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# Decreasing Global Transcript Levels over Time Suggest that Phytoplasma Cells Enter Stationary Phase during Plant and Insect Colonization

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**To highlight different transcriptional behaviors of the phytoplasma in the plant and animal host, expression of 14 genes of "*Candidatus* Phytoplasma asteris," chrysanthemum yellows strain, was investigated at different times following the infection of a plant host (*Arabidopsis thaliana*) and two insect vector species (*Macrosteles quadripunctulatus* and *Euscelidius variegatus*). Target genes were selected among those encoding antigenic membrane proteins, membrane transporters, secreted proteins, and general enzymes. Transcripts were detected for all analyzed genes in the three hosts; in particular, those encoding the antigenic membrane protein Amp, elements of the mechanosensitive channel, and two of the four secreted proteins (SAP54 and TENGU) were highly accumulated, suggesting that they play important roles in phytoplasma physiology during the infection cycle. Most transcripts were present at higher abundance in the plant host than in the insect hosts. Generally, transcript levels of the selected genes decreased significantly during infection of *A. thaliana* and *M. quadripunctulatus* but were more constant in *E. variegatus*. Such decreases may be explained by the fact that only a fraction of the phytoplasma population was transcribing, while the remaining part was aging to a stationary phase. This strategy might improve long-term survival, thereby increasing the likelihood that the pathogen may be acquired by a vector and/or inoculated to a healthy plant.**

Phytoplasmas are wall-less plant-pathogenic bacteria, classified as "*Candidatus* Phytoplasma" spp. (1). They belong to the class *Mollicutes* and infect a wide variety of plants, causing heavy crop losses in many different countries (2). Phytoplasmas are phloem limited in the infected plant, and they cause severe symptoms (yellowing, dwarfism, and phyllody), often leading to plant death. They are transmitted by phloem-feeding Hemipteran vectors (leafhoppers, planthoppers, and psyllids) in a persistent propagative manner (3).

Phytoplasmas are obligate parasites that depend on host cells for the uptake of essential compounds such as sugars, amino acids, ions, and nucleotide precursors (4). Consistent with this lifestyle, phytoplasmas have very small, A/T-rich genomes, ranging from 530 to 1,350 kb in size (5), that lack essential metabolic pathways, such as ATP synthesis. This genome condensation reflects the phytoplasma adaptation to nutrient-rich environments such as the plant phloem (6) and helps explain why these pathogens are not cultivable under axenic conditions (7).

Although the pathogenicity mechanisms are still largely unclear, phytoplasmas influence plant metabolism both directly, through a set of membrane proteins acting as molecular carriers (6), and indirectly, through secretion of effector proteins (8, 9). *In vitro* studies have also shown that phytoplasma immunodominant membrane proteins interact with vector proteins (10, 11) and plant proteins (12) and are subjected to strong positive selection (13–15). Moreover, phytoplasmas can modulate their genome expression according to the infection stage and the infected host species, as suggested by microarray analysis (16) and gene expression study of pathogen transcription factors (17) and of genes lying within potential mobile units (18).

Real-time reverse transcription-quantitative PCR (RT-qPCR) is routinely employed for gene expression studies due to its high sensitivity and accuracy (19–22). Strategies employed for bacterial transcript quantification through qPCR are currently based on relative (23–26) or absolute (27–29) quantification approaches. Phytoplasmas live their lives inside very different environments: the plant and the insect vector. The recent availability of phytoplasma genome sequences has provided tools to investigate phytoplasma-host relationships, but little is known about the molecular mechanisms involved in host switching and in the pathogen cycle in the two environments. These points are extremely important, both to provide the first insights into functional genomics of these pathogens and to start devising new tools for fine-tuned control strategies of these important plant pathogens for integration into the current control of vector populations by insecticide treatments. The aim of this work was to identify phytoplasma genes potentially involved in sensing the host environment, thereby discriminating between plant and insect hosts and, in an even more subtle way, between different insect vectors. As phyto-

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plasma colonization of the host is a continuous process from the original low-quantity inoculum to the final high-density population at the end of the infection cycle, a study was designed to measure transcript levels over time in the plant and in two vector insects. qRT-PCR protocols were set up to study the transcription profile of 14 "*Ca.* Phytoplasma asteris" chrysanthemum yellows phytoplasma (CYP) genes, during infection of *Arabidopsis thaliana* (L.) Heynh and of the two leafhopper vector species *Euscelidius variegatus* Kirschbaum and *Macrosteles quadripunctulatus* Kirschbaum. The two vectors were selected on the basis of their different characteristics with respect to transmitting CYP, as summarized in references 30 and 31: *M. quadripunctulatus* acquires (100% versus 88%) and transmits (100% versus 82%) CYP with higher efficiency than *E. variegatus* and supports multiplication of the phytoplasma at a rate higher than that seen with *E. variegatus*. Consequently, the CYP latent period in the former species is shorter than in the latter one (16 to 18 days versus 30 days). CYP genes were selected for transcript analyses from among predicted secreted proteins, known effectors, and general metabolism enzymes. In the absence of a phytoplasma endogenous control mRNA, the expression level of each pathogen transcript was correlated to the bacterial population measured by qPCR for each experimental date. Absolute quantification of bacterial transcripts was performed (27–29). For each phytoplasma gene, an expression index (EI) was calculated, indicating the transcript copy number per phytoplasma cell at each sampling date and in each infected host, according to the guidelines published for cultivable bacteria (27, 29). Regression analyses were also performed to compare the gene expression trends over time among the three hosts, irrespective of the absolute levels of the individual gene expression.

## MATERIALS AND METHODS

**Phytoplasma isolate, host plant, and insect vector.** Chrysanthemum yellows phytoplasma (CYP) was originally isolated in Italy from *Argyranthemum frutescens* (L.) Schultz-Bip and maintained by insect transmission on daisy, *Chrysanthemum carinatum* Schousboe, the phytoplasma source plant in this work. *Arabidopsis thaliana* ecotype Col-0 seeds were sown in single pots and kept at 4°C for 3 days. Pots were then placed in a growth chamber at 22 to 24°C with a photoperiod representing a short day (light, 9 h; dark, 15 h [L9:D15]) and were maintained under this condition during the whole experiment. Healthy colonies of *Euscelidius variegatus* and *Macrosteles quadripunctulatus*, vectors of CYP (32), were maintained on oat, *Avena sativa* L., inside plastic and nylon cages in growth chambers at 25°C and a photoperiod of L16:D8. To evaluate phytoplasma gene expression profiles in *A. thaliana*, experimental plants were inoculated with CYP by the use of *M. quadripunctulatus* vector. About 100 *M. quadripunctulatus* nymphs were fed on infected daisies for an acquisition access period (AAP) of 7 days and were then transferred on oat (immune to CYP) for a 25-day latency period (LP). Thirty-six *A. thaliana* plants were singly exposed to three infective insects for a 72-h inoculation access period (IAP) and were then treated with insecticide. Leaf samples were collected from 10 *A. thaliana* plants at 10, 14, 21, and 28 days postinoculation (dpi) for nucleic acid extraction. To evaluate the phytoplasma gene expression profile in insect vector, CYP-infected *E. variegatus* and *M. quadripunctulatus* were used. About 200 nymphs of each species were collected from healthy colonies, caged together for a 7-day AAP on CYP-source daisies, and then maintained on healthy oat plants. About 15 insects of each species were collected at 7, 14, 21, 28, and 35 days postacquisition (dpa) for nucleic acid extraction.

