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Differential Effects of Rare Specific Flavonoids on Compatible and Incompatible Strains in the *Myrica gale-Frankia* Actinorhizal Symbiosis▽†

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Plant secondary metabolites, and specifically phenolics, play important roles when plants interact with their environment and can act as weapons or positive signals during biotic interactions. One such interaction, the establishment of mutualistic nitrogen-fixing symbioses, typically involves phenolic-based recognition mechanisms between host plants and bacterial symbionts during the early stages of interaction. While these mechanisms are well studied in the rhizobia-legume symbiosis, little is known about the role of plant phenolics in the symbiosis between actinorhizal plants and *Frankia* genus strains. In this study, the responsiveness of *Frankia* strains to plant phenolics was correlated with their symbiotic compatibility. We used *Myrica gale*, a host species with narrow symbiont specificity, and a set of compatible and noncompatible *Frankia* strains. *M. gale* fruit exudate phenolics were extracted, and 8 dominant molecules were purified and identified as flavonoids by high-resolution spectroscopic techniques. Total fruit exudates, along with two purified dihydrochalcone molecules, induced modifications of bacterial growth and nitrogen fixation according to the symbiotic specificity of strains, enhancing compatible strains and inhibiting incompatible ones. Candidate genes involved in these effects were identified by a global transcriptomic approach using ACN14a strain whole-genome microarrays. Fruit exudates induced differential expression of 22 genes involved mostly in oxidative stress response and drug resistance, along with the overexpression of a *whiB* transcriptional regulator. This work provides evidence for the involvement of plant secondary metabolites in determining symbiotic specificity and expands our understanding of the mechanisms, leading to the establishment of actinorhizal symbioses.

Over the course of evolution, plants have developed many strategies to adapt to their environment and manage their biotic interactions. The production of secondary metabolites is an important tool in many of these strategies. In particular, secondary metabolites are involved in plant-microbe interactions both as weapons in plant defense mechanisms and as early signals in mutualistic as well as pathogenic relationships (22). For example, sesquiterpenes and flavonoids are involved in the early steps of arbuscular mycorrhizal symbiosis (1, 62). Similarly, in the early stages of the rhizobia-legume symbiosis, recognition of host flavonoids enhances rhizospheric competitiveness and root colonization and is the basic mechanism through which rhizobia induce the transcription of *nod* genes, leading to root hair deformation and nodulation in the plant tissues (13, 49, 55).

Actinorhizal symbiosis is a less well-known nitrogen-fixing interaction, resulting from the association between the actinobacterium *Frankia* and plants belonging to eight dicotyledonous families collectively called "actinorhizal": Betulaceae, Myricaceae, Rosaceae, Datiscaceae, Elaeagnaceae, Coriariaceae, Casuarinaceae, and Rhamnaceae (8). Due to the nitrogen-fixing ability of their symbionts, actinorhizal plants, together with legumes, are a major source of bioavailable reduced nitrogen in ecosystems. The woody perennial characteristics of actinorhizal plants, the isolation of the symbiont in pure culture only in 1978, the lack of genetic tools to study *Frankia*, and the very low growth rate of the bacterial isolates have restricted progress in understanding the mechanisms involved in this symbiosis. Recently, however, the genome sequences of several *Frankia* strains have been released and show that there are no canonical *nod* genes in *Frankia* (45), suggesting a novel interaction mechanism may be acting in actinorhizal symbioses.

Despite this difference, legumes and actinorhizal plants belong to the same rosid I phylogenetic group (61), and their symbioses share several central features at the plant or the microbial level. For example, *Frankia*, like rhizobia, produces an extracellular root hair-deforming factor, and in both cases, plant secondary metabolites likely play a role in host recognition. Previous studies of actinorhizal symbioses have shown that nodulation in *Alnus* may be inhibited or enhanced by different seed flavonoids (7), and cell-specific flavan biosynthesis and accumulation occur in *Casuarina glauca* nodules (36). At the genetic level, expression of *pal* (phenylalanine ammonia lyase) and *chs* (chalcone synthase) genes, involved in flavonoid biosynthesis, is enhanced in *Alnus glutinosa* root systems after inoculation with a *Frankia* strain (24). These studies strongly suggest the involvement of plant phenolics in the first steps of the actinorhizal symbiosis.

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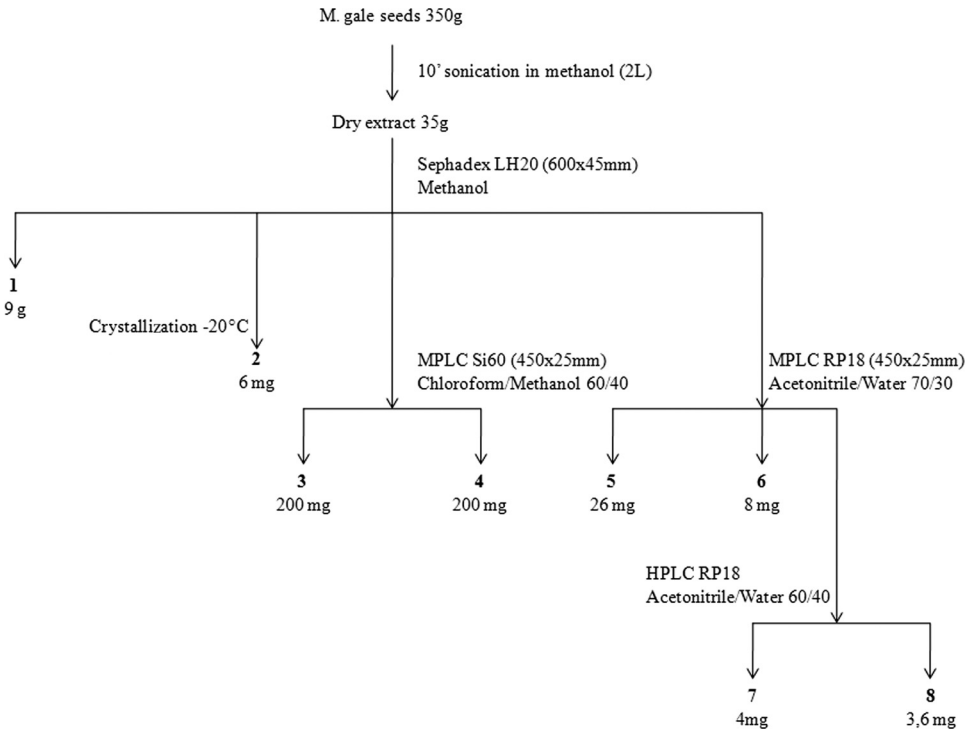

