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Distribution and Expression of Elicitin Genes in the Interspecific Hybrid Oomycete *Phytophthora alni*▽

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***Phytophthora alni* subsp. *alni*, *P. alni* subsp. *multiformis*, and *P. alni* subsp. *uniformis* are responsible for alder disease in Europe. Class I and II elicitin gene patterns of *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis*, *P. alni* subsp. *uniformis*, and the phylogenetically close species *P. cambivora* and *P. fragariae* were studied through mRNA sequencing and 3′ untranslated region (3′UTR)-specific PCRs and sequencing. The occurrence of multiple 3′UTR sequences in association with identical elicitin-encoding sequences in *P. alni* subsp. *alni* indicated duplication/recombination events. The mRNA pattern displayed by *P. alni* subsp. *alni* demonstrated that elicitin genes from all the parental genomes are actually expressed in this allopolyploid taxon. The complementary elicitin patterns resolved confirmed the possible involvement of *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* in the genesis of the hybrid species *P. alni* subsp. *alni*. The occurrence of multiple and common elicitin gene sequences throughout *P. cambivora*, *P. fragariae*, and *P. alni* sensu lato, not observed in other *Phytophthora* species, suggests that duplication of these genes occurred before the radiation of these species.**

The oomycete *Phytophthora alni* (stramenopile lineage) is a recently described highly aggressive pathogen specific to alder trees (*Alnus* spp.) that is spreading all over Europe, especially along rivers (13). *P. alni* sensu lato comprises three related taxa: *P. alni* subsp. *alni*, *P. alni* subsp. *uniformis*, and *P. alni* subsp. *multiformis* (5). *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* are scarce in comparison to *P. alni* subsp. *alni* (2, 15). Up to now, all three sibling taxa have been exclusively isolated from alder trees, but *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* are reported to be significantly less aggressive to *Alnus* than *P. alni* subsp. *alni* (4, 30). The three taxa are phylogenetically close to *P. cambivora* and *P. fragariae*, two species that, it was previously suggested, may be *P. alni* subsp. *alni*'s progenitors (3). However, it was recently inferred from nuclear and mitochondrial gene genealogies (15) and microsatellite patterns (16) that only *P. alni* subsp. *alni* is a genuine hybrid taxon, originating from hybridization between *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*. The status of *P. alni* subsp. *multiformis* is still questionable, while *P. alni* subsp. *uniformis*'s genetic features do not fit with a hybrid origin (15, 16). Up to now, reports of other natural hybrids within the genus *Phytophthora* are scarce and remain confined to limited geographical areas (19, 20), whereas the allopolyploid taxon *P. alni* subsp. *alni* is currently thriving throughout Europe. Since this hybrid taxon is significantly more aggressive than its putative parents, *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis* (4), heterosis or genetic rearrangement may be put forward as explanations for this kind of ecological advantage. Except for the observation of polymorphism in the internal transcribed spacer sequence (3), the extent of genetic rearrangement in the allopolyploid taxon *P. alni* subsp. *alni* remains unknown, and the expression of distinct genomes within this hybrid taxon has not been investigated yet.

In order to address these issues, multigenic families, such as elicitin genes, should be of great interest since they are particularly prone to duplication and recombination. Elicitin proteins are restricted to the oomycete genus *Phytophthora* and a few *Pythium* species (18, 25). They comprise a large family of polypeptides whose intrinsic function remains largely unknown and that can be divided into at least eight classes (18, 23). Elicitin genes are highly transcribed during vegetative growth, as deduced from their representation in a collection of expressed sequence tags from the broad-host-range pathogen *Phytophthora parasitica* (23). In particular, group I elicitins (ELI-1), characterized by the typical 98-amino-acid elicitin domain, were reported to be the most abundant secreted proteins in culture filtrates (18). Although elicitin genes are not appropriate for phylogenetic studies because of their multigenic family feature, they can be used as a tool for identification purposes since the amino acid sequence of a given elicitin may provide a signature at the species level (25). In addition, the 3′ untranslated regions (3′UTRs) of class I elicitin genes are strictly conserved within an individual species but diverge between species to such an extent that sequence alignment is almost impossible (1, 6, 11; Panabières et al., unpublished results).

The aims of the present work were, by taking advantage of the high expression of elicitin genes in the *Phytophthora* genome, (i) to test if the multiple genomes present in the allopolyploid hybrid *P. alni* subsp. *alni* are coexpressed or not

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TABLE 1. List of the *Phytophthora* spp. and *Pythium* spp. used in this study