**Extraction of nucleic acids.** Plant samples (about 200 mg of leaves), collected at different dpi, were pooled and divided into 100-mg aliquots stored at −80°C before DNA and RNA extraction.

Total DNA was extracted from 100 mg of plant material using a modified cetyltrimethyl ammonium bromide (CTAB) procedure originally described in reference 33, and the final DNA pellet was dissolved in 50 μl of sterile double-distilled water ($ddH_2O$). Total RNA from plant tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. Insect samples collected at different dpa were stored at −80°C before DNA and RNA extraction. Both total DNA and total RNA were extracted from single insects. A few liquid nitrogen drops were spilled into a 1.5-ml tube containing single leafhopper and the insect was then quickly crushed using a sterile micropestel in 200 μl of TE buffer (10 mM Tris, 1 mM EDTA) prepared with diethyl pyrocarbonate (DEPC) (0.1%) water. The resulting homogenate suspension was rapidly divided for DNA and RNA extraction; 100 μl was added to 400 μl of 3% CTAB buffer and treated as detailed before for DNA extraction, whereas 100 μl was added to 400 μl of TRIzol reagent (Invitrogen, USA) for RNA extraction following the manufacturer's instructions. Total RNA samples, extracted from both plants and insects, were treated with RNase-free DNase I (Life Technologies, Monza, Italy) in the supplied buffer to avoid residual DNA contamination. Following the digestion, the DNase was inactivated by phenol-chloroform extraction according to the manufacturer's instructions. RNA was finally suspended in 30 μl of RNase-free water containing 0.1% DEPC. Nucleic acid extracts were analyzed in a NanoDrop spectrophotometer to evaluate the concentration and purity and stored at −80°C.

**Phytoplasma detection and quantification.** The presence of CYP in *A. thaliana*, *E. variegatus*, and *M. quadripunctulatus* samples was verified by qPCR using the protocol described in reference 34. The DNA extracts from 10 infected plants and from 8 infected insects of both species for each sampling date (a total of 40 samples for each of the three species) were used as the template in qPCR to measure the absolute number of phytoplasma genome units (GU) per nanogram of host DNA (35). The quantification of phytoplasma cells was achieved by comparing the quantification cycles (Cqs) of the samples with those of three dilutions of pOP74 plasmid, containing a fragment of the CYP 16S rRNA gene (35). One fg of pOP74 contains 194 molecules of plasmid, with each containing a single copy of the CYP 16S rRNA gene. Because this gene is present in two copies in phytoplasma genomes, 1 fg of pOP74 corresponded to 97 CYP cells. The final concentration of CYP cells was expressed as CYP GU/100 mg of leaf sample or as CYP GU/insect.

**cDNA synthesis and mRNA quantification.** For absolute quantification of phytoplasma mRNAs during *A. thaliana* and insect vector infection, standard curves were produced using serial dilutions of recombinant plasmids carrying a fragment of the corresponding genes (recombinant plasmid DNA [recDNA]). CYP genes were selected based on sequences from a whole-genome shotgun sequencing project that have been deposited at DDBJ/EMBL/GenBank under accession number JSWH00000000. In the absence of a phytoplasma endogenous control mRNA, the expression level of each pathogen transcript was correlated to the bacterial population measured by qPCR for each experimental date. Fragments of 14 selected genes were amplified by conventional PCRs driven by specific primers designed through the use of Primer Express software v3.0.1 (Applied Biosystems, Branchburg, NJ, USA) and of sequences obtained by Illumina sequencing of the CYP genome according to the method described in reference 36 (Table 1). Amplicons were subjected to gel purification using a GeneClean Turbo kit (MP Biomedicals, Solon, OH, USA), cloned into pGEM-T Easy vector (Promega, Madison, WI, USA), and transformed in *Escherichia coli* DH5α. Plasmids were purified using a Fast Plasmid minikit (Eppendorf AG, Hamburg, Germany) and sequenced with universal primers M13F and M13R. The number of plasmid copies per microliter was derived from the concentration measured at the NanoDrop spectrophotometer, using the following formula: $M = C \times N/S$, where $M$ is the number of molecules per microliter, $C$ the RNA concen-

**TABLE 1** Chrysanthemum yellows phytoplasma genes selected for gene expression analysis and their function and product localization within the phytoplasma cell compartment, qPCR primer sequences and concentration in the reaction mix, amplicon melting temperature, and qPCR parameters[a]

| Product category and target gene | Gene product | Product localization | Primer: sequence (reference) | Primer concn (nM) (primer category) | Melting temp (°C) | $R^2$ | *E* (%) |
|---|---|---|---|---|---|---|---|
| Immunodominant membrane proteins | | | | | | | |
| *amp* | Antigenic membrane protein | Membrane | Amp64: GCTTTAATGTTTGTTGGCGTTC (37) | 100 | 82.5 | 0.993 | 78.7 |
| | | | CY02-101Rev: AGCTTTTCCAGCATCACCAT | 300 | | | |
| *imp* | Immunodominant membrane protein | Membrane | CY06-376Fw: ATTCCAAACTTGCCAGCATT | 300 | 78.5 | 1.000 | 96.0 |
| | | | CY06-376Rev: TCTTGGAGGTTTTTGGCATT | 100 | | | |
| Generic transporters | | | | | | | |
| *mscL* | Mechanosensitive channel of large conductance | Membrane | CY05-67Fw: ACTGGGGCTTTAAAAGATTTG | 200 | 77.5 | 0.993 | 75.3 |
| | | | CY05-67Rev: CCTTTTGCAAAGGATTTTTCC | 200 | | | |
| *mdlB* | Multidrug transporter | Membrane | CY04-67Fw: CGCAAATGCCTTTTCAAACT | 200 | 81.5 | 0.998 | 83.5 |
| | | | CY04-67Rev: AAGGCGAGGAATAAGCCCTA | 200 | | | |
| *oppC* | Oligopeptide transporter | Membrane | CY02-378Fw: CCAAGATGCCATTTCTAGCC | 200 | 80.0 | 0.996 | 84.3 |
| | | | CY02-378Rev: ATTCCGCCTGAAAGTTGATG | 200 | | | |
| *ftsY* | SecY translocase component | Membrane | CY03-2292Fw: TGAAGGCATCGAACTTGCTA | 100 | 81.5 | 0.999 | 83.3 |
| | | | CY03-2292Rev: GCTCCTGAAAGTGGAGTTGC | 300 | | | |
| Specific transporters | | | | | | | |
| *artI* | Arginine transporter | Membrane | CYartIFw: AATTCCTGTAGCTGCCCAAG | 200 | 80.0 | 1.000 | 88.1 |
| | | | CYartIRev: ATGAGCGGCTTCAATTTGTC | 200 | | | |
| *zntA* | Cation transporter | Membrane | CY036-368Fw: GACAAACAACCAGGCGATTT | 100 | 83.5 | 0.997 | 81.5 |
| | | | CY036-368Rev: CTGCAAGACCAAGGACAACA | 300 | | | |
| Secreted proteins | | | | | | | |
| *sap54* | Symptom effector | Secreted | CY01-1720Fw: TCAACAAGTAATGGGGATGAA | 200 | 76.5 | 0.999 | 90.5 |
| | | | CY01-1720Rev: AATTGTTGTATTTCGCTTTCTGT | 200 | | | |
| *sap67* | Unknown | Secreted | CY01-420Fw: TCAAGTGATGGCAATGGGTA | 100 | 78.0 | 0.992 | 78.8 |
| | | | CY01-420Rev1: TTGTATAGAACTTTCTGGTTGTTCAGA | 300 | | | |
| *sap68* | Unknown | Secreted | CY03-1700Fw: ATGGCAATGAATAACGGTCA | 200 | 79.0 | 0.999 | 88.5 |
| | | | CY03-1700Rev: TTGTTGAGCAGCGATACGAC | 200 | | | |
| *tengu* | Symptom effector | Secreted | CY04-87Fw1: ATTTGCTGGCTTTTGGGCTA | 200 | | | |
| | | | CY04-87Rev1: TTTCAATTAGAGTTATCACGTTTTCAA | 200 | 77.5 | 0.999 | 74.8 |
| General metabolism | | | | | | | |
| *pgsA* | Phosphatidylglycerol synthase | Cytoplasm | CY06-96Fw: TCGTTTGTCTGCTACGCAAG | 200 | 81.0 | 0.996 | 84.6 |
| | | | CY06-96Rev: AAAAGGCAAATGATGGCAAC | 200 | | | |
| *rpsU* | *rpsU* RNA ribosomal subunit | Cytoplasm | CY08-364Fw: GGAGAAACTATCGAAGAAACGCTAC | 200 | 81.5 | 0.995 | 86.0 |
| | | | CY08-364Rev: TTAACGCTTTTTACTACGCATATTTT | 200 | | | |

