8.2	0.0001
Ag164 protein	Y08436.1	1,030.7	0.011	Specific in nodules (8–12 weeks old; Pawlowski et al., 1997)	CG-R02f_009_G02	1.00E-12	0.45	0.002
Cys proteinase	U13940.1	253.6	0.029	Specific in nodules (Goetting-Minesky and Mullin, 1994)	CGCL1769Contig1	5.00E-112	762.0[a]	0.012
Acetyl-Orn transaminase	Y08680.1	22.0	0.018	About 20-fold enhanced expression in nodules (5–8 weeks old) versus roots (Guan et al., 1996)	–	–	–	–
Thiazole biosynthetic enzyme	X97434.1	14.2	0.002	About 15-fold enhanced expression in nodules (5–13 weeks old) versus roots (Ribeiro et al., 1996)	CG-R02f_022_P07	1.00E-107	0.71	–
Suc synthase	X92378.1	2.3	0.016	About 7-fold enhanced expression in nodules (5–13 weeks old) versus roots (van Ghelue et al., 1996)	CGCL129Contig1	0.0	1.8	–
Gln synthetase	Y08681.1	1.8	–	About 5-fold enhanced expression in nodules (5–8 weeks old) versus roots (Guan et al., 1996)	CGCL29Contig1	0.0	0.5	0.017
Ag13 protein	Y08435.1	1.6	–	About 5-fold enhanced expression in nodules (10 weeks old) versus roots (Guan et al., 1997)	CGCL280Contig2	4.00E-41	0.28	–
Enolase	X92377.1	0.8	–	About 3-fold enhanced expression in nodules (5–13 weeks old) versus roots (van Ghelue et al., 1996)	CGCL2897Contig1	1.00E-151	2.2	0.0067
Pathogenesis-related protein PR10A	AJ489323.1	0.1	0.0077	Down-regulated in nodules (Pawlowski et al., 2003)	–	–	–	–

[a]Probe flagged as present in nodules but absent in roots.

Frankia is a ubiquitous microbe that emerged about 150 to 200 million years ago (Normand et al., 1996), presumably from a rhizosphere-dwelling ancestor (Normand and Chapelon, 1997). In addition to its ability to synthesize an as yet uncharacterized root hair-deforming factor and specialized cells covered with a unique hopanoid lipid (Berry et al., 1993), it contains the sugar 2-*O*-methyl-D-Man (Mort et al., 1983), a determinant that is present in all the strains tested but otherwise very rare in the microbial world. *Frankia* is in symbiosis with plants scattered throughout the Fabid but that nevertheless share a pioneer lifestyle and the ability to tolerate nitrogen-poor soils and repopulate biotopes (Benson and Silvester, 1993). Although the fossil record indicates that the AM symbiosis indeed occurred around 400 million years ago (i.e. much earlier than the legume-rhizobia symbiosis, dated at about 55 million years ago [Lavin et al., 2005]), the

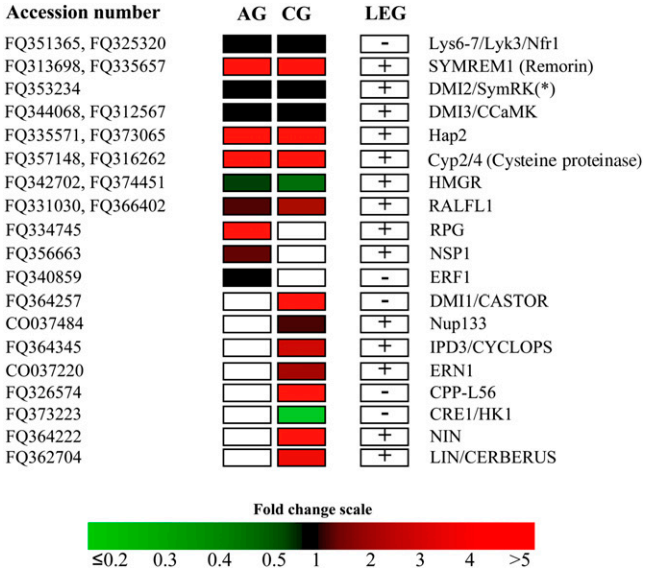

Figure 3. Transcriptional regulation of putative symbiotic signaling pathway genes in nodules of *A. glutinosa* (AG) and *C. glauca* (CG). Green/red color-coded heat maps represent relative transcript levels determined using the arrays (red, up-regulated; green, down-regulated, black, not regulated; white, not found in the database). The right column (LEG) shows whether change in gene expression in the legume is comparable (+) or different (−). Complete descriptions of BLAST results and related references are given in Supplemental Tables S5 and S6. * DMI2/SymRK was not found in the *C. glauca* unigene database, and expression results of Gherbi et al. (2008) were used for comparison with *A. glutinosa*.

actinorhiza-*Frankia* symbiosis is likely to have emerged earlier, although it is hard to date it precisely. Myricaceae and Betulaceae fossils have been reported around 80 to 90 million years ago, while Casuarinaeae, Elaeagnaceae, Coriariaceae, Datiscaceae, Rhamnaceae, and Rosaceae appeared later (Thomas and Spicer, 1987). More recently, Bell et al. (2010), studying *rbcL*, 18S rDNA, and *atpB* sequences, positioned the Rhamnaceae-Elaeagnaceae ancestor at 69 to 71 million years ago, as the most ancient actinorhizal lineage.