Taxon	Isolate[g]	Host	Origin	Yr	Isolator/supplier
P. alni subsp. *alni*	PAA2	*Alnus glutinosa*	France	2002	J. C. Streito (2N0685)
	PAA20	*Alnus glutinosa*	France	1997	J. C. Streito (71T1)
	PAA21	*Alnus glutinosa*	France	1997	J. C. Streito (77T4)
	PAA23	*Alnus glutinosa*	France	1997	J. C. Streito (82T1A)
	PAA24	*Alnus glutinosa*	France	1997	J. C. Streito (84T2)
	PAA34	*Alnus glutinosa*	France	1998	J. C. Streito (98-7-5)
	PAA35	*Alnus glutinosa*	France	1998	J. C. Streito (98-7-6)
	PAA38	*Alnus glutinosa*	France	2002	J. C. Streito (2N0529)
	PAA44	*Alnus glutinosa*	France	1998	J. C. Streito (DSFO98172)
	PAA47	*Alnus glutinosa*	France	1999	J. C. Streito (AUL026/1)
	PAA52	*Alnus glutinosa*	France	1999	J. C. Streito (9900783.4)
	PAA53	*Alnus glutinosa*	France	2001	J. C. Streito (1R0152)
	PAA58	*Alnus glutinosa*	France	2001	J. C. Streito (1N0201)
	PAA100	*Alnus glutinosa*	France	2003	R. Ioos (P1bisa)
	PAA103	*Alnus glutinosa*	France	2003	R. Ioos (P3a)
	PAA107	*Alnus glutinosa*	France	2003	R. Ioos (Priva)
	PAA108	*Alnus glutinosa*	France	2003	R. Ioos (Privb)
	PAA109	*Alnus glutinosa*	France	2003	R. Ioos (P6-2)
	PAA110	*Alnus glutinosa*	France	2003	R. Ioos (P6-1)
	PAA111	*A. glutinosa* soil	France	2003	C. Husson (Ainvelle Sol)
	PAA112	*Alnus glutinosa*	France	2003	C. Husson (2ALD03)
	PAA113	*Alnus glutinosa*	France	2003	C. Husson (102-1)
	PAA114	*Alnus glutinosa*	France	2002	C. Husson (Moselle)
	PAA115	*Alnus glutinosa*	France	2002	C. Husson (370-2)
	PAA116	*Alnus glutinosa*	France	2003	R. Ioos (3N10094-5a)
	PAA118	*Alnus glutinosa*	France	2003	R. Ioos (3N10094-5c)
	PAA120	*Alnus glutinosa*	France	2003	R. Ioos (3N10048-3a)
	PAA121	*Alnus glutinosa*	France	2003	R. Ioos (3N10048-3b)
	PAA125	*Alnus glutinosa*	France	2003	R. Ioos (3N10048-3f)
	PAA126	*Alnus glutinosa*	France	2003	C. Husson (Ainvelle4-4)
	PAA127	*Alnus glutinosa*	France	2003	C. Husson (Ainvelle1-2)
	PAA128	*Alnus glutinosa*	France	2003	C. Husson (Ainvelle1-1)
	PAA129*	*Alnus glutinosa*	France	2003	G. Capron (703)
	PAA130*	*Alnus glutinosa*	France	2003	R. Ioos (1429-6b)
	PAA131	*A. glutinosa*, soil	France	2003	C. Husson (Sol A15)
	PAA132	*A. glutinosa*, soil	France	2003	C. Husson (Sol A1)
	PAA133	*A. glutinosa*, soil	France	2003	C. Husson (Sol A7)
	PAA151*	*Alnus glutinosa*	France	2004	B. Thoirain (2051000-D12)
	PAA185	*Alnus glutinosa*	France	2004	R. Ioos (4N1605)
	PAA29	*Alnus glutinosa*	Belgium	1999	J. C. Streito (9900715.6)
	PAA86	*Alnus glutinosa*	Belgium	1999	D. De Merlier (2198[c])
	PAA88	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2295[c])
	PAA70	*Alnus* sp.	The Netherlands	Unknown	W. Man in't Veld (PD2010953)
	PAA74	*Alnus glutinosa*	Scotland	2000	G. Mackaskill (P1275)
	PAA75	*Alnus viridis*	Scotland	2000	J. Gibbs (P1272)
	PAA76	*Alnus glutinosa*	Scotland	2000	J. Gibbs (P1271)
	PAA77	*Alnus glutinosa*	Scotland	2000	J. Delcan (P1270)
	PAA78	*Alnus glutinosa*	England	1997	J. Delcan (P1960)
	PAA79	*Alnus glutinosa*	England	1997	J. Delcan (P957[a])
	PAA80	*Alnus glutinosa*	England	1997	J. Delcan (P950[a])
	PAA81	*Alnus glutinosa*	England	1997	J. Delcan (P937)
	PAA82	*Alnus glutinosa*	England	1996	S. Gregory (P850)
	PAA85	*Alnus glutinosa*	England	Unknown	C. Brasier (P834[e])
	PAA91	*Alnus glutinosa*	Hungary	2001	Z. Nagy (6[d])
	PAA92	*A. glutinosa*, soil	Hungary	2001	Z. Nagy (8[d])
	PAA93	*A. glutinosa*, soil	Hungary	2001	Z. Nagy (9[d])
	PAA94	*A. glutinosa*, soil	Hungary	2001	Z. Nagy (1a[d])
	PAA95	*Alnus glutinosa*	Hungary	2001	Z. Nagy (4-2[d])
	PAA134	*Alnus glutinosa*	Germany	2000	K. Kaminski (BBA 23/00)
	PAA162*	*Alnus glutinosa*	Germany	2004	R. Ioos (9a)
	PAA168	*Alnus glutinosa*	Germany	2004	R. Ioos (8b)
	PAA141	*Alnus glutinosa*	Austria	Unknown	T. Cech (Pucking B10)
	PAA143*	*Alnus glutinosa*	Poland	2002	G. Skuta (PO 192)
	PAA144	*Alnus glutinosa*	Poland	2003	G. Skuta (PO 193)
	PAA145	*Alnus glutinosa*	Poland	2004	G. Skuta (PO 203)
	PAA146	*Alnus glutinosa*	Poland	2002	G. Skuta (PO 205)

Continued on following page

TABLE 1—*Continued*

Taxon	Isolate[g]	Host	Origin	Yr	Isolator/supplier
	PAA189	*A. glutinosa*, soil	Poland	2004	L. Orlikowski (*P. alni* soil)
	PAA190	*Alnus glutinosa*	Poland	2004	L. Orlikowski (*P. alni* 5-yo)
P. alni subsp. *uniformis*	PAU60*	*Alnus glutinosa*	France	1999	J. C. Streito (AUL028)
	PAU84*	*Alnus glutinosa*	Sweden	1997	C. Olsson (P875[a,b,c,f])
	PAU87	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2271[c])
	PAU187	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2276[c])
	PAU188	*Alnus incana*	Belgium	2001	D. De Merlier (2277[c])
	PAU89*	*Alnus cordata*	Italy	2000	P. Capretti (CBS109280[e])
	PAU96	*Alnus glutinosa*	Hungary	1999	Z. Nagy (155-a[d])
	PAU97	*A. glutinosa*, soil	Hungary	1999	Z. Nagy (155-b[d])
	PAU98	*A. glutinosa*, soil	Hungary	1999	Z. Nagy (155-c[d])
	PAU142	*Alnus glutinosa*	Slovenia	2003	A. Munda (Phy-A-Slo)
P. alni subsp. *multiformis*	PAM54*	*Alnus glutinosa*	France	2000	J. C. Streito (DSFO/0125)
	PAM71*	*Alnus glutinosa*, soil	The Netherlands	Unknown	W. Man in't Veld (W1139)
	PAM90	*Alnus glutinosa*, soil	The Netherlands	Unknown	W. Man in't Veld (P972[a,c,f])
	PAM73*	*Alnus glutinosa*	United Kingdom	1996	S. Gregory (P841[a,c,f])
	PAM186	*Alnus glutinosa*	Belgium	2001	D. De Merlier (2274[c])
P. cambivora	PC463	*Castanea sativa*	France	1994	INRA Bordeaux
P. cambivora	PC643*	*C. sativa*, soil	France	2000	INRA Bordeaux
P. cambivora	PCjc17*	*Quercus* sp., soil	France	1999	C. Delatour
P. cambivora	PCGA1	*Quercus* sp., soil	France	1999	C. Delatour
P. cambivora	PC99428	*Castanea sativa*	France	1999	R. Ioos
P. cambivora	PCST3R1	*Quercus petraea*	France	1999	C. Delatour
P. cambivora	PC627	*Castanea sativa*	Italy	2000	INRA Bordeaux
P. cambivora	PC1A21	*Quercus* sp., soil	France	1999	INRA Bordeaux
P. cambivora	PC4N1425	*Castanea sativa*	France	2004	LNPV-UMAF
P. cambivora	PC4N444	*Castanea sativa*	France	2004	LNPV-UMAF
P. fragariae var. *fragariae*	PFF1	*Fragaria* × *ananassa*	United Kingdom	Unknown	K. Hughes
P. fragariae var. *fragariae*	PFF209.46	*Fragaria* × *ananassa*	United Kingdom	1946	CBS (CBS209.46)
P. fragariae var. *fragariae*	PFF309*	*Fragaria* × *ananassa*	United Kingdom	1962	CBS (CBS 309.62)
P. fragariae var. *rubi*	PFRVR 59	*Rubus* sp.	United Kingdom	Unknown	D. Cooke (FVR 59)
P. fragariae var. *rubi*	PFR163-2	*Rubus* sp.	France	Unknown	A. Baudry (163-2)
P. fragariae var. *rubi*	PFR2	*Rubus* sp.	United Kingdom	Unknown	K. Hughes
P. fragariae var. *rubi*	PFR967.95	*Rubus* sp.	United Kingdom	1985	CBS (CBS967.95)
P. fragariae var. *rubi*	PFR109*	*Rubus* sp.	United Kingdom	1991	CBS (CBS109.892)
P. cactorum	CAC4810/TJ	Unknown	France	Unknown	C. Delatour
P. cinnamomi	DSFO2N0964	*Castanea sativa*	France	2002	J. C. Streito
P. cinnamomi	DSFA970060	*Quercus suber*	France	1997	J. C. Streito
P. cinnamomi	DSFO990050	*C. sativa*, soil	France	1999	J. C. Streito
P. cinnamomi	P382	*Nothofagus procera*, soil	United Kingdom	1980	C. Brasier
P. citricola	2N0750-171	Unknown	France	2002	J. C. Streito
P. citricola	AUL 045 AP7	*Alnus glutinosa*	France	1999	J. C. Streito
P. citricola	2AE5	*Quercus* sp., soil	France	1998	C. Delatour
P. citricola	3N1345-17	*Alnus glutinosa*	France	2003	R. Ioos
P. citrophthora	2N1021	*Rosa* sp.	France	2002	J. C. Streito
P. cryptogea	990675	*Actinidia chinensis*	France	1999	J. C. Streito
P. erythroseptica	960713	*Polygonum oberti*	France	1999	J. C. Streito
P. europaea	AL5	*Quercus* sp., soil	France	1998	C. Delatour
P. europaea	2AU2	*Quercus* sp., soil	France	1999	C. Delatour
P. gonapodyides	Gonap 4	*Quercus* sp., soil	France	1998	C. Delatour
P. gonapodyides	AB4	*Quercus* sp., soil	France	1998	C. Delatour
P. humicola	3N1245-j	*A. glutinosa*, soil	France	2003	R. Ioos
P. ilicis	3N1245-l	*A. glutinosa*, soil	France	2003	R. Ioos
P. inundata	9500802	*A. glutinosa*, soil	France	1995	J. C. Streito
P. lateralis	98093.1-SPV	*Chamaecyparis* sp.	France	1998	J. C. Streito
P. megasperma	3N1245-m	*A. glutinosa*, soil	France	2003	R. Ioos
P. megasperma	BK1	*Quercus* sp., soil	France	1998	C. Delatour
P. megasperma	03–12	Water under *Quercus* sp.	France	1998	C. Delatour
P. megasperma	mega 1	Unknown	Germany	1998	T. Jung
P. megasperma	8RPOC3	*Quercus* sp., soil	France	1998	C. Delatour
P. nicotianae	960579	*Nicotiana tabacum*	France	1996	J. C. Streito
Phytophthora taxon forestsoil	8CARPPOC1	*Quercus* sp., soil	France	1998	C. Delatour
P. palmivora	970423	*Hedera* sp.	France	1997	J. C. Streito
P. parasitica	970029	*Lycopersicon esculentum*	France	1997	J. C. Streito
Phytophthora taxon Pgchlamydo	Haye,3,1	*Quercus* sp., soil	France	1998	C. Delatour