[a] $R^2$, correlation coefficient; E %, reaction efficiency.

tration (in ng/μl), *S* the molecular weight of the fragment, and *N* a factor derived from the Avogadro constant. recDNA was diluted, distributed in aliquots, and stored at −20°C to be used as a standard for qPCR. Standard curves were constructed by linear regression analysis of the Cq value of each standard dilution replicate over the log of the number of plasmid copies present in each sample. Data acquisition and analysis were handled by the use of CFX Manager software, version 3.0, which automatically calculates the Cq values and the parameters of the standard curves. qPCR efficiency (*E*) was calculated by the formula $E = e^{\ln 10/-s} - 1$, where *e* represents the base of the natural logarithm and a slope (*s*) value of −3.322 ($E = 2$) represents 100% efficiency. For each sampling date, cDNA was synthesized from total RNA (500 ng) using a High Capacity cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions and was stored in sterile microtubes until the qPCRs were performed. SYBR green-based qPCR protocols were optimized for each selected gene by adjusting the concentrations of forward and reverse primers from 100 μM to 300 μM. The final qPCR mix contained 1 μl of cDNA, 1× iQ SYBR green Supermix (Bio-Rad/Life Science Research, Hercules, CA, USA), 100 to 300 nM (each) primers (Table 1), and sterile double-distilled water added to reach a final volume of 25 μl. Reaction conditions were as follows: 5 min at 95°C and 45 cycles of 15 s at 95°C, 30 s at 59°C, and 30 s at 72°C. On each plate, samples were run in duplicate together with four 10-fold serial dilutions of the corresponding standard plasmid. The use of DNA standard curves for transcript quantification allowed comparisons of expression data from different genes (29, 38). Complete qPCR mix with total RNA and sterile distilled water instead of cDNA were used as negative controls in each plate. Reactions were carried out in a CFX Connect real-time PCR detection system (Bio-Rad, USA) supported by CFX Manager software, version 3.0. Melting curves were produced at the end of the PCR to assess the reaction specificity.

**Data analysis.** Absolute quantification of bacterial transcripts was performed. For each phytoplasma gene, an expression index (EI) was calculated, indicating the transcript copy number per phytoplasma cell at each sampling date and in each infected host, according to the guidelines published for cultivable bacteria (26, 28). Regression analyses were also performed to compare the gene expression trends over time among the three hosts, irrespective of the absolute levels of the individual gene expression. To compare the phytoplasma population sizes as well as the EI values of the genes at different times in each host, analysis of variance (ANOVA) was performed on ranks (Kruskal-Wallis test), followed by Tukey or Dunn tests for multiple comparisons. For each gene, in all the three host species, decreased expression from the early to the late sampling dates was observed. To compare the decreases in expression of genes within each functional category in the three host species, linear regression was calculated on the basis of the log-transformed EI of each gene and the sampling times. For *A. thaliana* samples, the first sampling date (10 dpi) was omitted from regression analysis, due to the high variances among the EI values measured at the initial phase of infection. To compare CYP gene expression levels among the different host species, the EI values from the first two sampling dates (10 and 14 dpi and 7 and 14 dpa for the plant and insect samples, respectively) were pooled, as they did not differ significantly, and were analyzed by ANOVA performed on ranks (Kruskal-Wallis test), followed by the Dunn test for multiple comparisons. For each gene, in all three species, decreased expression was observed from the early to the late sampling dates; to compare the decreases in expression of the genes within each functional category in the three species, linear regression was calculated on the basis of the log-transformed EI of each gene and the sampling times. For *A. thaliana* samples, the first sampling date (10 dpi) was omitted from regression analysis, due to the high variances among EI values measured at the initial phase of infection. All statistical analyses were performed with SigmaPlot 11.0 (SyStat).

**Nucleotide sequence accession numbers.** The strain CYP gene sequences from the whole-genome shotgun sequencing project have been deposited at DDBJ/EMBL/GenBank under accession number JSWH00000000.

## RESULTS

**Phytoplasma detection and quantification.** Diagnostic assays confirmed the presence of CYP in all *A. thaliana* plants at each sampling date. The phytoplasma population, expressed as CYP cells/100 mg of plant tissue, significantly increased from 10 dpi to 21 dpi (Fig. 1), and calculated values ranged from 2.23E+06 at 10 dpi to 2.93E+09 at 21 dpi (see Table S1 in the supplemental material). Diagnostic assays confirmed the presence of CYP in more than 80% of the *E. variegatus* samples at each sampling date and in all *M. quadripunctulatus* samples irrespective of the sampling date. The phytoplasma population increased from 7 to 21 dpa in both species (Fig. 1), and calculated values ranged from 1.75E+03 and 3.30E+03 at 7 dpa to 1.17E+07 and 3.57E+07 at 35 dpa for *E. variegatus* and *M. quadripunctulatus*, respectively (see Table S1 in the supplemental material).

**Optimization of qPCR assays.** For each target gene, specific primers were designed using CYP sequences. The primer list and the corresponding amplification conditions (annealing temperature and primer concentration) are reported in Table 1. A specific signal was obtained following melting analysis of the qPCR amplicons, while no amplification was obtained from no-reverse-transcribed-RNA and from no-template controls. Melting peak temperatures ranged from 76.5°C (*sap54*) to 83.5°C (*zntA*).

