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Genetic analysis of environmental strains of the plant pathogen *Phytophthora capsici* reveals heterogeneous repertoire of effectors and possible effector evolution via genomic island

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One sentence summary: Mobile genomic islands contribute to effector evolution in Phytophthora.
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ABSTRACT

Phytophthora capsici is a virulent oomycete pathogen of many vegetable crops. Recently, it has been demonstrated that the recognition of the RXLR effector AVR3a1 of *P. capsici* (PcAVR3a1) triggers a hypersensitive response and plays a critical role in mediating non-host resistance. Here, we analyzed the occurrence of PcAVR3a1 in 57 isolates of *P. capsici* derived from globe squash, eggplant, tomato and bell pepper cocultivated in a small geographical area. The occurrence of PcAVR3a1 in environmental strains of *P. capsici* was confirmed by PCR in only 21 of these pathogen isolates. To understand the presence-absence pattern of PcAVR3a1 in environmental strains, the flanking region of this gene was sequenced. PcAVR3a1 was found within a genetic element that we named PcAVR3a1-GI (PcAVR3a1 genomic island). PcAVR3a1-GI was flanked by a 22-bp direct repeat, which is related to its site-specific recombination site. In addition to the PcAVR3a1 gene, PcAVR3a1-GI also encoded a phage integrase probably associated with the excision and integration of this mobile element. Exposure to plant induced the presence of an episomal circular intermediate of PcAVR3a1-GI, indicating that this mobile element is functional. Collectively, these findings provide evidence of PcAVR3a1 evolution via mobile elements in environmental strains of *Phytophthora*.

Keywords: *Phytophthora capsici*; RXLR effector; AVR3a1; integrase; genomic island; vegetable crops

INTRODUCTION

Oomycetes are eukaryotic microbes that contain several species of plant pathogens. Some members of the genus oomycete *Phytophthora*, including *Phytophthora palmivora, P. cinnamomi, P. ramorum, P. parasitica*, *P. sojae*, *P. infestans* and *P. capsici*, are remarkably destructive to an extensive diversity of plant species, causing damages to both agricultural crops and natural ecosystems (Hansen, Reeser and Sutton 2012). Some of these species of *Phytophthora,* such as *P. sojae and P. infestans*, have a

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narrow host range (Tyler *et al.* 2006; Sanju *et al.* 2013), whereas other species, such as *P. parasitica* and *P. capsici*, have a wide host range (Panabieres *et al.* 2005; Lamour *et al.* 2012a). However, the molecular aspects of this differential plasticity to colonize plants are unknown. Particularly, *P. capsici* has revealed notable adaptation to chemical treatments (Lamour and Hausbeck 2000) and causes Phytophthora blight in many solanaceous hosts (Lamour *et al.* 2012b), inflicting significant losses in crop yield worldwide. The extreme plasticity of *P. capsici* could be associated with the presence of many mobile elements in its genome (Lamour *et al.* 2012a). Nevertheless, little is known of the molecular and ecological characteristics of *P. capsici* mobile elements, including their intrinsic mobilization capability and distribution among environmental strains.

To establish infection, *Phytophthora* plant pathogens translocate effectors into host plant cells (Armstrong *et al.* 2005; Wawra *et al.* 2012). Effectors can be recognized by plant disease resistance (R) proteins, resulting in modulation of the expression of genes involved in the hypersensitive response and plant cell death (Bos *et al.* 2010). Some effectors contain a conserved amino acid motif, RXLR (where R is arginine, X is any amino acid and L is leucine), necessary for their translocation (Whisson *et al.* 2007), and a C-terminal domain required to modulate plant defense (Dou *et al.* 2008). The best understood RXLR effector of Oomycetes is PiAVR3a from *P. infestans* (Armstrong *et al.* 2005; Bos *et al.* 2010; Wawra *et al.* 2012; He *et al.* 2015; Sanju *et al.* 2015), but its PsAVR1a and PcAVR3a homologous proteins from *P. sojae* and *P. capsici* respectively have also been characterized as functional effectors involved in plant pathogenesis (Dou *et al.* 2008; Vega-Arreguín *et al.* 2014). The recognition of PcAVR3a plays a critical role in the natural non-host resistance to *P. capsici* in *Nicotiana* species (Vega-Arreguín *et al.* 2014). In this study, the analysis of the occurrence of PcAVR3a1 and the PcAVR3a1 genetic region of environmental strains of *P. capsici* revealed that PcAVR3a1 is in a low frequency in natural isolates and is included into a mobile element, suggesting that this effector can rapidly evolve via mobile elements in natural conditions.