Continued on following page

TABLE 1—*Continued*

Taxon	Isolate[g]	Host	Origin	Yr	Isolator/supplier
P. pseudosyringae	EW5	*Quercus* sp., soil	France	1998	C. Delatour
P. psychrophila	FF20	*Quercus* sp., soil	France	1998	C. Delatour
P. quercina	FNA	*Quercus* sp., soil	France	1999	C. Delatour
P. quercina	Mers2	*Quercus* sp., soil	France	1999	C. Delatour
P. ramorum	2N0983	*Rhododendron* sp.	France	2002	C. Saurat
P. ramorum	3N0003	*Viburnum* sp.	France	2002	C. Saurat
P. sojae	443	*Glycine max*	Unknown	Unknown	F. Panabières
P. syringae	2JZ2	*Quercus* sp., soil	France	1999	C. Delatour
Pythium aphanidermatum	Ctsa	Unknown	France	2003	S. Verger
Pythium sylvaticum	0675/a	Unknown	France	2003	S. Verger
Pythium intermedium	02/84/1	Unknown	France	Unknown	S. Verger
Pythium irregulare	02/57/1	Unknown	France	Unknown	S. Verger
Pythium ultimum	433/3	Unknown	France	Unknown	S. Verger
Pythium sp.	3N1345-11	*A. glutinosa*, soil	France	2003	R. Ioos

[a] Also studied by Delcan and Brasier (9).
[b] Also studied by Brasier et al. (3).
[c] Also studied by De Merlier et al. (10).
[d] Also studied by Nagy et al. (22).
[e] Also studied by Santini et al. (30).
[f] Also studied by Brasier and Kirk (4).
[g] *, isolate used for mRNA production in this study and also studied by Ioos et al. (15).

and (ii) to assess the occurrence of genetic rearrangements. We studied the occurrence and the distribution of members of class I and II elicitin genes and their expression among the different *P. alni* taxa. This study included the phylogenetically close species *P. cambivora*, *P. fragariae* var. *fragariae*, and *P. fragariae* var. *rubi*, previously suggested as *P. alni* subsp. *alni*'s progenitors (3), for comparison of their elicitin gene patterns.

MATERIALS AND METHODS

***Phytophthora* isolates and culture.** French isolates of *Phytophthora alni* sensu lato and other *Phytophthora* spp. were collected on naturally infected tissues and isolated on PARBHY medium (28). Foreign isolates of *P. alni* sensu lato and *Phytophthora* spp. were obtained from the Centraalbureau voor Schimmelcultures (Utrecht, The Netherlands) or from collaborative researchers (Table 1). All the cultures were kept at 10°C in the dark on V8-agar slants (21) and as small V8-agar blocks flooded with sterile distilled water. Five isolates of *P. alni* subsp. *alni* (PAA129, PAA130, PAA143, PAA151, and PAA162), three isolates of *P. alni* subsp. *uniformis* (PAU60, PAU84, and PAU89), three isolates of *P. alni* subsp. *multiformis* (PAM54, PAM71, and PAM73), two isolates of *P. cambivora* (PC643 and PCjc17), one isolate of *P. fragariae* var. *fragariae* (PFF309), and one isolate of *P. fragariae* var. *rubi* (PFR109), selected from different geographical locations, were used for in vitro elicitin production and mRNA studies (Table 1).

Nucleic acid manipulation. Genomic DNA was extracted from 5-day-old cultures grown in shake culture in liquid V8 juice medium (21) at 20°C using a plant DNA extraction kit (DNeasy plant minikit; QIAGEN, Courtaboeuf, France) by following the manufacturer's instructions with slight modifications. Briefly, ca. 200 mg of fresh mycelium was harvested and mixed in a 2-ml tube with 400 μl of lysis buffer and 4 μl of the RNase A provided with the kit. The mixture was ground for 2 min with two 3-mm tungsten carbide beads at a frequency of 30 Hz, using a mixer mill grinder (Tissuelyser; QIAGEN). The ground solution was subsequently centrifuged for 5 min at 15,000 × *g* to compact the debris, and the resulting supernatant was treated in accordance with the manufacturer's instructions. Genomic DNA was stored at −20°C until used for PCR tests.

To enhance elicitin mRNA synthesis, oomycete cultures were grown for 3 days in shake culture in liquid elicitin secretion medium (ESM; M. Horta [Algarve University, Portugal], personal communication) at 20°C. The composition of the ESM was 0.05% (wt/vol) KH_2PO_4, 0.025% (wt/vol) $MgSO_4 \cdot 7H_2O$, 0.1% (wt/vol) asparagine, 1 mg/liter thiamine, 0.05% (wt/vol) yeast extract, and 2% (wt/vol) glucose. The medium was sterilized by filtration through a 0.2-μm membrane. mRNAs were extracted using a QuickPrep micro-mRNA purification kit with oligo(dT) cellulose (Amersham Biosciences, Orsay, France) in accordance with the manufacturer's instructions, resuspended in 40 μl of diethyl pyrocarbonate-treated molecular biology grade water, and stored at −80°C.