To estimate the expression levels of different CYP genes, the mRNA absolute quantity was divided by the phytoplasma titer measured in the corresponding sample. A plasmid standard curve, ranging from 10E+8 to 10E+4 gene copies, was set up for each target gene. Efficiencies of qPCRs ranged between 74.8% and 96.0% for primers amplifying the *tengu* and *imp* genes, respectively, whereas correlation coefficients ranged from 0.992 (*sap67*) to 1.000 (*imp*) (Table 1). Unbalanced primer final concentrations were optimized to improve the efficiency of reactions of primers amplifying *amp*-, *imp*-, *ftsY*-, *zntA*-, and *sap67*-specific amplicons (Table 1).

**Phytoplasma transcript levels in *Arabidopsis thaliana*.** Mean copy numbers of transcripts per CYP cell in leaf tissues of *Arabidopsis thaliana* sampled at 10, 14, 21, and 28 dpi are presented in Table S2 in the supplemental material.

**Immunodominant membrane proteins.** *amp* showed the highest transcript level among the analyzed CYP genes in *A. thaliana* challenged over time by phytoplasma infection (see Table S2 in the supplemental material). As with most of other CYP genes, *amp* transcripts decreased significantly in the late phase of infection (28 dpi) (Fig. 2 and Table 2). The mean expression level of *amp* was always higher than that of *imp*. On the other hand, *imp* transcript levels were constant from 10 to 28 dpi, *imp* being the most stable CYP gene during phytoplasma infection of *A. thaliana* as confirmed by the regression analyses (Fig. 2 and Table 2).

**Generic transporters.** *mscL* transcripts were the most abundant within this functional category, showing a mean expression level about 30 times higher than that of *mdlB* at each sampling date, from 70 to over 100 times higher than that of *ftsY*, and from 70 to over 500 times higher than that of *oppC* from 10 to 28 dpi (Fig. 2; see also Table S2 in the supplemental material). In line with this observation, *oppC* showed a rapid decrease of expression over time, as evidenced by the regression analysis (Fig. 2 and Table 2). Indeed, the *oppC* slope (absolute value) was the highest among those of the analyzed CYP genes in *A. thaliana*. Transcript levels of

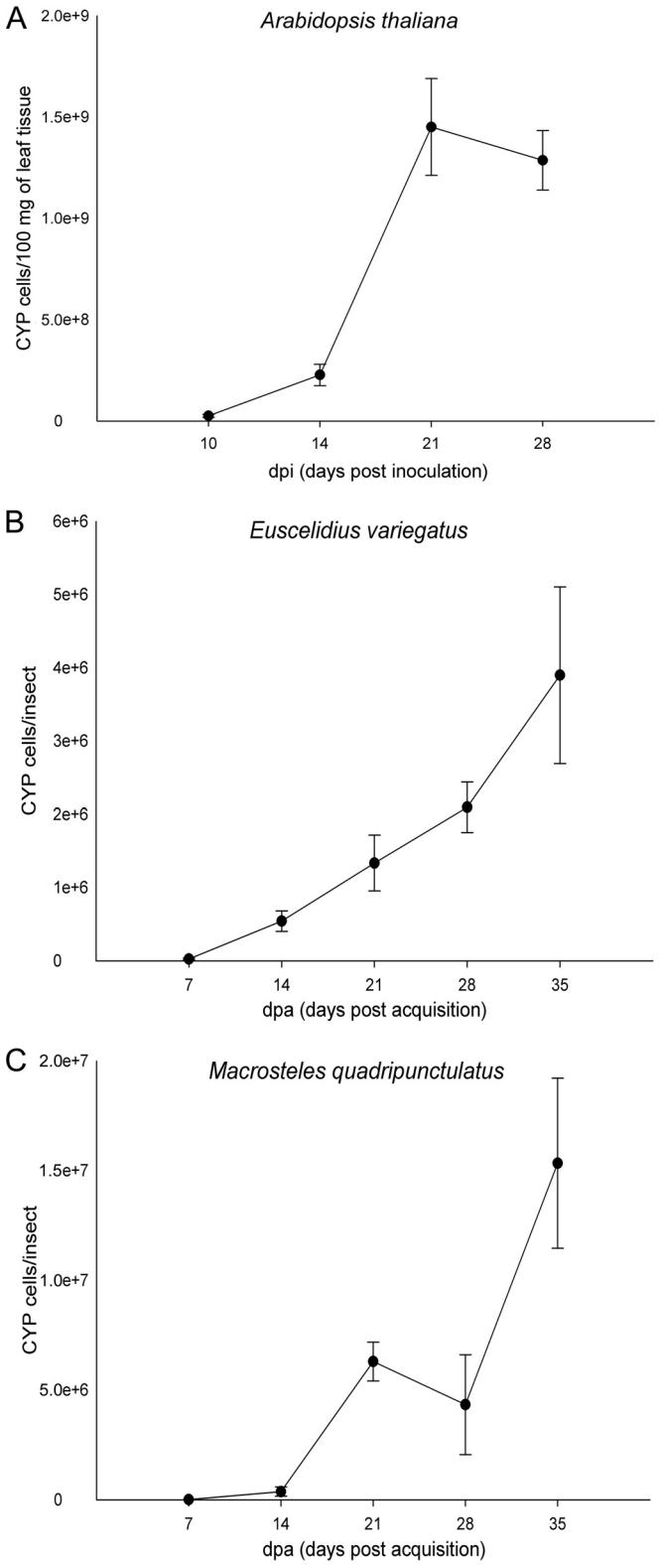


**FIG 1** CYP population measured in leaf tissues of *Arabidopsis thaliana* (A) as a function of sampling time (10, 14, 21, and 28 days postinoculation [dpi]) and in individuals of *Euscelidius variegatus* (B) and *Macrosteles quadripunctulatus* (C) as a function of sampling time (7, 14, 21, 28, and 35 days postacquisition [dpa]). In all cases, error bars indicate standard errors of the means.

the other generic and specific transporter genes significantly decreased over time but at lower rates (Fig. 2).

**Specific transporters.** The transcript levels of *zntA* were higher than those of *artI* under all conditions and time points (Fig. 2; see also Table S2 in the supplemental material). Mean expression levels of both *artI* and *zntA* decreased significantly in the late phase of infection (28 dpi). Regression analysis (Fig. 2 and Table 2) confirmed a significant decrease of transcript levels for both genes over time, indicating a correlation with sampling time for *zntA* stronger than that seen with *artI* and all other generic transporter genes except *oppC*.

**Secreted proteins.** Considering all 14 CYP genes analyzed, *tengu* and *sap54* were the second and the fourth most abundant transcripts under the *A. thaliana* host conditions, following *amp* and *mscL*, respectively. The *tengu* transcripts were the most abundant in this category (Fig. 2; see also Table S2 in the supplemental material), and *tengu* showed the second lowest regression slope after that of *imp* (Fig. 2 and Table 2). In contrast, a drop in the transcript levels of the other secreted proteins was clearly evident in the regression analysis, as slopes for these genes (absolute values) were the highest among the CYP genes analyzed in *A. thaliana*, just below the slope of *oppC*. *sap54* showed the second highest transcript level and the most evident expression decrease over time (Fig. 2 and Table 2).