Our data set comprises about 14,000 unigenes for *A. glutinosa* and 14,500 for *C. glauca* for which Gene Ontology classification in functional categories showed a similar distribution of genes in the two species, suggesting that the actinorhizal *C. glauca* and *A. glutinosa* are closely related. The use of arrays enabled us to identify with a high confidence level a collection of genes differentially regulated during nodulation in the two actinorhizal species. Around 1,500 unigenes (11%) for *A. glutinosa* and 2,000 unigenes (14%) for *C. glauca* were shown to be regulated or specifically induced during nodule development. In both species, it is worth noting that among the most highly induced genes (Table III), two previously characterized actinorhizal nodulins, a subtilase (*Ag12/Cg12*) and a dicarboxylate transporter (*Agdcta1*; Ribeiro et al., 1995; Laplaze et al., 2000; Svistoonoff et al., 2003; Jeong et al., 2004), were identified. More detailed data analysis enabled us to identify other known *A. glutinosa* nodulins with homologs in *C. glauca*. The fact that a series of well-characterized actinorhizal symbiotic gene markers exhibit expression patterns consistent with previous studies supports the hypothesis that new actinorhizal nodulin genes are putatively present in 50% of unidentified regulated genes.

Our analysis also revealed that the majority of *C. glauca* and *A. glutinosa* regulated genes are involved in transport, metabolism, protein synthesis machinery, cell wall, defense, and response to stress. During the switch from a root-specific to a nodule-specific gene expression program, important transcriptional changes would be expected to accompany bacterial recognition and invasion and plant morphological changes. This situation is extremely close to that described in different model legume transcriptomic studies (Asamizu et al., 2000; Journet et al., 2002; Colebatch et al., 2004; El Yahyaoui et al., 2004; Maunoury et al., 2010) with up-regulation of DCAT, a dicarboxylate transporter for delivery of photosynthates as TCA intermediates to the symbiont and a Gln synthetase, homolog of early nodulin *M. truncatula N6* (Mathis et al., 1999), for assimilation of the ammonium fixed and transferred to the plant cytosol, which is consistent with previous demonstrations that the assimilation of ^{13}N was through Gln synthetase synthesizing Gln (Lundberg and Lundquist, 2004). The level of expression of several defense genes (defensins, chitinases) as well as stress proteins (catalase, DnaJ, Mdr) was also modified in a similar way to what is known to occur in legume nodules (Pucciariello et al., 2009; Maunoury et al., 2010), in particular a γ-expansin natriuretic peptide that belongs to a novel class of peptide signal molecules (plant natriuretic peptides) involved in the response to biotic and abiotic stresses (Gottig et al., 2008; Meier et al., 2008). Interestingly, some defense/stress proteins were differentially expressed in *Casuarina* and *Alnus* nodules. This might be related to the different strategies for meeting the energy demand of nitrogen fixation and for nitrogenase protection (Pawlowski, 2008). Further studies are in progress aiming to understand global respiratory oxygen uptake and oxygen protection mechanisms in both species.

Although the two main types of RNS, legume/rhizobia and actinorhizal species/*Frankia*, differ in the bacterial partners involved, they share many features, such as their infection mechanisms (Pawlowski and Bisseling, 1996). Concerning the microbe, little is known about the symbiotic signals and their perception during actinorhizal symbiosis. Genome sequencing of *Frankia* revealed the absence of the canonical *nod* gene described in rhizobia (Normand et al., 2007a). Nevertheless, the *Frankia* root hair deformation signal shares functional similarities with the rhizobial Nod and fungal Myc factors, such as thermoresistance, a size below 1,400 D, sensitivity to some enzymes (Ceremonie, 1998), and hints that *N*-acetyl-D-glucosamine may be present (Ceremonie et al., 1999). Concerning the plant, numerous genes involved in the Nod factor signaling