Reverse transcription-PCR, cloning, and sequencing of elicitin mRNA. Polyadenylated RNAs were reverse-transcribed using a SuperScript first-strand synthesis system (Invitrogen, Cergy Pontoise, France) with NotI-oligo(dT) [5′-ATTCGCGGCCGCAGGA$(T)_{16}$-3′] (25). A PCR was performed on the cDNA template using a combination of the NotI-oligo(dT) primer and degenerate primer 1 (5′-ATGAACTTCCGCGCTCTSYTYGC-3′), initially designed from conserved sequences of class I elicitins located in the peptide signal region (25). This primer was assumed to efficiently anneal to the peptide signal region of every elicitin class I gene unraveled until now. PCRs were carried out in a 20-μl PCR mixture containing 1× polymerase buffer (Sigma-Aldrich, L'Isle d'Abeau, France), 0.9 mM $MgCl_2$, 0.3 μM of each primer, 180 μM deoxynucleoside triphosphates, 0.6 unit of *Taq* DNA polymerase (Sigma-Aldrich), and 2 μl of template cDNA. Molecular biology grade water was added to 20 μl. For each isolate, PCRs were also performed using genomic DNA as a negative control to test potential amplification of genomic DNA in cDNA amplification. The PCRs were carried out with a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA). The cycling profile included an initial denaturation step at 95°C for 2 min, followed by 35 cycles of denaturation, annealing, and elongation for, respectively, 20 s at 94°C, 30 s at 60°C, and 1 min at 72°C and a final extension step at 72°C for 7 min. PCR fragments were separated by a 1-h electrophoresis on a 1% agarose gel at 4 $V \cdot cm^{-1}$. Gels were stained with ethidium bromide, and images were recorded with a charge-coupled device camera and a GELDOC 2000 system (Bio-Rad, Marne-La-Coquette, France).

The PCR products generated with cDNAs were cloned for each of the 15 isolates tested using the pCR4-TOPO TA cloning kit (Invitrogen). Five microliters of the PCR product was transferred into a sterile 1.5-ml microcentrifuge tube, and the amplicons were ligated to a TOPO vector (Invitrogen) and used to transform TOP 10-competent cells (Invitrogen) according to the manufacturer's instructions. Positive clones were selected by PCR amplification of inserts with M13 sequencing primers. Positive clones were selected according to their expected PCR product sizes, corresponding to class I elicitin transcripts. PCR products were purified by centrifugation using a polyethylene glycol 8000 solution as described by Rosenthal et al. (29). Double-stranded DNA sequencing was performed by the dideoxy chain termination method using a T3-T7 sequencing kit on a CEQ 2000 XL DNA sequencer (Beckman, Fullerton, CA). Sequences were edited with Sequencher software (Gene Codes, Ann Arbor, MI) and aligned using ClustalW (33) (Table 2). The cDNA sequences were translated using Fast PCR software, version 3.6.62 (R. Kalendar, FastPCR, PCR primer design, DNA and protein tools, repeats and own database search program [www.biocenter.helsinki.fi/bi/programs/fastpcr.htm]). The isoelectric points (pI) of the deduced proteins were calculated using IEP online software (http://bioweb.pasteur.fr/seqanal/interfaces/iep.html). Multiple amino acid sequence alignments with hierarchical clustering were performed using MultAlin program, version 5.3.3 (8), with Blossum 62 as symbol comparison table. An unrooted

TABLE 2. Characteristics of all the different cDNA and genomic sequences obtained and assignation to a specific 3′UTR group

Taxon	Isolate	Elicitin	3′UTR group	GenBank accession no. of:	
				cDNA sequence	Genomic sequence
P. alni subsp. *alni*	PAA129	AE1.1	a2	DQ012518	
		AE1.1	a10′		EF158402
		AE1.2	a2		EF158401
		AE2	a3	DQ012517	
	PAA130	AE1.1	a1		EF158403
		AE1.1	a10′		EF158404
		AE1.2	a1	DQ012520	
		AE1.2	a5	DQ012519	
		BE1	b2		EF158405
	PAA143	AE1.2	a1	DQ012521	
		AE1.2	a2	DQ012522	
		AE2	a4		EF158406
	PAA151	AE1.2	a2	DQ012524	
		AE1.2	a3′	DQ012525	
		BE2	b1	DQ012523	
	PAA162	AE1.2	a2	DQ012527	
		BE1	b1	DQ012526	
P. alni subsp. *multiformis*	PAM54	AE1.1	a1	DQ012508	
		AE1.1	a2		EF158407
		AE1.2	a2		EF158408
		AE1.2	a3′		EF158409
		AE2	a3		EF158410
		BE1	b1		EF158411
		HAE1	ha1	DQ012509	
	PAM71	AE1.1	a8		EF158415
		AE1.2	a3′		EF158413
		AE2	a4	DQ012510	
		AE2	a3		EF158414
		BE1	b1	DQ012511	
		BE1	b1		EF158412
	PAM73	AE2	a3	DQ012513	
		BE1	b1	DQ012512	
P. alni subsp. *uniformis*	PAU60	AE1.1	a1		EF158417
		BE1	b2		EF158416
	PAU84	AE1.1	a1	DQ012514	
		AE1.1	a1		EF158418
		AE1.1	a10′		EF158419
	PAU89	AE1.1	a1	DQ012516	
		AE1.1	a1		EF158420
		HAE1	ha1	DQ012515	
	PAU142	AE1.1	a1		EF158421
	PAU188	AE1.1	a1		EF158422
P. cambivora	PC643	AE1.1	a6	DQ012529	
		AE1.1	a8	DQ012528	
		AE1.1	a2′		EF158424
		AE2	a10″		EF158423
	PCjc17	AE1.1	a6	DQ012530	
		AE1.1	a7	DQ012531	
		AE1.1	a8		EF158425
P. fragariae var. *fragariae*	PFF309	AE2	a9	DQ012532	
		AE2	a11	DQ012533	
P. fragariae var. *rubi*	PFR109	AE1.1	a9	DQ012535	
		AE1.1	a10		EF158426
		AE1.1	a10	DQ012534	

phylogram was built using a parsimony analysis and the neighbor-joining method in PAUP* 4.0b10 (32). Bootstrap values were computed on 10,000 replicates.

3′UTR-specific PCR detection and sequencing. Based on cDNA sequence alignment, a set of 11 reverse primers was designed to target 3′UTR-specific regions among elicitin-encoding sequences (Table 3). Primers were synthesized by Invitrogen and tested on the 15-isolate panel, then on the entire *Phytophthora* and *Pythium* collection listed in Table 1.