**General metabolism.** The ribosomal *rpsU* gene showed a mean transcript level about five times higher than that of *pgsA*, at each sampling date (Fig. 2; see also Table S2 in the supplemental material). Mean EI values of both *rpsU* and *pgsA* decreased significantly in the late infection phases. Regression analysis (Fig. 2 and Table 2) also showed a significant decrease in the transcription of both genes, characterized by very similar slopes.

**Phytoplasma gene transcript levels in *Euscelidius variegatus*.** Mean copy numbers of transcripts per CYP cell in individual *E. variegatus* sampled at 7, 14, 21, 28, and 35 dpa are presented in Table S3 in the supplemental material.

**Immunodominant membrane proteins.** *amp* showed the highest EI among the analyzed CYP genes in *E. variegatus* individuals (see Table S3 in the supplemental material). *amp* and *imp* EI values did not change significantly during the infection in this vector species as confirmed by the regression analyses (Fig. 2 and Table 2).

**Generic transporters.** Among the transporter genes, *mscL* produced the most abundant transcripts (see Table S3 in the supplemental material). Also, CYP generic transporter genes did not show significant changes of EI during phytoplasma infection of *E. variegatus* individuals (see Table S3). Regression analysis confirmed this result, as the EI values of all genes but *mscL* did not vary with time, and the regression of *mscL* transcript levels over time was barely significant (Fig. 2 and Table 2).

**Specific transporters.** The transcript levels of *zntA* were always higher than those of *artI* (see Table S3 in the supplemental material). The mean EI of *zntA*, but not that of *artI*, decreased significantly in the late phase of infection (35 dpa; see Table S4 in the supplemental material), and its regression showed the highest slope (absolute value) among the other CYP generic and specific transporter genes in the *E. variegatus* host condition (Fig. 2 and Table 1).

**Secreted proteins.** The transcripts of *tengu* and *sap54* were the second and the third most abundant under the *E. variegatus* host conditions, following those of *amp*. Indeed, *tengu* was the most

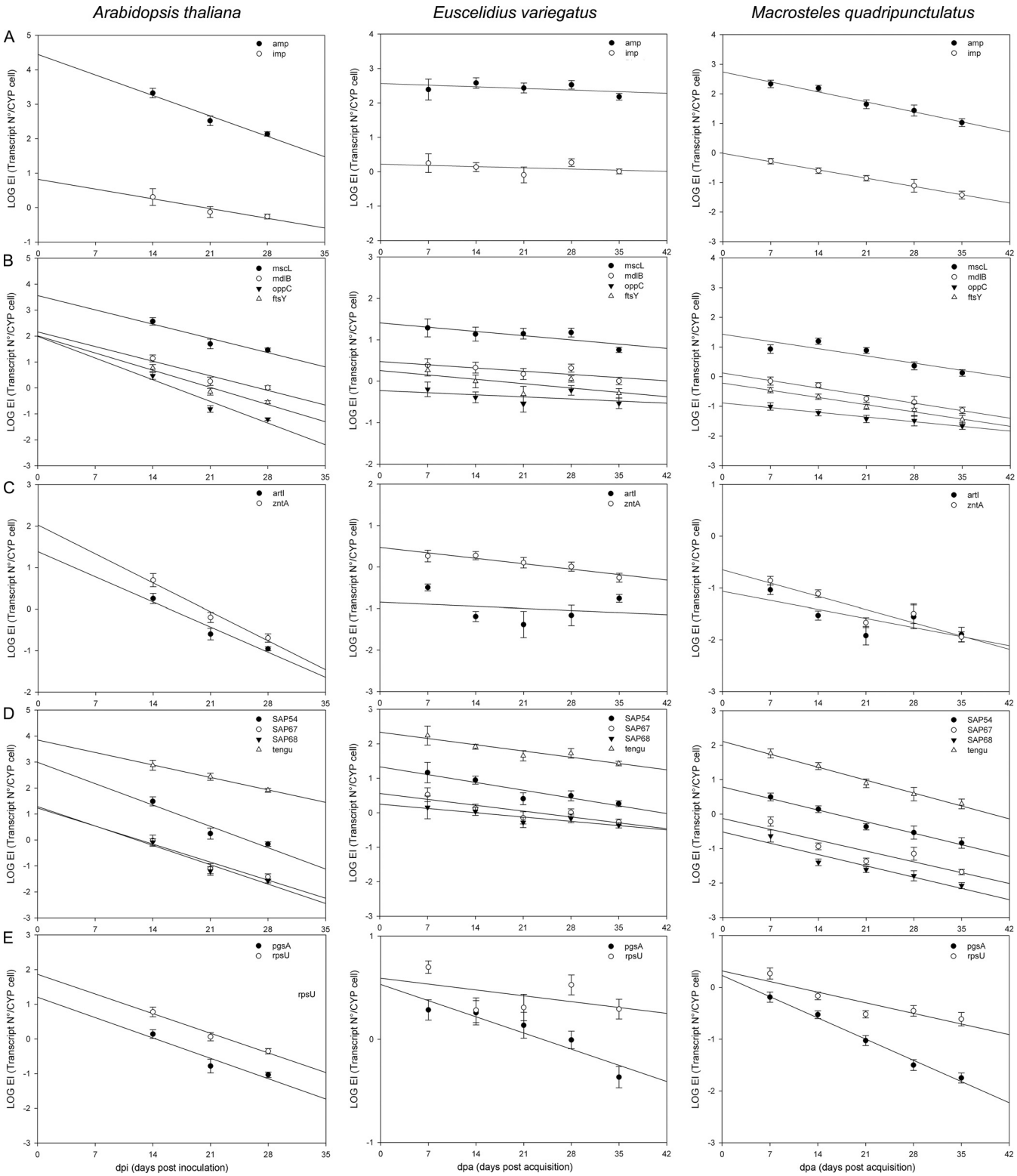


**FIG 2** Plot of linear regression analysis of log copy number of transcripts per phytoplasma cell (log expression index [LOG EI]) of chrysanthemum yellows phytoplasma (CYP) genes encoding immunodominant membrane proteins (A), generic transporters (B), specific transporters (C), secreted proteins (D), and proteins involved in general metabolism (E) measured in leaf tissues of *Arabidopsis thaliana* as a function of sampling time (14, 21, and 28 days postinoculation [dpi]) and in individuals of *Euscelidius variegatus* and *Macrosteles quadripunctulatus* as a function of sampling time (7, 14, 21, 28, and 35 days postacquisition [dpa]). In all cases, error bars indicate standard errors of the means.