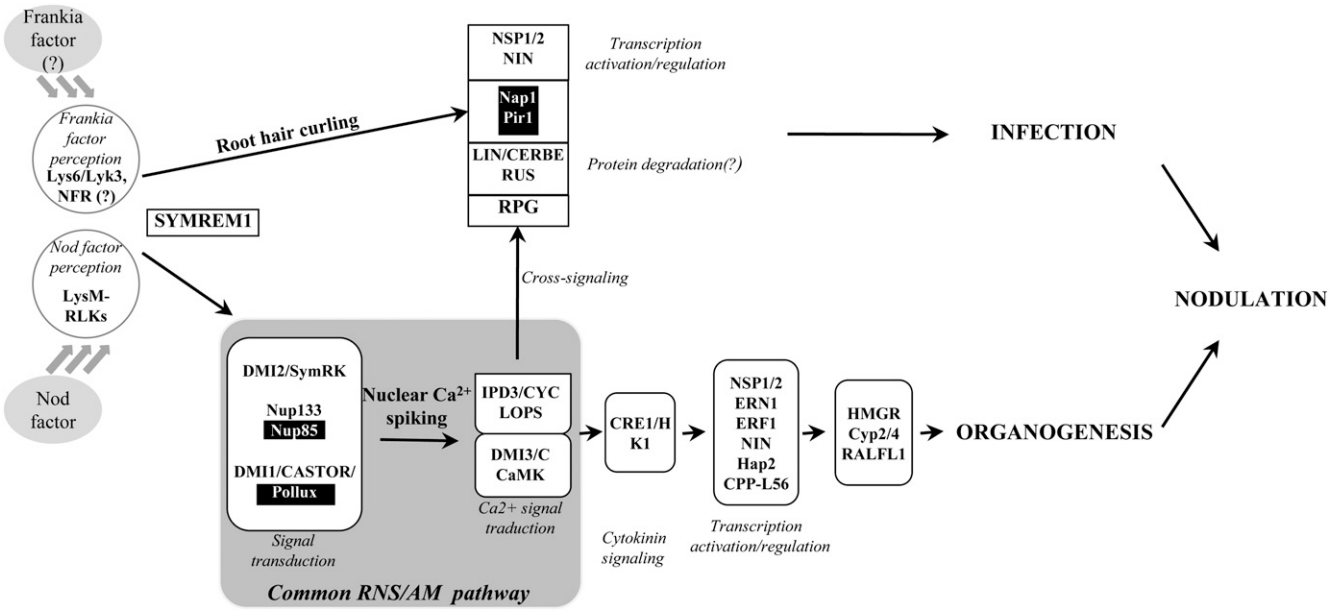

Figure 4. Proposed model for a common symbiotic signaling pathway between actinorhizal plants and legumes. The common genes identified in *A. glutinosa* and/or *C. glauca* were assigned to infection and organogenesis pathways. Black boxes indicate legume genes not yet identified in *A. glutinosa* or *C. glauca*. The common pathway between RNS and AM is indicated by the gray box. Adapted from Madsen et al. (2010).

cascade of model legumes have been characterized, and part of this cascade, called the SYM pathway, was also shown to be necessary for fungal signal transduction (Kistner and Parniske, 2002; Markmann and Parniske, 2009; Oldroyd et al., 2009). The recent demonstration that the common SYM pathway gene *SymRK* is also required for actinorhizal nodulation (Gherbi et al., 2008; Markmann et al., 2008) led us to reconsider to what extent the nodulation signaling pathway is conserved in legumes and actinorhizal plants. In this study, we were able to highlight 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. Furthermore, expression analyses demonstrated that transcriptional regulation of these genes in uninfected roots and nodules was comparable to that in legumes (Fig. 3). For example, nodule inception (*NIN*), remorin (*SYMREM1*), and *RPG*, shown to be involved in the legume/rhizobia infection process and nodule organogenesis (Schauser et al., 1999; Arrighi et al., 2008; Lefebvre et al., 2010), are strongly up-regulated in actinorhizals. 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, to our knowledge for the first time, points to the possibility of a similar nodule pathway between RNS (Fig. 4). 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).

Further studies to decipher genetic determinants of actinorhizal nodulation will thus be needed to fully identify the basic set of RNS determinants. It is crucial to undertake functional studies of these legume homolog symbiotic genes to help us reach a full understanding of the unique basic genetic program that governs RNS. This is a necessary prerequisite to define strategies to create artificial RNS. Our set of symbiosis-specific genes should also help focus the search for the *Frankia* symbiotic effectors.

MATERIALS AND METHODS

Plant and Bacterial Material

Casuarina glauca seeds were obtained from B&T World Seeds. *Alnus glutinosa* seeds were harvested from an *A. glutinosa* specimen growing on the left bank of the Rhône River in Lyon, France, used previously (Alloisio et al., 2010). Both species were grown and inoculated with a compatible *Frankia* strain: CcI3 for *C. glauca* (Gherbi et al., 1997) and ACN14a for *A. glutinosa* (Normand and Lalonde, 1982), as described previously (Gherbi et al., 1997; Alloisio et al., 2010). For construction of the EST library, noninoculated roots (controls), inoculated roots (2 and 7 d dpi), and nodules (3 weeks post inoculation) were collected and immediately frozen in liquid nitrogen.