The 3′UTR PCR tests were carried out in a 20-μl mixture containing 1× polymerase buffer (Sigma-Aldrich), 1.8 mM $MgCl_2$, 0.45 μM of degenerate primer 1, 0.45 μM of 3′UTR-specific primer, 180 μM deoxynucleoside triphos-

TABLE 3. List and sequences of the 3′UTR-specific reverse primers designed in this study

Elicitin class	Code	Sequence (5′–3′)
I, acidic	a2-R	AGG GTG GAT GGG GGA TTG CCA
	a3-R	CGA AGA CAC GTC GGT ATC CAT
	a4-R	GAC AAG TCG GCA TAA CAA AC
	a5-R	GCT CAG ACA ACA CTC AAG CT
	a7-R	GCT GAA ACA ATG CTC AAG A
	a8-R	GCT GAT CTG AAG ACG AGT C
	a10-R	GCT GCG TAC TTA GTC CAC GC
	a11-R	CTG CAT CGG AAT TCC AAC AAC
I, basic	b1-R	CTT CGA GTT AAT GGC GTA TTA
	b2-R	CCT TGA GTT TTA ATG GTA GA
II, highly acidic	ha1-R	GTG ACG TCG CGC CTG ATC CAG

phates, 0.7 $\mu g \cdot \mu l^{-1}$ bovine serum albumin (Sigma-Aldrich), 0.6 unit of *Taq* DNA polymerase (Sigma-Aldrich), and 2 μl of template genomic DNA or cDNA. Molecular biology grade water was added to 20 μl. PCR parameters were as indicated above except that the annealing temperature was lowered to 58°C. PCR products were resolved by agarose gel electrophoresis as described above.

In order to verify the specificity of the 3′UTR-specific PCR assays, some of the PCR products were subsequently sequenced using degenerate primer 1 as the sequencing primer.

Nucleotide sequence accession numbers. mRNA sequences generated in this study were deposited in GenBank under accession numbers DQ012508 to DQ012535. PCR products sequenced with degenerate primer 1 as the sequencing primer were deposited in GenBank under accession numbers EF158401 to EF158426.

RESULTS

Cloning, sequencing, and classification of elicitin-encoding sequences. A subset of 15 *Phytophthora* isolates (Table 1) was selected to study the elicitin expression pattern through mRNA sequencing. Using a combination of oligo(dT) primer and a degenerate oligonucleotide designed ahead of the 5′ end of the elicitin coding sequence, reverse transcription-PCR of the mRNA extracted for each of these strains showed a strong smeared signal corresponding to a 450- to 550-bp product. For each isolate, the entire amplicon was cloned and 32 individual clones were randomly selected and checked for insert size. Inserts ranged from ca. 480 to 570 bp. Therefore, for each isolate, one to three clones of different sizes were selected for sequencing. A total of 28 different sequences were obtained with clones generated from the 15-isolate panel that were translated along with two unpublished class I elicitin sequences from *P. cambivora* isolate 143 (PC_cam1) and *P. fragariae* var. *rubi* isolate 486 (PFR_fra1) (F. Panabières, unpublished data).

Translation of the 28 cDNA sequences resulted in five distinct amino acid sequences (Fig. 1), which belonged to the class I (98 amino acids [aa]) and class II (99 aa) elicitins, according to the classifications of Ponchet et al. (26) and Qutob et al. (27). The unrooted phylogram based on parsimony analysis separated the sequences into three classes, corresponding to acidic, basic, and highly acidic elicitins (Fig. 2).

First, two amino acid sequences deduced from five cDNA

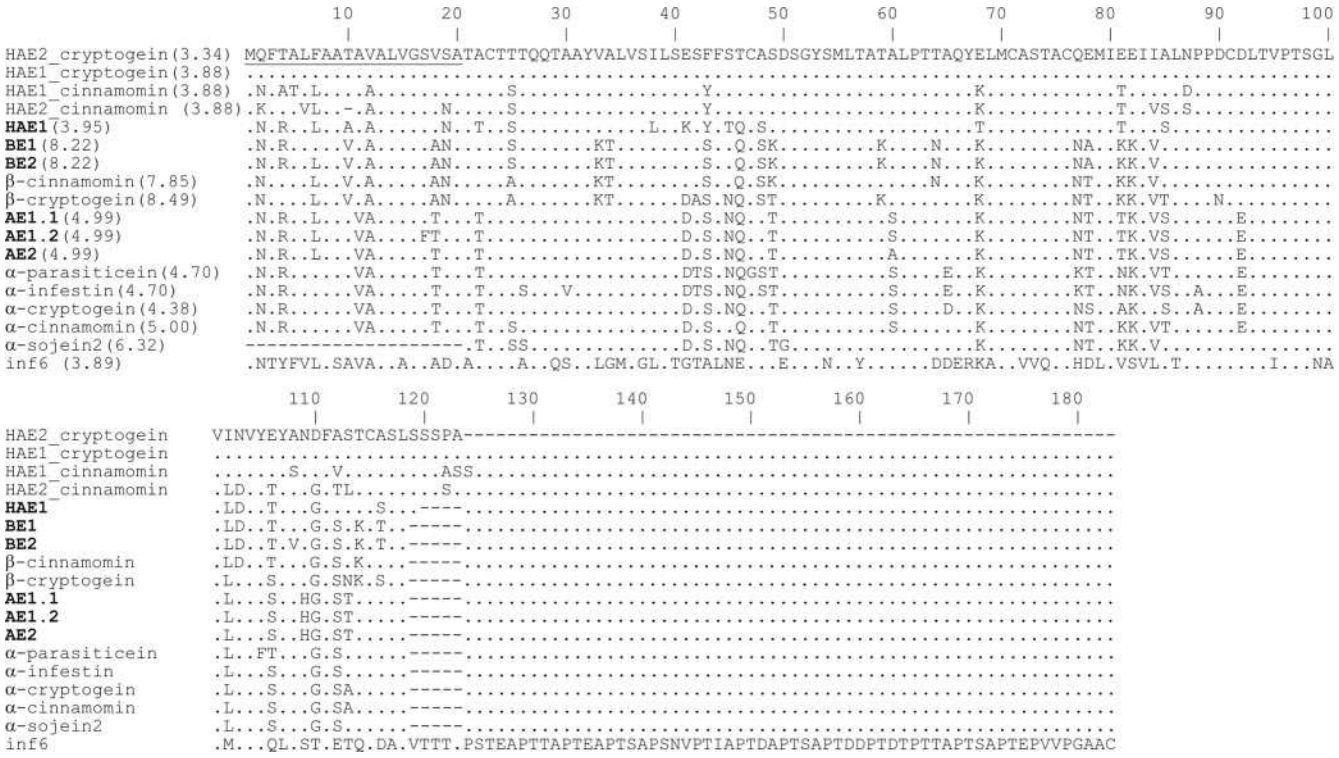

FIG. 1. Multiple-amino-acid sequence alignment of the elicitins characterized in this study for *P. alni*, *P. cambivora*, and *P. fragariae* (AE1.1, AE1.2, AE2, BE1, BE2, and HAE1) and well documented acidic (α), basic (β), and highly acidic (HAE) elicitins retrieved from the GenBank database: *P. cryptogea* Z34462, Z34459, Z34460, Z34461 (24); *P. infestans* AY830090 (17); *P. cinnamomi* AJ000071 (11); *P. sojae* AJ007859 (1); and *P. parasitica* S67432 (6). The sequence corresponding to the signal peptide is underlined. pI are indicated in parentheses, following sequence references.