**TABLE 2** Regression analysis parameters of log expression index values in function of sampling times of chrysanthemum yellows phytoplasma genes, grouped by functional category and measured in *Arabidopsis thaliana*, *Euscelidius variegatus*, and *Macrosteles quadripunctulatus* samples[a]

| Functional category | Gene name | *Arabidopsis thaliana* | | | | *Euscelidius variegatus* | | | | *Macrosteles quadripunctulatus* | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Slope | $R^2$ | *P* | *n* | Slope | $R^2$ | *P* | *n* | Slope | $R^2$ | *P* | *n* |
| Immunodominant membrane proteins | *amp* | −0.085 | 0.634 | <0.001 | 30 | **−0.007** | **0.023** | **0.357** | **39** | −0.048 | 0.611 | <0.001 | 40 |
| | *imp* | −0.040 | 0.164 | 0.026 | 30 | **−0.004** | **0.009** | **0.576** | **38** | −0.040 | 0.586 | <0.001 | 39 |
| Generic transporters | *mscL* | −0.079 | 0.497 | <0.001 | 30 | −0.015 | 0.134 | 0.028 | 36 | −0.036 | 0.488 | <0.001 | 39 |
| | *mdlB* | −0.081 | 0.560 | <0.001 | 30 | **−0.011** | **0.101** | **0.055** | **37** | −0.036 | 0.489 | <0.001 | 40 |
| | *oppC* | −0.120 | 0.752 | <0.001 | 30 | **−0.006** | **0.023** | **0.364** | **38** | −0.023 | 0.314 | <0.001 | 39 |
| | *ftsY* | −0.089 | 0.665 | <0.001 | 30 | **−0.013** | **0.082** | **0.089** | **36** | −0.035 | 0.477 | <0.001 | 39 |
| Specific transporters | *artI* | −0.087 | 0.655 | <0.001 | 29 | **−0.006** | **0.012** | **0.604** | **25** | −0.024 | 0.210 | 0.003 | 39 |
| | *zntA* | −0.099 | 0.680 | <0.001 | 29 | −0.020 | 0.281 | <0.001 | 37 | −0.036 | 0.524 | <0.001 | 39 |
| Secreted proteins | *sap54* | −0.118 | 0.613 | <0.001 | 30 | −0.032 | 0.330 | <0.001 | 38 | −0.048 | 0.630 | <0.001 | 40 |
| | *sap67* | −0.099 | 0.522 | <0.001 | 30 | −0.021 | 0.234 | 0.004 | 34 | −0.043 | 0.555 | <0.001 | 36 |
| | *sap68* | −0.107 | 0.680 | <0.001 | 30 | −0.017 | 0.150 | 0.018 | 37 | −0.047 | 0.645 | <0.001 | 38 |
| | *tengu* | −0.069 | 0.470 | <0.001 | 30 | −0.025 | 0.300 | <0.001 | 36 | −0.053 | 0.661 | <0.001 | 40 |
| General metabolism | *pgsA* | −0.084 | 0.555 | <0.001 | 29 | −0.024 | 0.371 | <0.001 | 36 | −0.058 | 0.843 | <0.001 | 38 |
| | *rpsU* | −0.081 | 0.639 | <0.001 | 30 | **−0.006** | **0.037** | **0.264** | **36** | −0.030 | 0.519 | <0.001 | 40 |

[a] Nonsignificant ($P > 0.05$) regression data are indicated in bold.

highly transcribed gene within its functional category (see Table S3 in the supplemental material). *sap54* was the only gene showing a significant decrease of its transcript levels over time (see Table S3), while regression analyses indicated a significant negative correlation of EI and time for all analyzed secreted protein genes (Fig. 2 and Table 2).

**General metabolism.** The ribosomal *rpsU* and *pgsA* genes showed analogous transcript levels in the early phase of infection (Fig. 2; see also Table S3 in the supplemental material). While the *rpsU* EI did not change over time (see Table S3 in the supplemental material), transcript levels of *pgsA* decreased significantly from the early to late infection phases (Fig. 2 and Table 1).

**Phytoplasma gene transcript levels in *Macrosteles quadripunctulatus*.** Mean copy numbers of transcripts per CYP cell in individual *M. quadripunctulatus* samples collected at 7, 14, 21, 28, and 35 dpa are presented in Table S4 in the supplemental material.

**Immunodominant membrane proteins.** In *M. quadripunctulatus*, *amp* showed the highest EI of all the analyzed CYP genes (see Table S4 in the supplemental material). The transcript levels decreased significantly in both late phases of infection (28 and 35 dpa; see Table S4), and regression analyses confirmed this result (Fig. 2 and Table 2). Mean transcript levels of *amp* were about 400 times higher than those of *imp* (see Table S4).

**Generic transporters.** *mscL* was the third most highly transcribed CYP gene in infected *M. quadripunctulatus*, following *amp* and *tengu*, and its transcripts were the most abundant within this functional category (see Table S4 in the supplemental material). All generic transporter genes showed a significant decrease of transcript levels over time (see Table S4), with *oppC* showing the lowest (absolute value) regression slope over time of all CYP genes in the *M. quadripunctulatus* host (Fig. 2 and Table 1).

**Specific transporters.** *zntA* and *artI* showed similar transcript levels in infected *M. quadripunctulatus* samples (see Table S4 in the supplemental material). The mean EI values of both genes decreased significantly in the late infection phase (Fig. 2 and Table 2). A more severe drop of transcript levels was recorded for *zntA* than for *artI*.

**Secreted proteins.** As seen in *A. thaliana*, *tengu* and *sap54* were the second and the fourth most highly expressed CYP genes in infected *M. quadripunctulatus* samples, and the *tengu* transcripts were the most abundant within this category (see Table S4 in the supplemental material). As seen in *A. thaliana*, all secreted protein-coding genes showed a significant EI decrease over time (see Table S4), with similar slope values in the regression analysis (Fig. 2 and Table 2).

**General metabolism.** As seen in *E. variegatus*, the ribosomal *rpsU* and *pgsA* genes showed similar transcript levels at the earliest sampling date (see Table S4 in the supplemental material). Regression analysis showed a stronger decrease over time for *pgsA*, with the highest (absolute value) slope of all the analyzed CYP genes in the *M. quadripunctulatus* host environment (Fig. 2 and Table 1).

**Comparison of gene transcript levels among species.** To compare CYP gene transcript profiles among the three hosts (see Table S5 in the supplemental material), the transcript levels of all analyzed CYP genes at the first two sampling dates (10 and 14 dpi and 7 and 14 dpa, for plant and insect samples, respectively) were pooled within each species, as they did not differ significantly (see Tables S2 to S4). Generally, CYP transcript levels were higher in the plant hosts than in the insect hosts and, for the two insect species, were higher in *E. variegatus* than in *M. quadripunctulatus*. However, CYP transcript levels did not significantly differ between *A. thaliana* and *E. variegatus* samples, except for *amp*, *mscL*, *artI*, and *tengu*. Transcripts of those four genes, together with *mdlB* and *rpsU*, were present at similar levels in the two insect vectors. The EI value for most CYP genes was significantly lower in *M. quadripunctulatus* than in *A. thaliana*, with the exception of *sap67* and *pgsA* (see Table S5 in the supplemental material). *amp*, *tengu*, and *mscL* were always the most abundant CYP transcripts in each host species (see Table S5). On the other hand, *sap*68, *pgsA*, and *sap67* produced the three least abundant CYP transcript levels in *A. thaliana*, while *artI*, *oppC*, and *ftsY* or *zntA* showed the lowest transcript levels in *E. variegatus* and *M. quadripunctulatus*, respectively. The timing and rate of decrease in CYP gene transcript levels over time differed among the three host species (Table 2).