FIG. 1. Purification strategy of *Myrica gale* fruit exudate compounds. Identified compounds are numbered from 1 to 8. 1, myrigalone A; 2, myrigalone E; 3, myrigalone B; 4, uvangoletin; 5, myrigalone P; 6, demethoxymatteucinol; 7, myrigalone D; 8, demethoxymatteucinol-7-methyl ether. MPLC Si60, medium-pressure liquid chromatography on Silica Gel 60; HPLC RP18, high-performance liquid chromatography on reverse-phase C_{18}; MPLC RP18, MPLC on reverse-phase C_{18} column.

Host specificity among *Frankia* strains has led to the description of the following three major host specificity groups (HSG) that correlate with strain 16S rRNA phylogeny: *Alnus-Comptonia*-infective strains (cluster 1a), Casuarinaceae-infective strains (cluster 1b), and Elaeagnaceae-infective strains (cluster 3) (44). Among actinorhizal plants, the family Myricaceae is a good experimental model to study the specificity of symbiotic interactions, as it includes species with either narrow or broad symbiont specificity. Most Myricaceae species are promiscuous (nonselective for *Frankia* strains), but *Myrica gale* exhibits a narrow symbiont specificity, being nodulated only by cluster 1a strains (5, 27). In addition, *M. gale* has the novel trait of exuding droplets of secondary metabolites at the fruit surface, and the phenolic contents of these metabolites are significantly different from those of other broad symbiont specificity Myricaceae species (our unpublished data).

In this study, we determined whether the responses of different *Frankia* strains to fruit exudates were correlated with symbiotic specificity. We hypothesize that the main parameters involved in symbiotic efficiency are (i) strain growth and competitiveness in the rhizosphere and (ii) nitrogen fixation activity. We thus purified and identified major *M. gale* fruit exudate constituents and evaluated their effects on growth and nitrogen fixation of compatible (cluster 1a) and noncompatible (cluster 1b and 3) *Frankia* strains. In addition, global transcriptional effects of total fruit exudates were assessed using *Frankia* whole-genome microarrays in order to identify the genes possibly involved in the molecular mechanisms of the *Frankia-M. gale* symbiosis.

MATERIALS AND METHODS

Plant material. *M. gale* fruits were collected in December 2004 on the shore of Biscarosse Lake, Bordeaux, France. A voucher specimen was deposited at the herbarium of the Université Claude Bernard Lyon 1, Villeurbanne, France, under the name "Collection Piola" and collector number 3. Air-dried fruits (350 g) were sonicated (40 kHz; Branson 2510) twice in 2 liters of methanol and evaporated, producing 35 g of dried residue. This extract was then diluted in methanol for the further steps of the analyses.

Chromatographic analysis, purification, and structural elucidation of flavonoids. Chromatographic analysis of the extracts was performed on an Agilent 1200 series high-performance liquid chromatograph (HPLC) equipped with a degasser (G132A), a quaternary pump module (G1311A), an automatic sampler (G1329A), and a diode array detector (DAD) (G1315B). Separations were carried out using a Kromasil reverse-phase C_{18} (RP18) column (250 by 4.6 mm, 5 μm, 100 Å), with a linear gradient of acetonitrile in water from 0% to 100% in 60 min supplemented with formic acid (0.4%). Chromatograms were recorded between 200 and 700 nm, and a specific channel settled at 280 nm was used to monitor chromatographic traces.

Gel filtration on Sephadex LH-20 and repeated chromatographic steps (medium-pressure liquid chromatography [MPLC] and HPLC) on RP18 and on silica of the fruit exudate extract led to the purification of 8 compounds (Fig. 1). The structural elucidation of the molecules was achieved by UV spectroscopy (Agilent 8453 UV spectrophotometer) recorded between 220 and 500 nm, HPLC-mass spectrometry (HPLC-MS), and by ^{1}H and ^{13}C mono- and bidimensional nuclear magnetic resonance (NMR) (Bruker DRX 500). For HPLC-MS analysis, the HPLC system described above was interfaced with an HP MSD (mass-selective detector) 1100 series, allowing use of the same chromatographic conditions as those used for HPLC-DAD analysis. Mass spectrometry with an atmospheric pressure chemical ionization (APCI) interface had the following operating conditions: gas temperature of 330°C at a flow rate of 9.0 liters min^{-1}, nebulizer pressure of 50 lb/in^2, quadripole temperature of 30°C, capillary voltage of 4,000 V, and fragmentor voltage of 100 V. Full-scan spectra from *m/z* 100 to 900 in both positive and negative ion modes were recorded.

TABLE 1. *Frankia* strains tested

Strain	Host plant	Cluster[a]	Growth response to *M. gale* fruit exudates[b]	Reference
Cj1-82	*Casuarina junghuniana*	1b	−	16
G2	*Casuarina equisetifolia*	1b	−	20
$Cg70_9$	*Casuarina glauca*	1b	−	30
$Cg70_4$	*Casuarina glauca*	1b	−	30
CcI3	*Casuarina cunninghamiana*	1b	0	65
ORS020608	*Casuarina equisetifolia*	1b	0	17
$Ea1_{12}$	*Elaeagnus angustifolia*	3	−	18
CH37	*Hippophae rhamnoides*	3	−	52
ACoN24d	*Alnus cordata*	1a	+	59
M16317	*Morella pensylvanica*	1a	+	60
M16467	*Morella pensylvanica*	1a	+	60
ACN14a	*Alnus viridis* subsp. *crispa*	1a	+	43
ArI3	*Alnus rubra*	1a	+	9
Ar24O2	*Alnus rubra*	1a	+	60

[a] Phylogenetic clusters (44).
[b] −, growth inhibited by fruit exudates; +, growth enhanced; 0, growth unaffected.