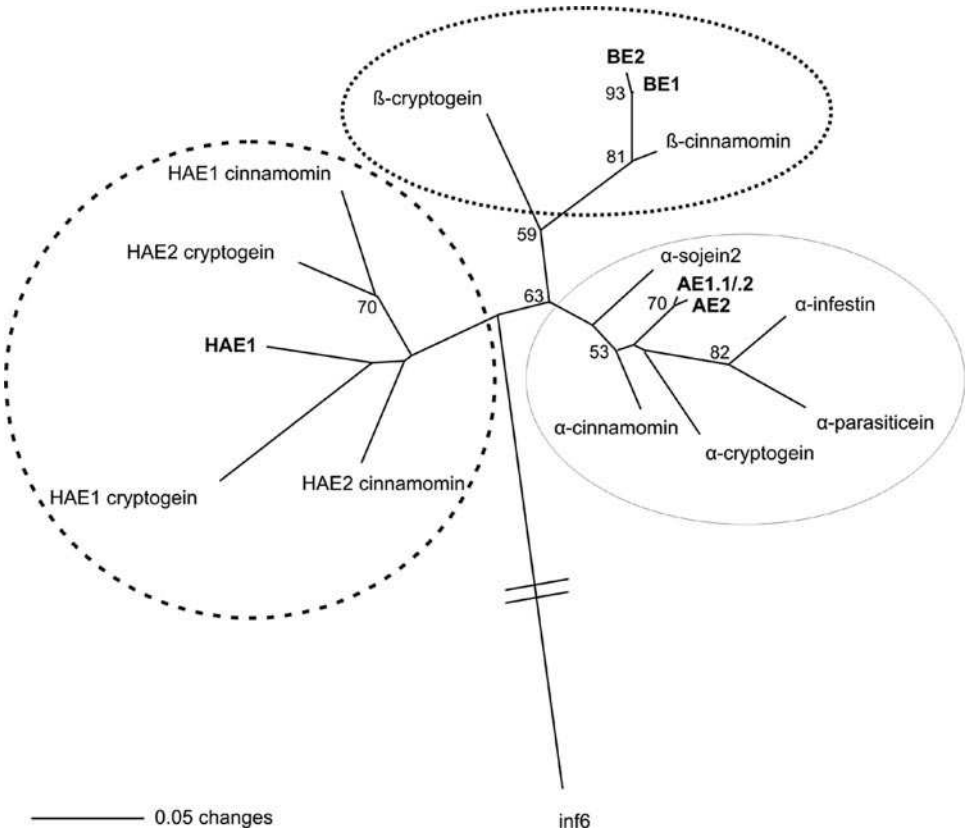

FIG. 2. Phylogenetic relationships between elicitins, inferred from the sequence alignment of the mature polypeptides. The unrooted phylogram was constructed using a parsimony analysis and the neighbor-joining method, based on the multiple alignment of elicitin sequences listed in Fig. 1 and using the *P. infestans* inf6 sequence as an outgroup. Bootstrap values (>50%) from 10,000 replicates are indicated. AE1.1/0.2 and AE2 fall into the class I acidic elicitin group (solid line), whereas BE1 and BE2 are class I basic elicitins (dotted line). HAE1 is closely related to class II highly acidic elicitins (dashed line).

clones, including PC_cam1, were identified as basic elicitins. They displayed a predicted pI of 8.22 and several key signatures such as the K13 frequently observed in basic elicitins (26). The two proteins differed only by a single A84V substitution and were therefore designated BE1 and BE2 (basic elicitin).

An alignment of the 98-aa core region corresponding to the mature protein (excluding the 20-aa peptide signal sequence) allowed the identification of two other proteins, differing by a single S40A substitution, among a set of 23 sequences. From alignment with published sequences and from their predicted pI of 4.99, they were clearly identified as acidic elicitins and named AE1 and AE2 (acidic elicitin). Extending the comparison to the entire amino acid sequence further split AE1 into two proteins, which differed by a single S17F mutation in the peptide signal sequence, and these were designated AE1.1 and AE1.2.

Last, a third type of elicitin protein was identified. The deduced peptide comprised 119 aa, including the peptide signal sequence, and displayed a predicted pI of 3.95. From alignment with known elicitin sequences, it was considered to belong to class II of highly acidic elicitins and was accordingly called HAE1 (11, 26).

Elicitin-encoding mRNAs display important 3′UTR variability. The diversity of elicitin-encoding sequences was also investigated at the nucleotide level on the 3′UTR (Fig. 3).

First, the two cDNA sequences encoding the putative highly acidic elicitin, including the 3′UTR, were identical. This 3′UTR region was consequently designated ha1.

Second, sequences encoding basic elicitins were highly similar, diverging by one to four synonymous substitutions and by a C/T transversion leading to the A84V substitution in the coding region. In addition, the 3′UTRs of the different clones derived from *P. alni* sensu lato were strictly identical over the entire 168-bp region, with the exception of a single G/C transversion in the transcript obtained from isolate PAA162 (data not shown). This 3′UTR sequence was designated b1. The PC_cam1 sequence was more divergent, displaying 8 synonymous substitutions in the coding region and 14 substitutions, as well as a 5-bp deletion, in the 3′UTR. This 3′UTR sequence was called b2.

Last, for acidic elicitins, 11 clones encoding AE1.1 and 7 cDNA clones encoding AE1.2 were examined. They displayed extensive conservation in the coding region, as only three synonymous mutations further differentiated the two groups. The polymorphism was much higher in the 3′UTRs, which could be