Generally, transcript levels of CYP analyzed genes dropped more severely after infection in the plant (regression slopes ranging from −0.081 to −0.120; Table 2) relative to colonization of the insects (regression slopes ranging from −0.004 to −0.580; Table 2) and more severely in *M. quadripunctulatus* (regression slopes ranging from −0.024 to −0.058; Table 2) than in *E. variegatus* (regression slopes ranging from −0.004 to −0.032; Table 2). Indeed, only seven CYP genes showed significantly decreased transcript levels related to increasing time in the latter species. However, for all the phytoplasma genes analyzed in *A. thaliana* and *M. quadripunctulatus*, decreased transcript levels were significantly related to increasing time after infection (Table 2). Secreted protein coding genes on average showed the strongest decrease of transcript levels in both vector and plant species, with the exception of *tengu* in *A. thaliana*. The transcript levels of *oppC* and *zntA* transporters decreased with time in *A. thaliana*, while the drop in the EI values of generic and specific transporter genes was less evident in the vector species. The *pgsA* and *rpsU* EI values displayed very similar decreases over time in *A. thaliana* samples, whereas, in insects of both species, that *pgsA* transcript level dropped more severely than that of *rpsU* (for *pgsA* and *rpsU* in *E. variegatus* and *M. quadripunctulatus*, regression slopes of −0.024 and −0.006 and regression slopes of −0.058 and −0.030, respectively, were determined; Table 2). Finally, while both the *amp* and the *imp* EI values decreased over time in *A. thaliana* and *M. quadripunctulatus*, the transcript levels of both genes did not vary in *E. variegatus*.

## DISCUSSION

We report a comparison of transcript levels of selected phytoplasma genes in plant and vector species at different moments of the infection cycle. Target genes were selected according to literature analyses (16) and their potential role in phytoplasma adaptation to different environments: the plant and the insect.

Target gene transcript levels were measured over time by reverse transcription (RT) and qPCR, which allows the quantification of mRNA levels in bacteria (39–42). For data normalization, the transcript levels of bacterial target genes may be related to the number of cells obtained through cell culture (28), to the total RNA mass input in the RT-PCR, or to the internal reference control genes (43). Normalization of bacterial transcripts in relation to genomic DNA (gDNA) from cell culture or recombinant plasmid DNA (recDNA) has also been used (22, 27, 29). For phytoplasmas, methods based on cell culture are not available and total RNA input in the qPCR is always contaminated by large amounts of host RNA. Although the use of internal reference genes has been suggested for phytoplasma transcript normalization (18), this may not be suitable for gene expression analyses over time and in different hosts, as evidenced in other studies of relative quantifications of bacterial gene expression levels (22, 28). In this work, quantification of CYP transcripts was performed through the use of standard recDNA curves (29, 44) and an EI, calculated by dividing the gene transcript copy number and phytoplasma cell number measured at the same sampling date.

The multiplication pattern of CYP in *A. thaliana* was similar to that in *C. carinatum* (45), with active phytoplasma multiplication at up to 3 weeks postinoculation, followed by the maintenance of a stationary phytoplasma population size until the end of the experiment (28 dpi).

All selected CYP gene transcripts were present at detectable levels in the three hosts at every sampling date, suggesting their active role in phytoplasma cell cycles. The presence of less than one transcript copy number per CYP cell recorded for all host species, usually at late sampling dates, indicates that not all CYP cells contained each gene transcript at that time of the infection. Generally, transcript levels of most of the 14 selected genes decreased more significantly in the plant and in *M. quadripunctulatus* from early to late samplings, while the decrease in *E. variegatus* was less evident. In the former species, phytoplasma multiplication is very active (30) and is associated with some degree of pathogenicity (46). In *E. variegatus*, transcript levels of most of the selected target genes did not vary significantly during the infection cycle. In this species, phytoplasma multiplication is less efficient (30); in fact, CYP population sizes were always below those measured in *M. quadripunctulatus* at each sampling date, and no pathogenic effects were recorded (46). In the case of CYP, due to the low phytoplasma population in the infected vectors and plant, the first sampling points were set at 7 and 10 days postinfection of the insect and plant hosts, respectively. Under these conditions, the transcript levels of most analyzed genes decreased with time irrespective of the host species, and our experimental conditions did not allow us to arrive at any conclusion with respect to the very early phases of infection.

**Antigenic membrane protein genes.** Phytoplasmas within the "*Ca.* Phytoplasma asteris" species carry genes that encode two immunodominant membrane proteins, Imp and Amp (14), and both of the genes encoding those proteins are objects of positive selection (13, 47). The *amp* transcripts were the most abundant of all the analyzed gene transcripts in all three host species, and the *amp* transcript levels decreased in *A. thaliana* and *M. quadripunctulatus* during the CYP infection cycle. Amp interacts specifically with the *M. striifrons* actin vector (10), possibly to enable phytoplasma motility (11). Indeed, *amp* transcript levels were stable during infection of *E. variegatus*, possibly implying a continuous need of the gene product to reach stable colonization of the insect body. Actually, CYP colonization of *E. variegatus* is slower than that of *M. quadripunctulatus* (30). Accordingly, *amp* transcript levels decreased during infection of *M. quadripunctulatus*. There is no information on the possible role of phytoplasma Amp in plant, but the low EI in *M. quadripunctulatus* at 28 dpa (when infective insects were caged for inoculation of healthy plants) and the high EI in *A. thaliana* at the first sampling date (10 dpi) suggest a role for Amp in the host switching for the colonization of the plant. Irrespective of the host species, *amp* transcripts were always more actively abundant than *imp* transcripts during the infection cycle, and this was in line with a previous finding determined for a closely related phytoplasma strain (13). *Imp* transcript levels were constant over time in *E. variegatus*, but not in *M. quadripunctulatus*, suggesting different gene regulation patterns in the two vector environments. *imp* was also the most stable gene with respect to transcripts among the 14 studied in *A. thaliana*. Nothing is known of the role of "*Ca.* Phytoplasma asteris" *imp* in the plant, although this gene is under positive selection in different phytoplasmas (13, 15). Imp of "*Ca.* Phytoplasma mali" binds to plant actin with a suggested role in phytoplasma motility (12).

**Transporter genes.** The "*Ca.* Phytoplasma asteris" genome includes several transporter genes (48, 49). Transcripts of genes in this category were abundant in the plant host on average, and, in particular, transcripts of *mscL* and *artI* were significantly more abundant in *A. thaliana* than in the two vectors. Also, the *mscL*