***Frankia* strains and growth measurement.** *Frankia* strains used in this study belong to the three major HSG (Table 1). Five-day-old cultures (end of exponential growth phase) were used as inocula. Nitrogen-free basal medium with propinate (BAP^- medium) (42) was inoculated with a normalized number of cell equivalent to 75 mg $liter^{-1}$ of bacterial lyophilized biomass. Homogenization of inocula was done by repeated forced passages through a 0.6-mm-diameter syringe. Total fruit exudate extract or purified compounds from *M. gale* were added to 20 ml BAP^- medium simultaneously with *Frankia* inoculation. Phenolic mixture or purified molecules were dissolved in 100 μl of methanol to final concentrations ranging from 10^{-4} to 10 mg $liter^{-1}$. The same volume of methanol was added to the controls and was shown to have no effect on *Frankia* growth and nitrogen fixation.

After 5 days, *Frankia* cells were sedimented by centrifugation at 5,000 × *g* for 15 min and washed three times with sterilized ultrapure water, and pellets were lyophilized. The effects of plant phenolic extracts on bacterial growth were tested by comparing the average weights of lyophilized *Frankia* cells for triplicates of each assay. Significant differences in means were assessed by Student's *t* test ($P < 0.05$). After bacterial growth in the presence or absence of the plant extracts, the resulting phenolic compounds present in the supernatants were extracted by solid-phase extraction (SPE; RP18) and eluted with methanol. Analyses of subsequent extracts were performed by HPLC in the same chromatographic system as that of the plant extracts described previously.

Nitrogen-fixing activity. Nitrogen-fixing activity was assessed by measuring the reduction of acetylene to ethylene (acetylene reduction assay [ARA]). *Frankia* strains were grown in 50 ml of BAP^- medium for 1, 3, and 5 days in the presence of 1 mg $liter^{-1}$ of *M. gale* fruit exudates. A total of 10 ml of acetylene corresponding to 10% in the atmosphere of the incubation flask was injected 24 h prior to the chromatographic analysis. Ethylene production was measured by injecting 0.4 ml of the culture atmosphere into a gas chromatograph equipped with a flame ionization detector (FID; Girdel). The injector temperature was 50°C, the oven temperature was maintained at 40°C, the vector gas used was nitrogen (2.5 bar), and the FID, functioning with pressurized air (1 bar) and hydrogen (2.3 bar), was heated to 150°C. The effect of *M. gale* fruit exudates on nitrogen-fixing activity was evaluated by comparing the average quantities of ethylene produced for triplicates of each assay. Quantifications were done by utilization of defined volumes of ethylene (Air Liquide). The amounts of ethylene produced for each assay were then normalized by the dry weight of the lyophilized bacterial cells. Significant differences in means were assessed by Student's *t* test ($P < 0.05$).

***Frankia* ACN14a gene expression. (i) Preparation of total RNA.** Assay and control cultures were performed in 250 ml of BAP^- medium inoculated with 0.4 g of cell pellets from a BAP^- medium mother culture. Assay cultures were grown in the presence of *M. gale* fruit exudates (1 mg $liter^{-1}$ in 0.5% methanol). Two controls were made, a methanol control (BAP^- medium with 0.5% methanol) and a BAP^- control (with neither exudates nor methanol) in order to assess the effect of methanol itself on ACN14a transcriptome (see the supplemental material). Three biological replicates were performed for assay cultures and BAP^- controls, and two biological replicates were performed for methanol controls. After 5 days of culture, stirring was stopped in order to gently sediment cells during the night. Supernatants were carefully discarded, and cells were treated with RNAprotect bacterial reagent (Qiagen, Courtaboeuf, France) for stabilization of RNA. To purify RNA, stabilized cells were processed by using the RNeasy plant minikit (Qiagen) and on-column DNase digestion with the RNase-free DNase set (Qiagen). In order to avoid contamination with any remaining DNA, a second DNase treatment was performed with RQ1 RNase-free DNase (Promega, Charbonnières-les-Bains, France), followed by RNA cleanup using the RNeasy minikit. Purity, concentration, and quality of RNA samples were checked using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Courtaboeuf, France) and agarose gel electrophoresis. A 0.24- to 9.5-kb RNA ladder (Invitrogen, Illkirch, France) was used as a standard.

(ii) Whole-genome expression microarray analysis. Based on the sequence and annotation data for the *Frankia alni* ACN14a genome (45), a whole-genome high-density microarray was designed and manufactured by Roche NimbleGen, Inc. (Madison, WI). Due to the high GC content (72.8%) of the *F. alni* genome, a 60-mer oligo length was chosen for probe design. NimbleGen design rules and analysis algorithms found probes for 6,607 out of 6,786 genes. For 6,340 genes, 18 probes per gene were obtained, and for 267 genes, 1 to 17 probes per gene were obtained. Probes were duplicated on the array. RNA quality analysis using the Agilent 2100 Bioanalyzer, double-stranded cDNA synthesis, and labeling by random priming with Cy3 dye-labeled 9-mers, hybridization at 42°C, array scanning, and data extraction were performed by the NimbleGen expression service lab (Reykjavik, Iceland), according to its standard protocol. Array normalization was performed using the quantile normalization method (10). Normalized gene expression values were generated using the robust multichip average (RMA) algorithm (28). Data were further analyzed using the GeneSpring gene expression analysis software version 7.3 (Agilent Technologies, Massy, France). Genes were considered differentially expressed between the two biological conditions if the increase/decrease was >80% in all pairwise comparisons yielded by biological replicates. Hence, six pairwise comparisons were generated when comparing cells in Bap^- medium with methanol (2 replicates) with exudates (3 replicates).