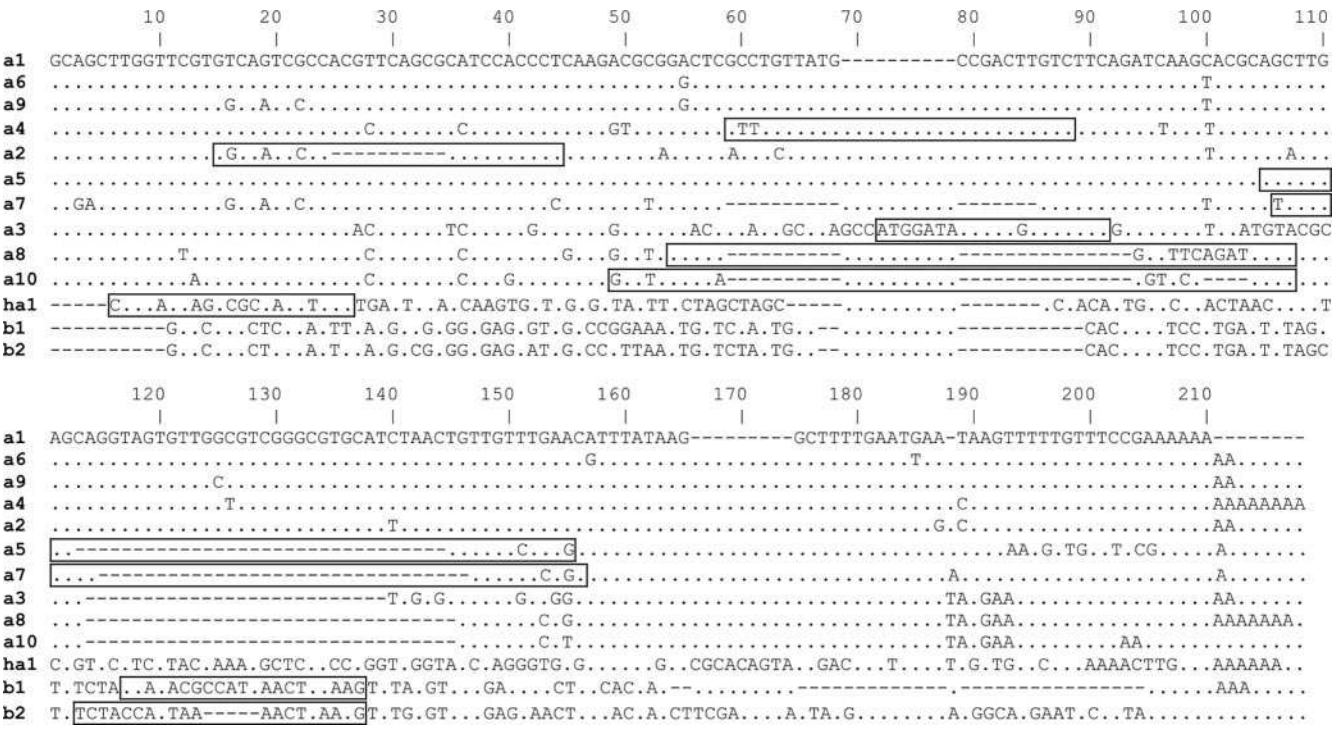

FIG. 3. Sequence alignment of the regions corresponding to the 3′UTRs of the mRNAs, deduced from cDNA sequencing. 3′UTR-specific groups are indicated on the left and correspond to the clustering of identical or nearly identical sequences. Fourteen different 3′UTR groups were defined among the 28 sequences obtained from our 15-isolate panel. The different 3′UTR groups were designated according to the elicitin class, i.e., a for acidic, ha for highly acidic, and b for basic elicitins. For each 3′UTR, the boxed sequence represents the polymorphic region from which a 3′UTR-specific primer could be designed. The PFF309b 3′UTR sequence was so divergent that it could not be properly aligned. PFF309b was therefore not represented, but a specific reverse primer could be designed (a11-R).

classified into 11 3′UTR groups, called a1 to a11. The diversity was the outcome of frequent insertions and deletions as well as single-nucleotide polymorphisms.

Overall, there was no obvious correlation between the classification of 3′UTRs and the protein sequence deduced from the coding region (Table 2). AE2 was associated with a3, a4, a9, and a11 3′UTRs. Similarly, AE1.1 was associated with a1, a2, a6, a7, a8, a9, and a10 3′UTRs, while AE1.2 was associated with a1, a2, a3′, and a5 3′UTRs. As a consequence, a given 3′UTR could be associated with two different protein sequences.

Expression and distribution of the different elicitin genes. The occurrence of the whole set of genes revealed by sequencing of cDNAs was further investigated through 3′UTR-specific PCR tests. Specific primers could be designed for 11 out of the 14 3′UTR groups (Table 3). PCR assays were carried out first with the cDNAs from the 15 isolates of the panel, then with genomic DNA extracted from a large set of 101 *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis*, *P. alni* subsp. *uniformis*, *P. cambivora*, *P. fragariae* var. *fragariae*, and *P. fragariae* var. *rubi* isolates and other *Phytophthora* and *Pythium* species (Table 1).

Results of the 3′UTR-specific PCR tests with cDNAs for *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis*, and *P. alni* subsp. *uniformis* were identical for all isolates of a given taxon, suggesting that the diversity observed in the elicitin-encoding sequences does not correspond to individual variation, but rather to a high level of elicitin complexity among the three taxa (Fig. 4).

In addition, the results of 3′UTR-specific PCR tests conducted on genomic DNA of the 101 *Phytophthora* and *Pythium* isolates (Table 1) were in complete agreement with those obtained on the cDNAs from the 15-isolate panel and showed that, except for *P. cambivora*, the elicitin gene patterns were conserved among the different isolates of each taxon (Fig. 4). Sequencing of a subset of 3′UTR-specific PCR products generated with genomic DNA extracts confirmed their assignation to specific 3′UTRs and resolved three additional 3′UTR sequences, called a2′, a3′, and a10′ and a10′′, slightly different from the original cDNA sequences a2, a3, and a10, respectively (Table 2).

The distributions of the various acidic elicitin-related 3′UTRs with cDNA and with genomic DNA were identical, demonstrating that the whole elicitin gene content was actually expressed in the current culture conditions. As a single exception, ha1 was amplified from the genomic DNA of *P. cambivora* isolates, whereas it was not detected in the cDNA, indicating that, for this species, this class of genes was not expressed during our vegetative-growth conditions.

Overall, *P. alni* subsp. *alni* displayed an elicitin gene pattern that combined those observed for *P. alni* subsp. *multiformis* and for *P. alni* subsp. *uniformis*, with the exception of the a8 3′UTR, which could not be observed in *P. alni* subsp. *alni*, due to unexpected cross-annealing of the a8-R PCR

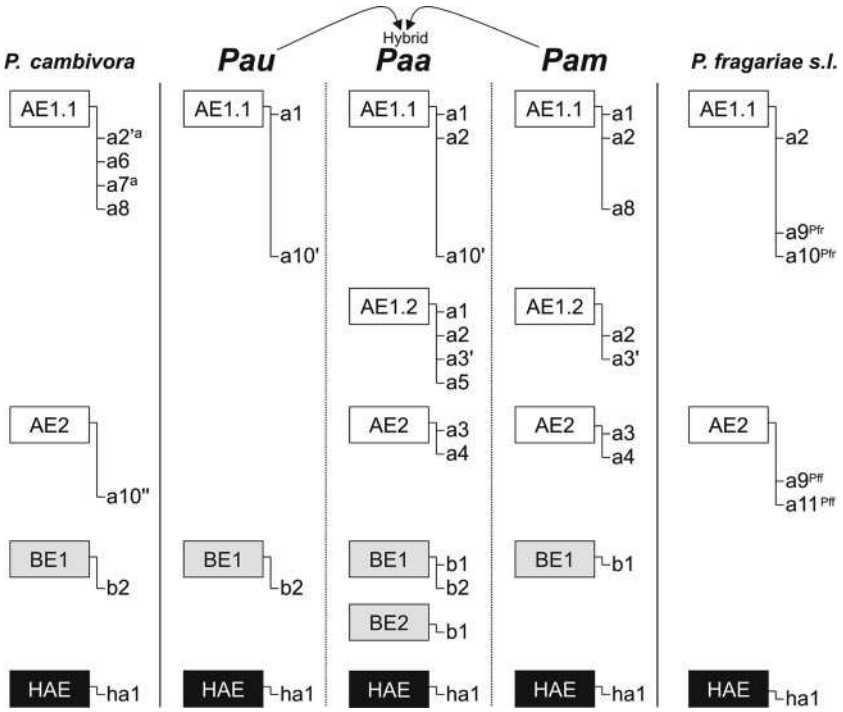

FIG. 4. Distribution of the acidic, basic, and highly acidic elicitin genes resolved in this study for *P. alni* subsp. *alni* (*Paa*), *P. alni* subsp. *multiformis* (*Pam*), *P. alni* subsp. *uniformis* (*Pau*), and the phylogenetically close species *P. cambivora* and *P. fragariae* sensu lato, as inferred from sequencing of cDNA and 3′UTR-specific PCR tests conducted on genomic DNA and cDNA libraries. Coding sequences are boxed and were found to be associated with different 3′UTRs. The occurrence of a1, a6, and a9 3′UTRs is deduced only from sequencing, as no 3′UTR-specific primer could be designed from these sequences. With the exception of the HAE1 associated with the ha1 3′UTR in *P. cambivora*, all the elicitin genes we resolved were shown to be expressed in our 15-isolate panel by 3′UTR-specific PCR with cDNA libraries. [a], only for *P. cambivora* isolate PCjc17; [Pff], obtained for *P. fragariae* var. *fragariae*; [Pfr], obtained for *P. fragariae* var. *rubi*.

