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

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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).

Řhizobial 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

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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 \leq 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.

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.

	itinosa	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	NÁ		
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_l06	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 3 β -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_103	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		

^aSame sequence as for reported actinorhizal nodulin GHRP (Table IV). ^bSame sequence as for *A. glutinosa* γ-expansin natriuretic peptide cDNA (GU062393). ^cEST 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.

		inosa	C. glauca			
Clone Name Fold Change NOD/NIR		Product Description	Clone Name Fold Char NOD/NI		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_010	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_001	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 debydrogenase-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
	AGCI 103Contig1	0.04	Suspensor-specific protein	CG-R02f 001 N20	0.00	Pollen-specific kinase partner protein
	AG-107f 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

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,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 micro-array (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/CER-BERUS, 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

	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	CG-N02f_009_P18	6,703.78	NA	
			peptideª				
	AG-N01f_036_K05	8,311.45	NA	CGCL126Contig1	5,735.54	Aquaporin, MIP family,	
						PIP subtamily ^u	
	gi_757521_emb_X85975.1	7,823.16	Ag12, subtilisin-like	CGCL488Contig1	5,627.12	τ -Glutathione S-transferase	
		6 071 43	protease	CC N02(007 N11	F (17 (2		
	AGCL2186Contig1	6,0/1.43	Dicarboxylate transporter	CG-N02f_00/_NTT	5,617.62	Aquaporin, MIP family,	
	ACCI 24Contig1	E 901 26	(AgDCTAT)	CC NO2F 012 422	E E1E 40	FIF Subiamity	
	AGCL24CONUST	5,091.30		CCCL1727Contig1	5,515.40	Subiliase Cytochromo P450	
	AG-N011_015_L15	4 584 00		CC NO2f 027 M02	4 592 71	Cytochrome P450	
	AC N01f 011 E15	4,304.00	NA Ag12 subtilisin like	CC NO2f 021 107	4,302.71	Protoin kinaso family	
	AG-NOT_0TT_TTS	4,550.75	nrotease ^b	CG-N021_031_107	4,320.42	protoin	
	ACCI 240Contig1	3 761 25	NA	CC-N02f 028 D21	4 276 36	Male sterility 2-like	
	Adde240contign	nugi 3,701.23 10/1		CG-11021_020_D21	protein		
	AGCI 10Contig1	3 537 90	NA	CGCL 3025Contig1	4 217 26	NA	
	AGCI 806Contig1	3,390,73	NA	CGCL 58Contig1	4.083.10	NA	
	AG-N01f 027 M10	2.971.80	Dicarboxylate transporter	CGCL672Contig1	3,984,77	NA	
		2,57 1100	(AgDCTA1) ^c	20010, 20011.lg.	5,50 117		
	AGCL581Contig1	2.941.04	NA	CG-N02f_013_C02 3,898.22		Dicarboxylate transporter	
	8	2/311101 101			-,	(AgDCTA1)	
	AG-N01f_032_L02	AG-N01f 032 L02 2.678.19 Plastocyanin-lik		CGCL491Contig1	3,762.19	Integral membrane family	
		domain-containing	Ū.		protein		
protein		protein			·		
	AGCL716Contig1	2,592.40	Integral membrane	CGCL484Contig1	3,652.93	Receptor protein kinase	
			protein			related	
	AGCL507Contig1	2,470.97	γ-Expansin natriuretic	CG-N02f_029_C14	3,203.59	NA	
			peptide ^a				
	AG-N01f_043_P03	2,354.12	pdr3_tobac, pleiotropic	CG-N01f_010_G05	3,053.58	NA	
			drug resistance				
			protein 3				
	AG-N01f_026_P02	2,109.03	Ag12, subtilisin-like	CGCL125Contig1	3,008.36	NA	
			protease				

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.

^aEST 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 <i>A. glutinosa</i>	Clone Name	E Value	Fold Change NOD/NIR	Student's t	
Dicarboxylate transporter	AJ488290.1	12,186.0 ^a	0.010	Specific in nodules (6–8 weeks old;	CG-N02f_009_D12	3.00E-79	850.9 ^a	0.004	
(<i>AgDcat1</i>) Subtilisin-like protease (<i>Ag12</i>)	X85975.1	7,823.2 ^a	0.006	Jeong et al., 2004) Specific in nodules (5–13 weeks old; Bibairo 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	-	_	-	_	
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)	-	_	-	_	

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 wellcharacterized 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 upregulation 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 ¹³N 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 symbiosisspecific 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 Nano-Drop 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 μ g 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 μ g of total RNA.

For both species, about 2 μ g 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'-CCGCTGACCTACTCA-3') and reverse (5'-GGGATCCATAGAT-CCAACGCTCAATCCATCA-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 a sabsent) 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.

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