RESULTS

Characterization of the major compounds of *M. gale* fruit exudates. Ultrasound-assisted washing of 350 g of *M. gale* fruits in methanol led to 35 g of dry extract. HPLC-DAD-MS chromatograms indicated a majority of flavonoids along with terpenes among detectable compounds. After fractionation of the extract, eight compounds were purified, and their absolute structures were obtained by NMR analysis and by comparing NMR experimental data with the literature for the known molecules (Fig. 2). All structures are also in accordance with UV and MS spectra (data not shown). Seven of these compounds have previously been isolated from various plants and described in the literature, and one is a new natural product.

The known compounds were identified as myrigalone A (MyA) (37), myrigalone E (MyE) (3), myrigalone B (MyB) (37), uvangoletin (26), demethoxymatteucinol (64), myrigalone D (MyD) (37), and demethoxymatteucinol-7-methyl ether (31), on the basis of a comparison of their ^1H and ^{13}C NMR spectroscopic data with those reported previously.

The fifth compound was obtained as a white powder. Its APCI-negative and positive MS spectra gave a molecular weight of 286. Its ^1H and ^{13}C NMR spectroscopic data are given in Table 2. All assignments are based on bidimensional heteronuclear multiple-quantum coherence (HMQC) and heteronuclear multiple-bond coherence (HMBC) experiments. This compound is described here for the first time as a natural product, and the name myrigalone P (MyP) was assigned to this molecule. Three major compounds represented 80% of the total extract, MyA, MyB, and uvangoletin, with 40%, 20%, and 20%, respectively. The others are minor molecules (<1% to 3%).

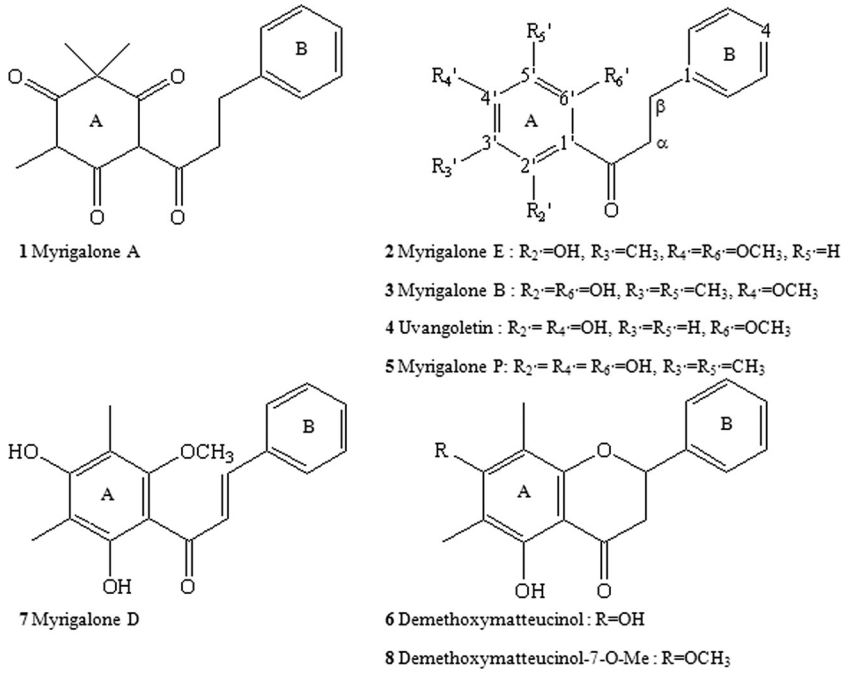

FIG. 2. Structures of compounds purified from *M. gale* fruit exudates. Compounds 1 to 5, dihydrochalcones; compound 7, chalcone; compounds 6 and 8, flavanones.

Effect of total fruit exudates on *Frankia* growth. A total of 14 *Frankia* strains belonging to the three major HSG were tested for their responsiveness to *M. gale* fruit exudates. All but two strains (assigned to group C) were significantly affected, six strains (assigned to group A) showed inhibited growth, and six other strains (assigned to group B) showed enhanced growth compared with that of controls (Fig. 3).

All the affected strains (inhibited or enhanced) respond at 1 mg liter^{-1} total exudates. In order to determine the minimal active concentration of total fruit exudates, further experiments using concentrations from 0.1 μg liter^{-1} to 1 mg liter^{-1} were conducted on the following four strains, representative of the two affected groups: $Ea1_{12}$ and $Cg70_4$ (inhibited strains) and ACN14a and M16467 (enhanced strains) (Table 3). The lowest concentration at which growth of $Ea1_{12}$ and $Cg70_4$ was inhibited was 100 μg liter^{-1}, whereas the lowest concentration at which growth of ACN14a and M16467 was enhanced was 1 μg liter^{-1}.

In order to determine whether these effects were due to

TABLE 2. Myrigalone P ^{1}H and ^{13}C NMR spectroscopic data[a]

Position	δH	δC	Correlation with ^{1}H-^{13}C HMBC
1		142.3	
2	ca. 7.24 m	128.4	128.5; 31.5; 142.3
3	ca. 7.24 m	128.5	31.5; 125.8; 128.4; 142.3
4	ca. 7.24 m	125.8	128.5; 128.4; 31.5; 142.3
5	ca. 7.24 m	128.5	31.5; 125.8; 128.4; 142.3
6	ca. 7.24 m	128.4	128.4; 125.8; 31.5; 142.3
α	3.39 t (7.9)	46.3	31.5; 142.3; 205.9
β	2.96 t (8.2)	31.5	46.3; 128.4; 128.5; 142.3; 205.9
CO		205.9	
1′		105.4	
2′		159.1	
3′		102.8	
4′		160.8	
5′		102.8	
6′		159.1	
R3′ R5′	2.03 s	7.3	102.8; 105.4; 159.1; 160.8

[a] Recorded in methanol-d_4 at 500 and 125 MHz for ^{1}H and ^{13}C NMR, respectively. *J* values in Hz are in parentheses. C positions are consistent with the numbering shown in Fig. 2.