primer with the a1 3′UTR sequence, and of the a5 3′UTR, which could not be observed in *P. alni* subsp. *multiformis*, due to unexpected cross-annealing with the a3′ 3′UTR sequence. These cross-annealing events were suggested by in silico annealing tests and further unambiguously demonstrated by sequencing the PCR products (data not shown). Additionally, the occurrence of the BE2 elicitin gene in *P. alni* subsp. *multiformis* or in *P. alni* subsp. *uniformis* could not be verified since all the sequenced products generated by b1-specific PCR corresponded to a gene encoding BE1 (Table 2). The BE2-encoding sequence may be either (i) present in the hybrid while not present in the progenitors, thus representing an autapomorphic feature (a derived characteristic unique to a given taxon or monophyletic group) that would have been generated during or after the hybridization event, or (ii) not detectable by sequencing PCR products because of underrepresentation in comparison with BE1-encoding sequences.

In contrast with *P. alni* sensu lato, analysis of *P. cambivora* and *P. fragariae* cDNAs revealed a patchy distribution of the various acidic elicitin-related 3′UTRs. The a8 3′UTR was detected in the cDNAs from the two strains of *P. cambivora* (the sequence of a6 previously obtained from these strains did not allow the design of specific primers), but only isolate PCjc17 appeared to express additional 3′UTRs, namely, a2′ and a7 3′UTRs. Similarly *P. fragariae* var. *fragariae* and *P. fragariae* var. *rubi* isolates shared a2 and a9 3′UTRs (whose sequences did not allow the design of specific primers), but only the *P. fragariae* var. *rubi* isolate expressed an additional gene containing a10, while a11 appeared to be specific to the *P. fragariae* var. *fragariae* isolate.

Finally, 3′UTR-specific PCRs did not yield any positive signal with other *Phytophthora* or *Pythium* species (Table 1), confirming that the primers designed in this study are specific to *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis*, *P. alni* subsp. *uniformis*, *P. cambivora*, and *P. fragariae* and that these five taxa share particular evolutionary relationships.

DISCUSSION

Taken as a whole and inferred both from sequence data and from 3′UTR-specific PCR amplifications, the elicitin gene family is more diverse in *P. alni* subsp. *alni* and *P. alni* subsp. *multiformis* than in the diploid or nearly diploid taxa *P. alni* subsp. *uniformis*, *P. cambivora*, *P. fragariae* var. *fragariae*, and *P. fragariae* var. *rubi*. This complexity is consistent with the hybrid status for *P. alni* subsp. *alni* but also supports the hypothesis that *P. alni* subsp. *multiformis* is probably also an ancient allopolyploid taxon (15). In addition, most of the mRNA patterns observed in the allopolyploid hybrid *P. alni* subsp. *alni* are a composite of the mRNA patterns from its putative progenitors, *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis*. In this respect, we showed that, at least for the elicitin genes, the different genomes are currently expressed in the hybrid *P. alni*

subsp. *alni*. However, additional data would be needed to demonstrate that the entire distinct genome sets are still coexpressed in *P. alni* subsp. *alni*. Garcia-Olmedo et al. (12) and Volkov et al. (34) showed that, in the course of evolution of protein-coding genes in allopolyploid plants, the alleles of one parental species are mainly transcriptionally active, whereas the alleles from the other parent are gradually transformed into pseudogenes. By contrast, the results presented here suggest that gene silencing is not yet observed for this highly expressed family of elicitin genes in the allopolyploid hybrid *P. alni* subsp. *alni*. These results strengthen the hypothesis that this taxon is of recent origin and still evolving (3), which is in good accordance with its recent emergence as an aggressive alder pathogen (14).

More unexpected, from an evolutionary point of view, is the association of given 3′UTRs, namely, those of a1 and a2, with two distinct coding regions in the hybrid *P. alni* subsp. *alni* and in *P. alni* subsp. *multiformis*, since a higher selection pressure is assumed to have been exerted on the coding regions. In this respect, it may be hypothesized that recent (*P. alni* subsp. *alni*) or likely more ancient (*P. alni* subsp. *multiformis*) hybridization events may have been followed by recombination with coding sequences from paralogs present in the putative parental species. In polyploid plants and animals, chromosomal reorganization and gene silencing generally occur rapidly and may be so extensive that the genome is no longer structured as an allopolyploid (31). *P. alni* subsp. *alni* still appears structured as an allopolyploid taxon since previous studies demonstrated that *P. alni* subsp. *alni* combined the alleles of its progenitors, *P. alni* subsp. *uniformis* and *P. alni* subsp. *multiformis*, for a series of single-copy genes (15) and for microsatellite loci (16). However, this study demonstrates that, similar to the additivity observed in the ribosomal DNA internal transcribed spacer (3), at least for the allopolyploid taxon *P. alni* subsp. *alni*, genetic recombination also occurred between paralogs of the elicitin genes.

Except for the observed new combinations between coding sequences and 3′UTRs, the genomic elicitin pattern of *P. alni* subsp. *alni* combined as expected those of the putative parental taxa, *P. alni* subsp. *multiformis* and *P. alni* subsp. *uniformis*. In addition, while *P. alni* subsp. *uniformis* shared several 3′UTRs with *P. cambivora*, *P. alni* subsp. *alni* and *P. alni* subsp. *multiformis* possessed private sequences, e.g., a3, a4, and b1. These sequences were found neither in *P. cambivora* nor in *P. fragariae*. These findings confirm the close relationship between *P. cambivora* and *P. alni* subsp. *uniformis* (3, 15). Conversely, both *P. cambivora* and *P. fragariae* displayed specific elicitin sequences not found in *P. alni* subsp. *alni*, in agreement with previous results rejecting the hypothesis that these species may have been the putative parents of the hybrid *P. alni* subsp. *alni* (15).

Furthermore, *P. alni* subsp. *alni*, *P. alni* subsp. *multiformis*, *P. alni* subsp. *uniformis*, *P. cambivora*, and *P. fragariae* seem to have retained identical genes independently of the speciation. This phenomenon has been partially shown for *P. cactorum* and *P. pseudotsugae* (26), but cactorein and pseudotsugaein genes possess distinct 3′UTR sequences (F. Panabières, unpublished data). Jiang et al. (18) demonstrated that the main diversification events of elicitin genes occurred before *Phytophthora* radiation and that elicitin genes of a given clade, such as the one analyzed in the present work, are under purifying selection. In this respect, the duplication of elicitin genes, creating paralogs, could also explain the multiplicity of divergent 3′UTR sequences for a given elicitin gene, with the assumption of a lower selection pressure on this part of the gene. It is likely that the diversity of elicitin genes in *P. cambivora*, *P. fragariae*, and *P. alni* reflects duplication events prior to the radiation of these species from their common ancestor. It would also indicate that the radiation of these species is of particularly recent origin (7), which seems to be supported by the cross-amplification of microsatellite markers in all three species (16). However, the unexpected conservation of several 3′UTR regions for these different species (e.g., ha1, b2, a8) could also be the outcome of reticulation or introgression events. Gene transfer after speciation should not be ruled out as a possibility to explain the elicitin gene patterns observed within this clade.

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