RNA Extraction

Total RNA was purified from roots and nodules by ultracentrifugation (Hocher et al., 2006) for *C. glauca* and using the RNeasy plant mini kit (Qiagen; Alloisio et al., 2010) for *A. glutinosa*. Residual DNA was removed from RNA samples using the Turbo DNA free kit (Ambion), quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific), and qualitatively assessed using a Bioanalyzer 2100 according to the manufacturer's instructions (Agilent).

Unigene Database Construction

Construction of cDNA Libraries

Poly(A$^+$) RNA was isolated from *A. glutinosa* total RNA using the MicroPoly(A) Purist kit (Ambion/Applied Biosystems). About 0.5 and 0.8 μg of poly(A$^+$) RNA from nodules and noninoculated roots, respectively, were used to construct two *A. glutinosa* EST libraries with the Creator Smart cDNA Library Construction kit (Clontech) and *Escherichia coli* DH10B-T1 host cells (Invitrogen Life Science). For *C. glauca* root and nodule libraries, the same protocol was followed using 2 μg of total RNA.

For both species, about 2 μg of poly(A$^+$) RNA from inoculated roots (2 and 7 dpi) was used to construct two subtraction libraries using the PCR-Select cDNA Subtraction Kit from Clontech according to the manufacturer's instructions. Poly(A$^+$) RNA from inoculated roots (2 or 7 dpi) was used as testers, and poly(A$^+$) RNA from noninoculated roots was used as drivers. For each species, subtraction efficiency was validated using primers specific to a gene encoding a metallothionein-like protein: for *A. glutinosa*, forward (5′-CCGCTGACCTGAGCTACTCA-3′) and reverse (5′-GGGATCCATAGATCCAACCAT-3′); for *C. glauca*, forward (5′-TGTCTTCCTGTGGCTGTG-3′) and reverse (5′-TCCTCCTTCAACGCTCATC-3′).

EST Sequencing and Annotation

For root and nodule libraries, around 15,000 clones were randomly selected and processed through the Genoscope robotic and genomic platform (http://www.cns.fr/spip/). Single-read sequencing from the 5′ end was undertaken. For each subtraction library, the same approach was used on approximately 2,000 randomly selected clones. A total of 32,591 valid EST sequences obtained for *A. glutinosa* and 34,569 for *C. glauca* and each set of sequence data were processed and annotated (E value cutoff at 10^{-10}) using a multimodule custom pipeline as described previously (Hocher et al., 2006). For *C. glauca*, previously obtained EST data were included for clustering (Hocher et al., 2006). For both species, a few sequences present in databases were included. After the removal of identified bacterial sequences, EST clustering was done using TGICL software (http://compbio.dfci.harvard.edu/tgi/software/). All resulting data (sequences, clustering results, and BLAST results) were automatically integrated into a relational database, searchable via a local Web browser-based interface. For both species, unigenes were then classified according to Gene Ontology using Blast2GO (Conesa et al., 2005).

Array Design and Analysis: Agilent Platform

Microarrays were manufactured and hybridized by Imaxio (http://www.imaxio.com/index.php), accredited by Agilent Technologies (http://www.home.agilent.com/agilent/home.jspx) as a certified service provider for microarray technologies.

Microarray Design

Based on the unigenes, annotation data, and sequences deposited in the National Center for Biotechnology Information database for *C. glauca* and *A. glutinosa*, 60-mer probes were designed using eArray software (one probe per unigene) and custom 8 × 15 K oligonucleotide slides. Optimal probes were designed for 14,543 unigenes for *C. glauca* and 13,909 unigenes for *A. glutinosa*. Microarrays were manufactured using Agilent Technologies.

Microarray Experiment and Analysis

Two biological conditions were selected for each plant: noninoculated roots and 3-week-old nodules. The reliability and reproducibility of the analyses were ensured by the use of biological triplicates for each selected condition. Total RNA was used to synthesize copy RNA, incorporating the Cy-3 dye using Agilent's Low RNA Input Linear Amplification Kit, one-color. The Cy-3 copy RNA fragments were hybridized to custom microarrays using reagents and protocols provided by the manufacturer. The microarrays were scanned with the Agilent G2505B Scanner. The Feature Extraction software (Agilent; version 9.1) was used to quantify the intensity of fluorescent images, and microarray data were analyzed using GeneSpring GX version 7.3 software (Agilent Technologies). Normalization per chip (normalized to the 50th percentile) and per gene (normalized to the median) were performed to allow comparison of the three independent biological replicates performed for each set of experiments. Microarray data were filtered according to flag parameters (marginal, absent, present). After microarray analysis, 15% of probes for *C. glauca* and 11% for *A. glutinosa* revealed no signal in all samples and were discarded. Only those transcripts that were declared present in at least two-thirds of the chips were taken into account. A Student's test comparing nodules versus roots was applied, and only those genes with an average fold change greater than 2 (up-regulated) or less than 0.5 (down-regulated) at $P < 0.01$ were considered. In order to identify genes specifically expressed in nodules (induced) or in roots (repressed in nodules), probes flagged as present in only one of the two biological conditions were also considered in a complementary list. To assess gene expression level for this second list, Student's test was applied using background average as the lower value (probes flagged as absent) and genes were selected using the same criteria as in the first gene category described above.