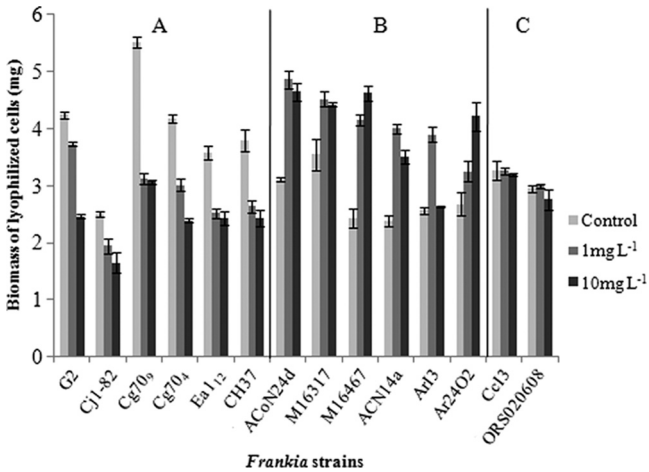

FIG. 3. Biomass of *Frankia* strains in the presence of *M. gale* fruit exudates. (A) Growth inhibition; (B) growth enhancement; (C) growth unaffected. Methanolic exudate solutions were added at 1 and 10 mg liter^{-1} to *Frankia* cultures, and exudate-free controls contained only methanol. Each value is the average result from three independent assays. The biomass of *Frankia* strains was measured after 5 days of culture.

TABLE 3. Biomass of *Frankia* strains grown in the presence of *M. gale* fruit exudates

Strain	Biomass at the indicated fruit exudate concns[a]					
	0	0.1 μg/liter	1 μg/liter	10 μg/liter	0.1 mg/liter	1 mg/liter
Inhibited strains						
$Cg70_4$	3.18	3.18	3.20	3.21	2.46A	2.23A
$Ea1_{12}$	3.14	3.17	3.09	3.12	2.48A	2.27A
Enhanced strains						
M16467	2.96	2.88	3.26B	3.36B	3.50B	3.54B
ACN14a	2.82	2.80	3.21B	3.32B	3.44B	3.52B

[a] Biomass shown in mg of lyophilized cells. Values followed by A and B are significantly lower and higher, respectively, than the values of fruit exudate-free controls at P values of <0.05.

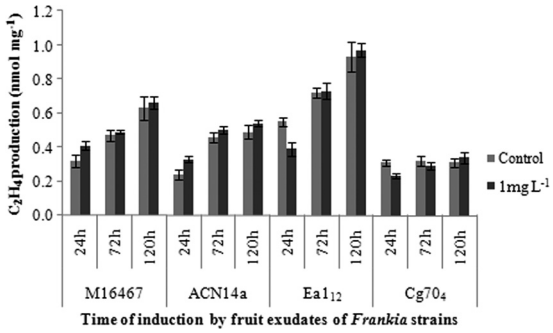

FIG. 4. Effect of *M. gale* fruit exudates on nitrogen fixation activity of four *Frankia* strains. Nitrogen fixation activity was assessed by the acetylene reduction assay (10% volume). Ethylene production in nmol of ethylene per mg of lyophilized cell produced in 24 h after 24, 72, and 120 h of induction by fruit exudates. Methanolic exudate solutions were added at 1 mg liter^{-1} to *Frankia* cultures, and exudate-free controls contained only methanol. Each value is the average result from three independent assays.

trophic utilization of fruit exudates or response to a signal produced by exudates, phenolic compounds in the culture supernatants of the four strains were analyzed after bacterial growth. The HPLC chromatograms showed no quantitative or qualitative differences between *Frankia* culture supernatants and controls (culture media plus fruit exudates without bacterium) (data not shown), indicating that no metabolization, chemical transformation, or degradation of exudates via exoenzymes occurred. Besides, measures performed on fruit exudates showed very small amounts of total nitrogen, indicating that those exudates are not a significant source of nitrogen for *Frankia* (our unpublished data). These results suggest signal processes and not trophic functions of fruit exudates on growth enhancement of compatible strains.

Effect of purified compounds on *Frankia* growth. In order to identify compounds responsible for the activity of total fruit exudates, the three major molecules and the new natural product (MyA, MyB, MyP, and uvangoletin) were evaluated for their effect on the growth of four *Frankia* strains, $Ea1_{12}$, $Cg70_4$, ACN14a, and M16467 (Table 4).

Whereas MyA and uvangoletin had no significant effect on *Frankia* growth, MyB and MyP affected *Frankia* growth similarly to total exudate extract: inhibition of $Ea1_{12}$ and $Cg70_4$ and enhancement of ACN14a and M16467. In order to determine the lowest active concentration, those two molecules were also tested at 1, 10, and 100 μg liter^{-1}. None were active below 1 mg liter^{-1}.

Effect of *M. gale* fruit exudates on nitrogen fixation activity. Fruit exudates at 1 mg liter^{-1} were tested for their effect on the nitrogen fixation activity of the four representative *Frankia* strains by ARA. At 24 h after inoculation, ARA of incompatible strains ($Ea1_{12}$ and $Cg70_4$) was significantly inhibited, whereas ARA of compatible strains (ACN14a and M16467) was significantly enhanced (Fig. 4). However, whichever the strain, these differences were no longer significant at 3 and 5 days after induction by plant extracts.