MATERIALS AND METHODS

Sample collection and microbial strains

The microbial strains used in this study were 57 strains of *P. capsici* isolated from commercial vegetable production of globe squash (*Cucurbita maxima* Duchesne ssp. maxima var. Zapallitina Grebensc), eggplant (*Solanum melongena*), tomato (*Solanum lycopersicum*) and bell pepper (*Capsicum annuum*) collected in the Pampa Region of Argentina (Cuyeu *et al.* 2013), specifically in a small area located between 34°19′14.05″S, 59°11′58.87″W and 34°41′33.75″S, 58°54′4.25″W (Table 1).

For strain isolation, infected plants were thoroughly washed with tap and sterile distilled water. Small sections of tissue were then excised from the edge of the expanding lesion at every organ and transferred to V8 agar (V8A) plates. Plates were incubated for 2 days at 24°C. Hyphal tips were subcultured from

Table 1. PcAVR3a1 occurrence in environmental *P. capsici* strains.

Phytophthora capsici		Plant host			*Phytophthora capsici*		Plant host		
Strains	ITS locus (accession number)	Species	Organs	PCR-AVR3a1	Strains	ITS locus (accession number)	Species	Organs	PCR-AVR3a1
CT1	KF746518	Eggplant	Shoot	+	CT30	KF746546	Globe squash	Fruit	–
CT2	KF746519	Eggplant	Fruit	–	CT31	KF746549	Globe squash	Fruit	–
CT3	KF746520	Eggplant	Fruit	+	CT32	KF746551	Eggplant	Fruit	+
CT4	KF746521	Eggplant	Fruit	–	CT33	KF746559	Globe squash	Fruit	–
CT5	KF746523	Globe squash	Fruit	–	CT34	KF746574	Globe squash	Fruit	–
CT6	KF746524	Globe squash	Stem	–	CT35	KF746576	Tomato	Fruit	–
CT7	KF746525	Globe squash	Stem	+	CT36	KF746577	Tomato	Fruit	+
CT8	KF746526	Globe squash	Fruit	–	CT37	KF746579	Tomato	Fruit	–
CT9	KF746535	Tomato	Fruit	+	CT38	KF746582	Globe squash	Stem	–
CT10	KF746528	Globe squash	Fruit	–	CT39	KF746547	Globe squash	Stem	+
CT11	KF746529	Globe squash	Fruit	–	CT40	KF746561	Globe squash	Stem	–
CT12	KF746532	Eggplant	Fruit	–	CT41	KF746564	Globe squash	Stem	–
CT13	KF746537	Globe squash	Fruit	+	CT42	KF746565	Globe squash	Stem	–
CT14	KF746538	Globe squash	Fruit	–	CT43	KF746567	Globe squash	Stem	+
CT15	KF746539	Pepper	Stem	+	CT44	KF746570	Globe squash	Stem	–
CT16	KF746540	Globe squash	Stem	–	CT45	KF746578	Eggplant	Fruit	–
CT17	KF746541	Globe squash	Fruit	–	CT46	KF746580	Eggplant	Fruit	+
CT18	KF746542	Tomato	Fruit	–	CT47	KF746584	Globe squash	Fruit	+
CT19	KF746543	Globe squash	Fruit	+	CT48	KF746585	Globe squash	Fruit	–
CT20	KF746544	Eggplant	Shoot	–	CT49	KF746586	Globe squash	Stem	+
CT21	KF746545	Eggplant	Fruit	+	CT50	KF746587	Globe squash	Stem	–
CT22	KF746548	Globe squash	Fruit	–	CT51	KF746558	Globe squash	Stem	–
CT23	KF746552	Eggplant	Fruit	+	CT52	KF746560	Pepper	Stem	+
CT24	KF746553	Eggplant	Fruit	–	CT53	KF746569	Globe squash	Fruit	–
CT25	KF746557	Globe squash	Stem	–	CT54	KF746581	Globe squash	Fruit	–
CT26	KF746562	Eggplant	Stem	–	CT55	KF746568	Globe squash	Fruit	+
CT27	KF746571	Globe squash	Stem	–	CT56	KF746583	Globe squash	Fruit	–
CT28	KF746572	Globe squash	Stem	+	CT57	KF746586	Globe squash	Fruit	+
CT29	KF746573	Tomato	Fruit	+					–