gene transcripts were the most abundant transporter gene transcripts during the CYP infection cycle in the three hosts. Microbial cells constitutively express the large mechanosensitive (MS) conductance channel that opens in response to stretch forces in the lipid bilayer, and the gene is upregulated in the presence of osmotic downshocks to protect from cell lysis (50). Phytoplasmas move from the plant to the vector body during feeding and are therefore subjected to severe osmotic stresses, as the osmotic pressure of the plant phloem is, on average, two to five times higher than that of insect body fluids (51). *mscL* transcription might be induced in the plant, when phytoplasma cells are exposed to high osmolarity, to prepare for the eventuality of hypo-osmotic stress conditions when phytoplasma cells are acquired by the vector. Indeed, *de novo* gene expression cannot modulate the levels of MS channel proteins on a short time scale (50). Moreover, Oshima et al. (16) showed that phytoplasma growth *in planta* was partially suppressed by gadolinium chloride, an inhibitor of the MscL osmotic channel, emphasizing its additional role in facilitating phytoplasma growth in plant. Transcripts of other genes in this category (*artI* and *oppC*) were among the least abundant during the CYP infection cycle in both vectors. In bacteria, Opp transport systems participate in a wide range of biological events, including biofilm formation (52), antimicrobial-compound production (53), and adaptation to different environments (54–57), through the modulation of the cell membrane lipid profile (58). In bacteria, *artI* is the periplasmic binding component of the arginine transporter system, specifically binding arginine and ornithine. Arginine is a key amino acid for the bacterial cell, and its metabolism and regulation are linked to the virulence of several pathogenic bacterial species such as *Mycobacterium tuberculosis*, *M. bovis*, *Listeria monocytogenes*, and *Legionella pneumophila* (59–62). Phloem sap has, in general, a lower concentration of essential amino acids than that found in optimal diets for some phloem feeders (63). We can speculate that the presence of arginine in the insect hosts downregulates the transcription of CYP *artI* and that the transcription resumes upon inoculation of the phytoplasma in the plant host. Interestingly, *oppC*, *artI*, *ftsY*, and *mdlB* transcript levels were also stable during the CYP infection cycle in *E. variegatus* and were among those that decreased least in *M. quadripunctulatus*. This suggests that basal transcription of these genes occurs in the insect milieu. In contrast, genes in this category were subjected to severe transcript reduction over time in the plant, *oppC* being the most dramatically affected. Zinc, together with iron, manganese, and copper, is required by all living organisms, and maintaining adequate intracellular levels of transition metals is fundamental to the survival of all organisms. Transcript levels of *zntA*, predicted to encode the soluble periplasmic metallochaperone that captures zinc and delivers it to the transmembrane component of the transporter, decreased in the three hosts during infection. Pathogens use low-metal conditions as a signal to recognize and respond to the host environment (64), and *Salmonella* exploits the ZnuABC zinc transporter to maximize zinc availability during growth within the infected animal under conditions where amounts of free metals available for bacterial growth are limited (65). The *zntA* EI value was lower for *M. quadripunctulatus* than for *E. variegatus*, and the transcript levels increased by 10 days after phytoplasma inoculation in the plant host, suggesting a role of zinc in the infection process. There is no information on the possible role of phytoplasma transporter genes in plant host, although the low EI value for *M. quadripunctulatus* at 28 dpa (when infective insects were caged on healthy plants for IAP) and the high EI value for all of the genes, except *ftsY*, in *A. thaliana* at the first sampling date (10 dpi) suggest a role in the colonization of the plant host. Recently, the involvement of transport proteins as additional bacterial cell sensors has been explored (66), as these proteins are well informed about the presence of substrates outside the cell. FtsY protein is involved in cellular secretion, as part of the internal channel of the Sec secretion system that is functional in the "*Ca*. Phytoplasma asteris" OY strain (67) and in the virulence of *Rickettsia* spp. (68) and *Pseudomonas aeruginosa* (69).

**Secreted proteins.** Effector proteins are secreted from phytoplasmas via the Sec translocation system and function directly in the host cells (70). About 50 putative secreted proteins are present in phytoplasma genomes, and these effector genes differ from those found in other plant-pathogenic bacteria (71). Some of these have been shown to encode functional effectors, including SAP11, SAP54, SAP67, SAP68, and TENGU (8). SAP11, known to induce a bushy morphology and to enhance vector fitness by blocking jasmonic acid biosynthesis in plants (72), was not found in the draft genome of CYP, and it was not detected by PCR with specific primers designed for the closely related AY-WB phytoplasma (73). Transcripts encoding the four secreted proteins were all present in the three hosts, suggesting a role of these effectors during the CYP life cycle in both plant and insect hosts. Interestingly, *sap67* transcripts were present at the same levels in *A. thaliana* and in the two vector species, while the *tengu* and *sap54* transcripts were among the most abundant transcripts in the two insect vectors. SAP54 and its homolog, PHYL1, of "*Ca*. Phytoplasma asteris" AY-WB and OY, respectively, induce phyllody-like flower abnormalities (74), probably through the ubiquitin-mediated proteasome-dependent degradation of MADS domain proteins involved in floral development (75, 76). TENGU is a small, secreted peptide encoded by OYP that affects plant morphology with the production of typical phytoplasma witches' broom symptoms, through the inhibition of the auxin-related pathways (77). The high EI values of *sap54* and *tengu* during the infection cycle of the two vectors together with the comparable EI values of *sap67* in plant and in the two insects indicate that these genes must have a role in the infection of the animal host.

**General metabolism.** Transcripts encoding PgsA, the limiting enzyme in the synthesis of phosphatidylglycerol, the major constituent of bacterial membranes, were present at comparable levels at early phases of infection of the plant and both insect species, supporting its role in the maintenance of essential bacterial phospholipids irrespective of the host species. Transcripts of *rpsU*, coding 30S ribosomal subunit protein S21, were more abundant in *A. thaliana* than in *M. quadripunctulatus*, this being in line with previous results determined for OYP (16). Nevertheless, *rpsU* transcript levels in *A. thaliana* and in *E. variegatus* were similar and were constant in the latter species at all analyzed time points. This gene is known to be stably transcribed at different time points through the entire *in vitro* life cycle of *Bacillus cereus* (78), and it can be considered, together with the general and specific transporter genes *mdlB*, *oppC*, *ftsY*, and *artI*, a candidate for future normalization of CYP gene expression in *E. variegatus*.

Expression of 14 CYP phytoplasma genes at different times postinfection of *A. thaliana* and the leafhopper vectors *M. quadripunctulatus* and *E. variegatus* was addressed to highlight the different transcription profiles of the bacteria in the plant and animal host. The transcription patterns of genes within the four analyzed

categories differed according to the host species, suggesting that the bacteria are able to sense diverse environments and respond accordingly. Moreover, CYP transcript profiles of genes within the same category differed between the two leafhoppers, indicating the ability of CYP to distinguish between leafhopper host environments. Transcripts encoding Amp and the four secreted proteins were present in the three hosts, suggesting the important role of this immunodominant protein and unpredicted functions of these secreted phytoplasma proteins also during leafhopper infection. To explain the observed decrease of phytoplasma transcript levels during the CYP infection cycles in the plant and insect hosts starting from 7 to 10 days postinfection onwards, we might speculate that, during host colonization, new phloem elements and insect cells or organs are progressively invaded, possibly by actively multiplying and transcribing phytoplasma cells, while the transcriptional activity of older cells may slow down. In this hypothesis, phytoplasmas invading the plant phloem would form aggregates (79) that might enter a stationary phase, possibly to improve long-term survival, thereby increasing the likelihood of transmission. In this hypothesis, the number of cells (EI denominator) entering the stationary phase would increase with time after inoculation, while the number of new colony-forming cells would be low on average (as it would represent only a fraction of the measured total phytoplasma DNA). A similar mechanism may happen during colonization of the insect vectors, when phytoplasmas would not be able to escape the vector body and invade new tissues without inoculation to a new plant. In line with this hypothesis, phytoplasmas, being obligate parasites of plant and insects, sense the environment and switch their metabolism accordingly. Despite obvious differences, a very recent pioneer study has proposed that the xylem-limited bacterium *Xylella fastidiosa* switches its life style from adhesive cells capable of insect transmission to an "exploratory" lifestyle for systemic spread within the plant by production of outer membrane vesicles (80). Interestingly, the release of these vesicles is suppressed by a diffusible signal factor-dependent quorum-sensing system (80).

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