Quantitative Real-Time RT-PCR

Reverse transcription-PCR and qRT-PCR were performed with the same three biological replicates of nodules and noninoculated roots used for microarray experiments. For *C. glauca* analyses, single-strand cDNA was synthesized from 500 ng of total RNA using the SuperScript III Reverse Transcriptase kit (Invitrogen Life Science). Three independent reverse transcription reactions were pooled to minimize potential heterogeneity in reverse transcriptase yield. qRT-PCR was performed on a Stratagene MX 3005P apparatus (Agilent) with the Brilliant II SYBR Green QPCR Master Mix (Agilent) under the following conditions: 95°C for 5 min, 40 cycles of 95°C for 10 s, and 60°C for 30 s. For *A. glutinosa* analyses, reverse transcription was performed with 9 μg of total mRNA using Transcriptor Reverse Transcriptase and oligo(dT)$_{15}$ primer (Roche). qRT-PCR was run on a LightCycler 480 (Roche) using LightCycler 480 SYBR Green I Master (Roche) under the following conditions: 95°C for 5 min; and 45 cycles of 95°C for 20 s, 60°C for 20 s, and 72°C for 15 s. The primer set was designed using Beacon Designer software (Premier Biosoft International) and can be seen in Supplemental Table S7. In both experiments, three qRT-PCRs were run for each biological replicate. Expression values were normalized using the expression level of the *ubi* gene, which encodes ubiquitin: *CgUbi* for *C. glauca* (Hocher et al., 2006) and *AgUbi* for *A. glutinosa*.

EST sequences reported in this paper have been deposited in the GenBank (accession nos. CO036851–CO0388878) and EMBL (accession nos. FQ312199–FQ377516) databases. The normalized and raw microarray data values have been deposited in the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo; accession nos. GPL10929 for *C. glauca* and GSE24153 for *A. glutinosa*, respectively).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. *A. glutinosa* and *C. glauca* EST and cluster collection statistics.

Supplemental Table S2. Genes differentially or specifically regulated during nodulation in *A. glutinosa*.

Supplemental Table S3. Genes differentially or specifically regulated during nodulation in *C. glauca*.

Supplemental Table S4. Validation of microarray results by qRT-PCR.

Supplemental Table S5. *A. glutinosa* homologs of *L. japonicus* or *M. truncatula* genes involved in Nod factor signal transduction

Supplemental Table S6. *C. glauca* homologs of *L. japonicus* or *M. truncatula* genes involved in Nod factor signal transduction

Supplemental Table S7. *C. glauca* and *A. glutinosa* primers used for qRT-PCR validation.

ACKNOWLEDGMENTS

We thank D. Abrouk (University of Lyon 1) for help in data processing, D. Desbouchages (Institut Fédératif de Recherche 41) for use of the greenhouse,

and the Technical Platform (Développement de Techniques et Analyses Moléculaires de la Biodiversité; Institut Fédératif de Recherche 41) for use of the qRT-PCR and the environmental genomics platform (UMR CNRS 5557).

Received February 11, 2011; accepted March 30, 2011; published April 4, 2011.

LITERATURE CITED

Alloisio N, Queiroux C, Fournier P, Pujic P, Normand P, Vallenet D, Médigue C, Yamaura M, Kakoi K, Kucho K-I (2010) The *Frankia alni* symbiotic transcriptome. Mol Plant Microbe Interact **23:** 593–607

Arrighi JF, Godfroy O, de Billy F, Saurat O, Jauneau A, Gough C (2008) The RPG gene of *Medicago truncatula* controls *Rhizobium*-directed polar growth during infection. Proc Natl Acad Sci USA **105:** 9817–9822

Asamizu E, Nakamura Y, Sato S, Tabata S (2000) Generation of 7137 non-redundant expressed sequence tags from a legume, *Lotus japonicus*. DNA Res **7:** 127–130

Bell CD, Soltis ED, Soltis PS (2010) The age and diversification of the angiosperms re-revisited. Am J Bot **97:** 1296–1303

Benson DR, Silvester WB (1993) Biology of *Frankia* strains, actinomycete symbionts of actinorhizal plants. Microbiol Rev **57:** 293–319