actively expanding mycelium and transferred to V8A plates. For long-term storage, agar plugs of mycelium were stored in 2-ml Eppendorf tubes with 1 ml of sterile distilled water and three sterile and pregerminated bird seeds. All *P. capsici* isolates were morphologically identified based on zoosporangium and oogonium characters and the growth at specific temperatures (Erwin and Ribeiro 1996). The sporangia used for identification purposes were produced by growing cultures on V8A at 24°C under natural light conditions for 2 days. As described above, the preliminary taxonomic assignation of these isolates to *P. capsici* based on phenotype characteristics was confirmed by molecular phylogenetic analyses.

DNA extractions

For DNA isolation from *in vitro* cultures of *P. capsici*, strains were transferred to fresh V8A and grown in the dark at 24°C for 7 days. The mycelium of each isolate was then harvested and freeze-dried, and high-molecular weight DNA was extracted according to Ristaino *et al.* (1998). To detect the existence of circularized forms of PcAVR3a1-GI, tomato plants were infected with *P. capsici* strain CT21. For the inoculation of tomato plants, leaves attached to the plant were infiltrated in the abaxial side, using a needleless syringe with 15 μl of zoospore solution containing 2500 zoospores, and then the plants were kept at 25°C for 1 week. DNA extraction from plants infected with pathogens was carried out according to Garcia *et al.* (2014).

Sequence analyses of strains

The fragments of the ITS loci from the environmental *P. capsici* strains were obtained by PCR amplification using ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GGAAGTAAAAGTCGTAACAAGG-3′) primers according to White *et al.* (1990). The full length of the PcAVR3a1 gene from *P. capsici* strains was obtained by PCR amplification using primers AVR3a1-up (5′-ATGCGTCTTTCCTTCCTGTTG-3′) and AVR3a1-low (5′-TCAATAAACCAGGTGGAGCG-3′) and a program of 1 cycle of 94°C for 3 min, 34 cycles of 94°C for 1 min, 63°C for 30 s and 72°C for 2 min, and a final cycle of 72°C for 10 min. The amplification fragments were cloned into the pGEM-t easy vector (Invitrogen), and sequenced using T7 and SP6 universal primers by Macrogen (Korea). To study the genomic region flanking the PcAVR3a1 gene from *P. capsici* strain CT21, a genomic walking assay (Ayub *et al.* 2015) was performed using walk1 (5′-GACATCTTGTTTTGGTCGGAAGTTGCGTTACAAG-3′) and walk2 (5′-GGCAAGTACGATCGGGTCTACAACGGGTA-3′) primers. For detection of the episomal circular intermediate (ECI), PCR was performed by using ECI-1 (5′-TTATGCAATTTGCTTTACCATACA-3′) and ECI-2 (5′-TCTTAATCAGCTCATGCTTGCACTG-3′) primers and a PCR condition of 1 cycle of 94°C for 5 min, 34 cycles of 94°C for 1 min, 65°C for 30 s and 72°C for 1 min and a final cycle of 72°C for 10 min. The amplification fragment was sequenced and found to be identical (100%) to that expected for the ECI of PcAVR3a1-GI. The sequencing reactions were performed at the Centro de Investigación en Ciencias Veterinarias y Agronómicas (CICVyA) of the National Institute of Agricultural Technology (Argentina). Sequence search was performed using BLASTP tools. Evolutionary analysis was conducted using MEGA version 5.0 (Tamura *et al.* 2011). Protein sequences were aligned using the ClustalW program (Thompson, Higgins and Gibson 1994). Phylogenetic trees were constructed using the neighbor-joining (NJ) method with genetic distances computed using the p-distance model and bootstrap analysis of 1000 resamples and root on mid-point.