***Frankia* ACN14a gene expression.** *M. gale* exudates at 1 mg liter^{-1} induced differential modification of the growth and nitrogen fixation of *Frankia*, depending on the compatibility statuses of strains. We have previously shown (4) that this concentration also induced substantial modifications of bacterial protein expression. To investigate the effects of these exudates at the transcriptional level, we performed whole-genome microarrays on *Frankia* ACN14a cells growing in the presence of the extract of fruit exudates. Fruit exudates induced differential expression of 22 genes compared to that induced by controls (Table 5). Most were downregulated. Eight genes had an annotated function: a cytochrome *c* oxidase (FRAAL6078), a putative membrane-bound *O*-acyltransferase (FRAAL6221), a putative lyase (FRAAL6071), and a putative

TABLE 4. Biomass of *Frankia* strains grown in the presence of purified compounds from *M. gale* fruit exudates

Strain	Control biomass[a]	Biomass[a] with indicated concn (mg/liter) of purified compound							
		MyA		Uvangoletin		MyB		MyP	
		1	10	1	10	1	10	1	10
Inhibited strains									
$Cg70_4$	2.83	2.73	2.77	2.86	2.82	2.73	2.51A	2.41A	2.26A
$Ea1_{12}$	2.74	2.76	2.68	2.81	2.74	2.54A	2.49A	2.35A	2.22A
Enhanced strains									
M16467	2.76	2.83	2.73	2.82	2.77	3.32B	3.38B	3.48B	3.47B
ACN14a	2.53	2.64	2.57	2.67	2.51	3.29B	3.30B	3.48B	3.45B

[a] Biomass shown in mg of lyophilized cells. Values followed by A and B are significantly lower and higher, respectively, than the values of fruit exudate-free controls at P values of <0.05.

TABLE 5. Differentially expressed *Frankia* ACN14a genes in the presence of *M. gale* fruit exudate[a]

Gene	Fold change	Gene annotation
FRAAL2935	0.2	Hypothetical protein, putative signal peptide
FRAAL2757	0.2	Hypothetical protein
FRAAL2483	0.3	Hypothetical protein
FRAAL6078	0.3	CytB cytochrome *c* oxidase
FRAAL1757	0.3	Hypothetical protein
FRAAL1800	0.3	Hypothetical protein
FRAAL6221	0.3	Putative membrane-bound *O*-acyltransferase
FRAAL2936	0.3	Hypothetical protein, putative signal peptide
FRAAL5444	0.3	Hypothetical protein
FRAAL3032	0.4	Conserved hypothetical protein
FRAAL2135	0.4	Hypothetical protein, putative signal peptide
FRAAL0467	0.4	Hypothetical protein
FRAAL2669	0.4	Hypothetical protein, putative membrane protein
FRAAL0519	0.4	Hypothetical protein
FRAAL6071	0.5	Putative lyase
FRAAL4284	0.5	Putative multicopper oxidase, putative signal peptide
FRAAL1540	1.9	Conserved hypothetical protein
FRAAL5833	2.3	Hypothetical protein, putative signal peptide
FRAAL5116	2.1	Rubrerythrin
FRAAL5117	2.9	Putative peroxide operon regulator (Fur family)
FRAAL2286	3.4	Putative WhiB family transcriptional regulator
FRAAL1973	19.0	Putative multidrug export protein

[a] Transcription levels assessed by whole-genome microarrays (NimbleGen). Gene expression of *Frankia* cells grown in the presence of 1 mg liter^{-1} of methanol solution of *M. gale* fruit exudates (3 biological replicates) compared to that of control cells grown in the presence of methanol (2 biological replicates). Genes were considered differentially expressed between the two biological conditions if the fold change was >1.8 or <0.55 in all pairwise comparisons yielded by biological replicates.

multicopper oxidase-encoding gene (FRAAL4284) were downregulated, whereas genes coding for rubrerythrin (FRAAL5116), a putative Fur family peroxide operon regulator (FRAAL5117), a putative WhiB family transcriptional regulator (FRAAL2286), and a putative multidrug export protein (FRAAL1973) were upregulated.

DISCUSSION

Phytochemical characterization of *M. gale* fruit exudates. In this work, fruit exudates were studied for their involvement in the *Frankia-M. gale* symbiosis. We chose fruit extracts instead of root exudates because of the difficulty of obtaining sufficient quantity and reproducibility with root exudates. Moreover, the flavonoids that are active in *Rhizobium nod* gene induction have been identified from seeds and seed coats of alfalfa (25), and flavonoid-like compounds from *Alnus rubra* seeds have been shown to influence nodulation of that host by *Frankia* (7). Finally, those fruit exudates are the first compounds released in the soil when the germination of the seeds occurs and thus might be involved in the first steps of the interactions between the root plantlets and the rhizospheric environment, in particular with *Frankia* strains, as *M. gale* is rapidly nodulated by its symbiont after germination.

The quantity and chemical diversity of *M. gale* fruit exudates were such as to represent 10% of fruit dry weight and more than 30 different HPLC signals. These results imply a strong investment of plant photosynthates into exudate production and suggest that fruit exudates may impact deeply the rhizospheric environment. However, in spite of the great quantity of secondary metabolites present on the fruits, the degree to which they permeate the soil remains unclear, as it is affected by many parameters, such as the water solubility of each molecule and the nature of the soil matrix. Phenolic bioavailability in soil is poorly documented, and more data are needed concerning spatial and temporal distribution of flavonoids in soils and their effects on rhizospheric microorganisms (57, 58).

The compounds identified in this study are flavonoids. Among the 8 identified, 5 are dihydrochalcones (Fig. 2). This class of flavonoids is quite rare in the plant kingdom. Interestingly, dihydrochalcones have previously been identified in a few Fabaceae species, but no *nod*-inducing activity has been reported for those compounds, whereas several chalcones are known to be *nod* inducers (2, 41, 50, 54).

Among the compounds described here, four were previously identified in other Myricaceae species as follows: demethoxymatteucinol in *Morella pensylvanica*, a species with broad symbiont specificity, MyB, MyE, and MyD in *Morella serrata*, and MyB in *Comptonia peregrina* (both hosts with unknown symbiotic specificity) (19, 64). The other compounds (MyA, MyP, uvangoletin, and demethoxymatteucinol-7-methyl ether) have been described only in *M. gale* among actinorhizal plants. The presence of these four metabolites in the global phenolic content of *M. gale* (a narrow-specificity species) and their absence in *Morella pensylvanica* and *Morella cerifera* (two broad-specificity Myricaceae spp.) suggest that these compounds are good candidates for an involvement in the restricted symbiont range of *M. gale*.