Berg RH, McDowell L (1988) Cytochemistry of the wall of infected cells in Casuarina actinorhizae. Can J Bot **66:** 2038–2047

Berry AM, Harriott OT, Moreau RA, Osman SF, Benson DR, Jones AD (1993) Hopanoid lipids compose the *Frankia* vesicle envelope, presumptive barrier of oxygen diffusion to nitrogenase. Proc Natl Acad Sci USA **90:** 6091–6094

Capoen W, Den Herder J, Sun J, Verplancke C, De Keyser A, De Rycke R, Goormachtig S, Oldroyd G, Holsters M (2009) Calcium spiking patterns and the role of the calcium/calmodulin-dependent kinase CCaMK in lateral root base nodulation of *Sesbania rostrata*. Plant Cell **21:** 1526–1540

Ceremonie H (1998) Molecular and genetic interaction in *Frankia-Alnus* symbiosis. PhD thesis. Lyon 1 University, Lyon, France

Ceremonie H, Debelle F, Fernandez MP (1999) Structural and functional comparison of *Frankia* root hair deforming factor and rhizobia Nod factor. Can J Bot **77:** 1293–1301

Colebatch G, Desbrosses G, Ott T, Krusell L, Montanari O, Kloska S, Kopka J, Udvardi MK (2004) Global changes in transcription orchestrate metabolic differentiation during symbiotic nitrogen fixation in *Lotus japonicus*. Plant J **39:** 487–512

Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics **21:** 3674–3676

Doyle JJ (1998) Phylogenetic perspectives on nodulation: evolving views of plants and symbiotic bacteria. Trends Plant Sci **3:** 473–478

El Yahyaoui F, Küster H, Ben Amor B, Hohnjec N, Pühler A, Becker A, Gouzy J, Vernié T, Gough C, Niebel A, et al (2004) Expression profiling in *Medicago truncatula* identifies more than 750 genes differentially expressed during nodulation, including many potential regulators of the symbiotic program. Plant Physiol **136:** 3159–3176

Gherbi H, Duhoux E, Franche C, Pawlowski K, Nassar A, Berry AM, Bogusz D (1997) Cloning of a full-length symbiotic hemoglobin cDNA and *in situ* localization of the corresponding mRNA in *Casuarina glauca* root nodule. Physiol Plant **99:** 608–616

Gherbi H, Markmann K, Svistoonoff S, Estevan J, Autran D, Giczey G, Auguy F, Péret B, Laplaze L, Franche C, et al (2008) *SymRK* defines a common genetic basis for plant root endosymbioses with arbuscular mycorrhiza fungi, rhizobia, and *Frankia* bacteria. Proc Natl Acad Sci USA **105:** 4928–4932

Goetting-Minesky MP, Mullin BC (1994) Differential gene expression in an actinorhizal symbiosis: evidence for a nodule-specific cysteine proteinase. Proc Natl Acad Sci USA **91:** 9891–9895

Gottig N, Garavaglia BS, Daurelio LD, Valentine A, Gehring C, Orellano EG, Ottado J (2008) *Xanthomonas axonopodis* pv. *citri* uses a plant natriuretic peptide-like protein to modify host homeostasis. Proc Natl Acad Sci USA **105:** 18631–18636

Guan C, Akkermans ADL, van Kammen A, Bisseling T, Pawlowski K (1997) *ag13* is expressed in *Alnus glutinosa* nodules in infected cells during endosymbiont degradation and in the nodule pericycle. Physiol Plant **99:** 601–607

Guan C, Ribeiro A, Akkermans AD, Jing Y, van Kammen A, Bisseling T, Pawlowski K (1996) Nitrogen metabolism in actinorhizal nodules of *Alnus glutinosa*: expression of glutamine synthetase and acetylornithine transaminase. Plant Mol Biol **32:** 1177–1184

Hammad Y, Nalin R, Marechal J, Fiasson K, Pepin R, Berry AM, Normand P, Domenach A-M (2003) A possible role for phenylacetic acid (PAA) in *Alnus glutinosa* nodulation by *Frankia*. Plant Soil **254:** 193–205

Hocher V, Auguy F, Argout X, Laplaze L, Franche C, Bogusz D (2006) Expressed sequence-tag analysis in *Casuarina glauca* actinorhizal nodule and root. New Phytol **169:** 681–688

Jeong J, Suh S, Guan C, Tsay YF, Moran N, Oh CJ, An CS, Demchenko KN, Pawlowski K, Lee Y (2004) A nodule-specific dicarboxylate transporter from alder is a member of the peptide transporter family. Plant Physiol **134:** 969–978

Journet EP, van Tuinen D, Gouzy J, Crespeau H, Carreau V, Farmer MJ, Niebel A, Schiex T, Jaillon O, Chatagnier O, et al (2002) Exploring root symbiotic programs in the model legume *Medicago truncatula* using EST analysis. Nucleic Acids Res **30:** 5579–5592

Kistner C, Parniske M (2002) Evolution of signal transduction in intracellular symbiosis. Trends Plant Sci **7:** 511–518

Kucho K, Hay A, Normand P (2010) The determinants of the actinorhizal symbiosis. Microbes Environ **25:** 241–252

Laplaze L, Ribeiro A, Franche C, Duhoux E, Auguy F, Bogusz D, Pawlowski K (2000) Characterization of a *Casuarina glauca* nodule-specific subtilisin-like protease gene, a homolog of *Alnus glutinosa ag12*. Mol Plant Microbe Interact **13:** 113–117

Lavin M, Herendeen PS, Wojciechowski MF (2005) Evolutionary rates analysis of Leguminosae implicates a rapid diversification of lineages during the tertiary. Syst Biol **54:** 575–594

Lefebvre B, Timmers T, Mbengue M, Moreau S, Hervé C, Tóth K, Bittencourt-Silvestre J, Klaus D, Deslandes L, Godiard L, et al (2010) A remorin protein interacts with symbiotic receptors and regulates bacterial infection. Proc Natl Acad Sci USA **107:** 2343–2348

Lundberg P, Lundquist PO (2004) Primary metabolism in N2-fixing *Alnus incana-Frankia* symbiotic root nodules studied with 15N and 31P nuclear magnetic resonance spectroscopy. Planta **219:** 661–672

Madsen LH, Tirichine L, Jurkiewicz A, Sullivan JT, Heckmann AB, Bek AS, Ronson CW, James EK, Stougaard J (2010) The molecular network governing nodule organogenesis and infection in the model legume *Lotus japonicus*. Nat Commun **1:** 1–12

Maillet F, Poinsot V, André O, Puech-Pagès V, Haouy A, Gueunier M, Cromer L, Giraudet D, Formey D, Niebel A, et al (2011) Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. Nature **469:** 58–63

Markmann K, Giczey G, Parniske M (2008) Functional adaptation of a plant receptor-kinase paved the way for the evolution of intracellular root symbioses with bacteria. PLoS Biol **6:** e68

Markmann K, Parniske M (2009) Evolution of root endosymbiosis with bacteria: how novel are nodules? Trends Plant Sci **14:** 77–86

Mathis R, Grosjean C, de Billy F, Huguet T, Gamas P (1999) The early nodulin gene *MtN6* is a novel marker for events preceding infection of *Medicago truncatula* roots by *Sinorhizobium meliloti*. Mol Plant Microbe Interact **12:** 544–555

Maunoury N, Redondo-Nieto M, Bourcy M, Van de Velde W, Alunni B, Laporte P, Durand P, Agier N, Marisa L, Vaubert D, et al (2010) Differentiation of symbiotic cells and endosymbionts in *Medicago truncatula* nodulation are coupled to two transcriptome-switches. PLoS ONE **5:** e9519

Meier S, Bastian R, Donaldson L, Murray S, Bajic V, Gehring C (2008) Co-expression and promoter content analyses assign a role in biotic and abiotic stress responses to plant natriuretic peptides. BMC Plant Biol **8:** 24

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N (2007) *CERK1*, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. Proc Natl Acad Sci USA **104:** 19613–19618

Mort A, Normand P, Lalonde M (1983) 2-O-Methyl-D-mannose, a key sugar in the taxonomy of *Frankia*. Can J Microbiol **29:** 993–1002

Normand P, Chapelon C (1997) Direct characterization of *Frankia* and of close phyletic neighbors from an *Alnus viridis* rhizosphere. Physiol Plant **99:** 722–731

Normand P, Lalonde M (1982) Evaluation of *Frankia* strains isolated from provenances of two *Alnus* species. Can J Microbiol **28:** 1133–1142

Normand P, Lapierre P, Tisa LS, Gogarten JP, Alloisio N, Bagnarol E, Bassi CA, Berry AM, Bickhart DM, Choisne N, et al (2007a) Genome

characteristics of facultatively symbiotic *Frankia* sp. strains reflect host range and host plant biogeography. Genome Res **17:** 7–15

Normand P, Orso S, Cournoyer B, Jeannin P, Chapelon C, Dawson J, Evtushenko L, Misra AK (1996) Molecular phylogeny of the genus *Frankia* and related genera and emendation of the family Frankiaceae. Int J Syst Bacteriol **46:** 1–9

Normand P, Queiroux C, Tisa LS, Benson DR, Rouy Z, Cruveiller S, Médigue C (2007b) Exploring the genomes of *Frankia* sp. Physiol Plant **13:** 331–343

Oldroyd GE, Harrison MJ, Paszkowski U (2009) Reprogramming plant cells for endosymbiosis. Science **324:** 753–754