Several biological activities have been reported for those dihydrochalcones, and among them, antioxidant and radical scavenging properties were described (38, 40). The highest radical scavenger, MyB, and MyA have also been reported to perturb the oxidative electron transfers in the respiratory chain of rat mitochondria and to inhibit ATP synthesis, indicating a high effect of those flavonoids on the membrane redox environment (39).

The effect of *M. gale* fruit exudates on *Frankia* growth and nitrogen fixation is related to strain symbiotic compatibility. The symbiotic specificity of *M. gale* has been previously studied using cross-inoculation experiments as well as in field studies (27), and only cluster 1a strains have ever been reported to establish a symbiosis with *M. gale*. All but two of the strains studied here have their growth either inhibited or enhanced by *M. gale* fruit exudates (Fig. 3). All the growth-enhanced strains belong to the *Alnus-Comptonia* HSG (cluster 1a), which includes all the strains nodulating *M. gale*, whereas all the growth-inhibited strains belong to clusters 1b and 3. The minimal active concentration for which there is still a growth enhancement of the compatible strains is 1 μg liter^{-1}. Considering the structures of the purified compounds and an average molecular mass of 300 g mol^{-1} for a flavonoid, the molar concentration is approximately 10^{-9} M, which is a typical active-signal concentration, as observed for example in the rhizobial symbiosis. It is worthwhile to note that the minimal active concentration for which there is still a growth inhibition is about 100 μg liter^{-1}, corresponding to 10^{-7} M. This 100-fold

difference between these two minimally active concentrations suggests either different targets or different signal molecules involved in the process.

Similarly, aqueous extracts of *Casuarina cunninghamiana* tissues stimulate the growth of several *Frankia* strains (66). Flavonoids released from host plants have been also described as involved in the microbial growth modulation in other symbiosis. Indeed, daidzein and luteolin not only are *nod* inducers but also increase *Bradyrhizobium japonicum* and *Sinorhizobium meliloti* growth, respectively (15, 25). Flavonoids released from host plants have also been described as hyphal growth inductors in the mycorrhizal symbiosis (6). On the contrary, depending on concentration, rhizobial growth inhibition by flavonoids has been reported with various selective effects. For example, medicarpin, a phytoalexin produced by *Trifolium* species and *Medicago sativa*, inhibits growth of incompatible strains (*Bradyrhizobium japonicum*) but has no effect on compatible rhizobia, thus participating in the selection of rhizospheric bacterial populations (46). Besides, glyceollin from *Glycine max* inhibits its own symbionts, indicating the complexity of regulation between symbiosis induction and plant defense (47).

Among the four tested flavonoids, MyB and MyP show similar patterns of biological activity on compatible and incompatible *Frankia* strains as the total extract. This strongly suggests the involvement of dihydrochalcones in the molecular mechanisms of the symbiosis. However, these pure compounds are not active below 1,000 μg liter^{-1}, whereas total extract remains active at 1 μg liter^{-1}. This difference between thresholds suggests that single compounds are not individually responsible for the effect observed with the total extract. A synergistic effect between different compounds could be at the root of growth modulation of *Frankia* strains. In the rhizobial symbiosis, such a synergy has previously been described. Combinations of inducers like eriodictyol and naringenin together with genistein and liquiritigenin on pea rhizobia generate higher *nod* gene expression than each compound alone (13). However, no synergistic effect was observed in our experiments with any combination of flavonoids tested, even with MyB and MyP together (data not shown). Other plant compounds might be involved in the activity of *M. gale* total fruit exudate extract. Furthermore, the activity of the total extract could be due to two groups of compounds: on the one hand, molecules specifically responsible for the growth induction of compatible strains and, on the other hand, other molecules involved only in growth inhibition of incompatible strains. A bioguided fractionation of the fruit exudates based on the monitoring of growth modulation would be an efficient way to determine exactly which molecules are responsible for those effects.

In this study, we show that nitrogen fixation activity is affected by *M. gale* fruit exudates in the same manner as bacterial growth: induction of nitrogen fixation in compatible strains and inhibition in incompatible ones. Growth induction or inhibition could thus be at least partly the consequence of modifications in nitrogen supply for the bacteria in the early perception of plant chemicals. Indeed, after 3 days of induction, nitrogen fixation of both compatible and incompatible *Frankia* cells is no longer affected by the flavonoid mixture. These transitory effects on nitrogen fixation could result from the oxidative stress generated by *M. gale* flavonoids, as we have previously described (4). In this previous work, we have shown deep modifications of the hydrosoluble intracellular proteomic content of several *Frankia* strains induced by *M. gale* fruit exudates. Among the proteins differentially affected, many involved in the oxidative stress response (e.g., superoxide dismutase, peroxidases, and tellurium resistance proteins) were also detected in *Frankia* after induction by *Alnus glutinosa* extracts (23). As nitrogenase is highly sensitive to the cellular oxygen level, a bacterial redox environment perturbation could affect its functioning. Due to the previously described antioxidant and radical scavenging properties of dihydrochalcones, such compounds might interfere with the redox environment of *Frankia* cells, and a specific management of those compounds by compatible strains could be part of the molecular mechanisms of host specificity. The possibility for *Frankia* strains to efficiently protect their nitrogenase could lead to a successful infection. This hypothesis is supported by our microarray experiments (discussed below). We note that in plant-microorganism interactions, oxidative stress commonly occurs, for example, in rhizobial symbiosis (56) and also in phytopathogenic infections (35). More generally, it could be a common and fundamental mechanism occurring widely in many prokaryote-eukaryote interactions, as it has been described recently in the *Aedes albopictus-Wolbachia* symbiosis (11).

Effects of *M. gale* fruit exudates on ACN14a *Frankia* gene expression. In order to identify bacterial genes possibly involved in the physiological changes induced by *M. gale* fruit exudates as part of the very early mechanisms of the *Frankia-M. gale* interaction, transcriptomic analysis of the compatible strain ACN14a was performed. Surprisingly, only 22 genes were differentially expressed between cells induced with *M. gale* fruit exudates and methanol controls. Considering the large phenotypic effects of *M. gale* fruit exudates that we saw on growth and proteomic expression (4), we expected to observe correspondingly deep modifications of the bacterial transcriptome. However, the slight effect on gene expression could be explained by a different time-dependent response at the transcriptional and physiological levels. *Frankia* ACN14a RNA extraction was performed after 5 days of growth in the presence of plant compounds, in order to be under the same conditions that we used for biomass and proteomic analyses (4). It is possible that the transcriptional reaction of the bacteria to exudates occurred earlier and then decreased to a lower level over 5 days, when stationary growth phase is achieved. Similarly, the nitrogen fixation capacity of strains is affected by fruit exudates 24 h after induction but not after 5 days. Moreover, methanol cell toxicity could mask a few differentially transcribed genes, even though a 0.5% (vol/vol) concentration is classically used for biological cellular assays. Fruit exudates induce a downregulation of most of the 22 differentially regulated genes. Only eight affected genes have an annotated function. The highest overexpression (19-fold change) was observed for a multidrug resistance (MDR) protein (FRAAL1973), which is an efflux pump with high homology to the *Nocardia lactamdurans* cephamycin export protein CmcT (14). Mutations of genes encoding multidrug efflux pumps of *Rhizobium etli* CFN42 lead to the reduction of 40% of the numbers of nodules on its host plant *Phaseolus vulgaris* (21). MDR efflux pumps are also implicated in the virulence of phytopathogens such as *Erwinia amylovora*, *Botrytis cinerea*, or *Fusarium graminearum* (12). The synthesis of such a trans-

porter could explain the resistance of ACN14a and compatible strains to the antibacterial activity of *M. gale* fruit exudates (51). However, MDR homolog genes, and all the other differentially transcribed genes, are also present in EaN1pec and CcI3 *Frankia* genomes, which are both considered to be incompatible with *M. gale*. Differential activity of MDR pumps between compatible and incompatible strains could thus result either from a specific molecular interaction between flavonoids and MDR proteins or from genetic regulation of related encoding genes rather than from the presence/absence of the latter in the genomes of *Frankia* strains.

Three differentially expressed genes are involved either in oxidative stress response or in oxidative process in respiratory chains, metabolic pathways that were shown to be affected by plant extracts (4). FRAAL6078 is a cytochrome *c* AA3 subunit-encoding gene whose transcription is reduced by *M. gale* compounds. A significant effect of dihydrochalcones such as MyA and MyB on electron transfers in rat mitochondria inhibiting ATP synthesis has been reported in the literature (39). Respiratory phosphorylation effects of flavonoids could partly explain the toxicity observed on bacterial strains and the modification of electron transfers, resulting probably in reactive oxygen species (ROS) generation. The two sequences encoding rubrerythrin (FRAAL5116) and a Fur family transcriptional repressor (FRAAL5117) are colocalized in the ACN14a genome and are involved in peroxide regulation and in iron metabolism. Oxidative stress and iron metabolism are often coregulated, particularly during the Fenton reaction, which produces hydroxyl radicals (33). Moreover, flavonoids are known as iron chelators, and their presence in *Frankia* medium could thus modify siderophore production and iron availability and consequently alter the redox environment of the bacterial cell generating ROS. Consequently, these two proteins could be involved in the management of such ROS to avoid toxic effects. Besides, ROS generated by the oxidative stress induced by the flavonoid mixture could also play a role in signal mechanisms. ROS and RNS (reactive nitrogen species) usually associated with biotic and abiotic stress were first regarded as damaging for cells but appear more and more as ubiquitous signaling molecules. In legumes, those reactive species are considered to play a key role in the symbiosis (48). ROS production in *Frankia* cells induced by plant flavonoids could thus be involved as a signal in the interaction with the host plant, while the bacteria would manage the plant's oxidative burst (63).

Finally, the last upregulated gene (FRAAL2286) induced by *M. gale* fruit exudates is homologous to *whiB*, a transcriptional regulator-encoding gene present in *Actinomycetales*. Regulators of the Whi family are involved in *Streptomyces* cell differentiation, sporulation, or antibiotic production and are highly suspected to be involved in *Mycobacterium smegmatis* virulence (32, 53). A conserved role in biotic interactions can thus be suspected for Whi family regulators in the *Actinomycetales*. WhiB could be at the origin of growth enhancement observed for compatible strains in the presence of *M. gale* fruit exudates. The presence of four cysteine residues suggests that these WhiB proteins may be sensitive to redox changes perhaps through a metal atom bound or through direct sensitivity to oxidation via disulfide-bound formation. It has been shown that *Streptomyces* WhiD, a member of the WhiB family, binds a [4Fe-4S] cluster and that all four cysteine residues are essential for its activity (29). *Corynebacterium glutamicum* WhcE, also a member of the WhiB family, is important for survival following heat and oxidative stress (34). These observations support the hypothesis that the members of this protein family are likely to be associated with intracellular redox-sensing pathways. *Frankia* ACN14a WhiB could thus be induced by ROS signals generated after induction by flavonoids and causing a specific cell multiplication related to the symbiotic process.

The work presented in this paper demonstrates for the first time, to our knowledge, a role for plant secondary metabolites in the specificity of actinorhizal symbioses. Phytochemical characterization of fruit exudates of *M. gale* revealed the occurrence of many flavonoids, particularly from the rare class of dihydrochalcones. They induce phenotypic (growth and nitrogen fixation) and transcriptional effects in *Frankia* strains congruent with the symbiotic compatibility statuses of the strains, supporting the involvement of these molecules in the symbiotic interaction. The physiological effects of *M. gale* fruit exudates on *Frankia* strains indicate the bivalent role of plant phenolic compounds, acting as positive chemical signals on compatible strains and as chemical weapons on incompatible ones. Whole-genome microarrays allowed the identification of several ACN14a genes that could be involved in the molecular mechanisms of the interaction. The Myricaceae-*Frankia* symbiosis is a model of interest for the study of the interactions occurring between actinorhizal partners. More generally, the *Frankia*/actinorhizal plant symbiosis is an original and crucial model for progress in the understanding of the evolution of nitrogen-fixing symbioses.

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