Pawlowski K (2008) Nodules and oxygen. Plant Biotechnol **25:** 291–298

Pawlowski K (2009) Induction of actinorhizal nodule by *Frankia*. *In* K Pawlowski, ed, Prokaryotic Symbionts in Plants. Microbiology Monographs. Springer, Heidelberg, pp 127–154

Pawlowski K, Bisseling T (1996) Rhizobial and actinorhizal symbioses: what are the shared features? Plant Cell **8:** 1899–1913

Pawlowski K, Swensen S, Guan C, Hadri AE, Berry AM, Bisseling T (2003) Distinct patterns of symbiosis-related gene expression in actinorhizal nodules from different plant families. Mol Plant Microbe Interact **16:** 796–807

Pawlowski K, Twigg P, Dobritsa S, Guan C, Mullin BC (1997) A nodule-specific gene family from *Alnus glutinosa* encodes glycine- and histidine-rich proteins expressed in the early stages of actinorhizal nodule development. Mol Plant Microbe Interact **10:** 656–664

Perrine-Walker F, Doumas P, Lucas M, Vaissayre V, Beauchemin NJ, Band LR, Chopard J, Crabos A, Conejero G, Péret B, et al (2010) Auxin carriers localization drives auxin accumulation in plant cells infected by *Frankia* in *Casuarina glauca* actinorhizal nodules. Plant Physiol **154:** 1372–1380

Perrine-Walker F, Gherbi H, Imanishi L, Hocher V, Ghodhbane-Gtari F, Lavenus J, Benabdoun FM, Nambiar-Veetil M, Svistoonoff S, Laplaze L (2011) Symbiotic signaling in actinorhizal symbioses. Curr Protein Pept Sci (in press)

Pucciariello C, Innocenti G, Van de Velde W, Lambert A, Hopkins J, Clément M, Ponchet M, Pauly N, Goormachtig S, Holsters M, et al (2009) (Homo)glutathione depletion modulates host gene expression during the symbiotic interaction between *Medicago truncatula* and *Sinorhizobium meliloti*. Plant Physiol **151:** 1186–1196

Ribeiro A, Akkermans ADL, van Kammen A, Bisseling T, Pawlowski K (1995) A nodule-specific gene encoding a subtilisin-like protease is expressed in early stages of actinorhizal nodule development. Plant Cell **7:** 785–794

Ribeiro A, Praekelt U, Akkermans AD, Meacock PA, van Kammen A, Bisseling T, Pawlowski K (1996) Identification of *agthi1*, whose product is involved in biosynthesis of the thiamine precursor thiazole, in actinorhizal nodules of *Alnus glutinosa*. Plant J **10:** 361–368

Schauser L, Roussis A, Stiller J, Stougaard J (1999) A plant regulator controlling development of symbiotic root nodules. Nature **402:** 191–195

Simon L, Bousquet J, Levesque RC, Lalonde M (1993) Origin and diversification of endomycorrhizal fungi and coincidence with vascular land plant. Nature **363:** 67–69

Soltis DE, Soltis PS, Morgan DR, Swensen SM, Mullin BC, Dowd JM, Martin PG (1995) Chloroplast gene sequence data suggest a single origin of the predisposition for symbiotic nitrogen fixation in angiosperms. Proc Natl Acad Sci USA **92:** 2647–2651

Svistoonoff S, Laplaze L, Auguy F, Runions J, Duponnois R, Haseloff J, Franche C, Bogusz D (2003) *cg12* expression is specifically linked to infection of root hairs and cortical cells during *Casuarina glauca* and *Allocasuarina verticillata* actinorhizal nodule development. Mol Plant Microbe Interact **16:** 600–607

Swensen SM (1996) The evolution of actinorhizal symbioses: evidence for multiple origins of the symbiotic association. Am J Bot **83:** 1503–1512

Thomas BA, Spicer RA (1987) The Evolution and Paleobiology of Land Plants. Croom Helm, London

Tjepkema JD (1978) The role of oxygen diffusion from the shoots and nodule roots in nitrogen fixation by root nodules of *Myrica gale*. Can J Bot **56:** 1365–1371

Tjepkema JD (1979) Oxygen relations in leguminous and actinorhizal nodules. *In* JC Gordon, ed, Symbiotic Nitrogen Fixation in the Management of Temperate Forests. Oregon State University Press, Corvallis, OR, pp 175–186

Torrey JG (1976) Initiation and development of root nodules of *Casuarina* (Casuarinaceae). Am J Bot **63:** 335–344

van Ghelue M, Ribeiro A, Solheim B, Akkermans AD, Bisseling T, Pawlowski K (1996) Sucrose synthase and enolase expression in actinorhizal nodules of Alnus glutinosa: comparison with legume nodules. Mol Gen Genet **250:** 437–446