Nucleotide sequence accession number

The nucleotide sequences of ITS locus and PcAVR3a1-GI obtained here have been deposited in the Genbank, accession numbers: KF746518-KF746586 and KT441013, respectively.

RESULTS AND DISCUSSION

PcAVR3a1 occurrence in environmental strains of *P. capsici*

We explored the presence of environmental strains of *P. capsici* in different organs, including fruit, shoot and stem of globe squash, eggplant, tomato and bell pepper (Table 1). Phylogenetic analysis of the ITS locus confirmed that the 57 isolates belong to *P. capsici* (Fig. 1). In addition, the isolates obtained were closely related (ITS nucleotide identity of >99%), which is expected for isolates from vegetable crops cocultured in a very narrow geographical region. Interestingly, the occurrence of PcAVR3a1 in environmental strains of *P. capsici* was confirmed by PCR in only 21 of the 57 pathogen isolates (Table 1), and we found no association between strain relationships and occurrence of PcAVR3a1 (Fig. 1 and Table 1), suggesting a high degree of evolution of this effector in natural conditions, and at least two populations of *P. capsici* strains containing different repertoires of virulence factors. These DNA fragments share 100% identity among environmental strains of *P. capsici*, and the coding sequences of these genes share 99% identity with the PcAVR3a1 protein from the genome-sequenced strain *P. capsici* LT1534 (Fig. 2). Hence, they were named PcAVR3a1.

PcAVR3a1 is located within a genomic island

To understand the particular PcAVR3a1 pattern in environmental strains of *P. capsici*, the flanking region of PcAVR3a1 PCR-positive *P. capsici* strain CT21 was sequenced (GenBank accession number KT441013). PcAVR3a1 was found within a mobile genetic element (3739 bp) that we termed PcAVR3a1-GI (PcAVR3a1 genomic island) (Fig. 3A). PcAVR3a1-GI was flanked by a 22-bp (5′-TCCTTTTAAAAAGTTCAAAATC-3′) direct repeat (Fig. 2A), which is part of its site-specific recombination site (Fig. 3A). Analysis of PcAVR3a1-GI allowed the identification of a phage integrase, suggesting that PcAVR3a1-GI could have a certain degree of autonomy (Fig. 3A). This integrase was found at the left end of PcAVR3a1-GI, upstream from PcAVR3a1 and in the same orientation (Fig. 3A), and showed similarity to gene products from other *Phytophthora* species (Table S1, Supporting Information).

The identification of a genomic island containing AVR3a1-like effectors in other sequenced *Phytophthora* species was not possible, probably due to the intrinsic instability of these types of mobile elements (Juhas *et al.* 2009). In addition, the evolution of *Phytophthora* effectors could also be mediated by other specialized genetic elements under environmental conditions, including the extensive repertoire of retrotransposons and transposons present in their genomes, which probably play a critical role in genomic instability and pathogenicity (Tyler *et al.* 2006; Haas *et al.* 2009; Lamour *et al.* 2012a).

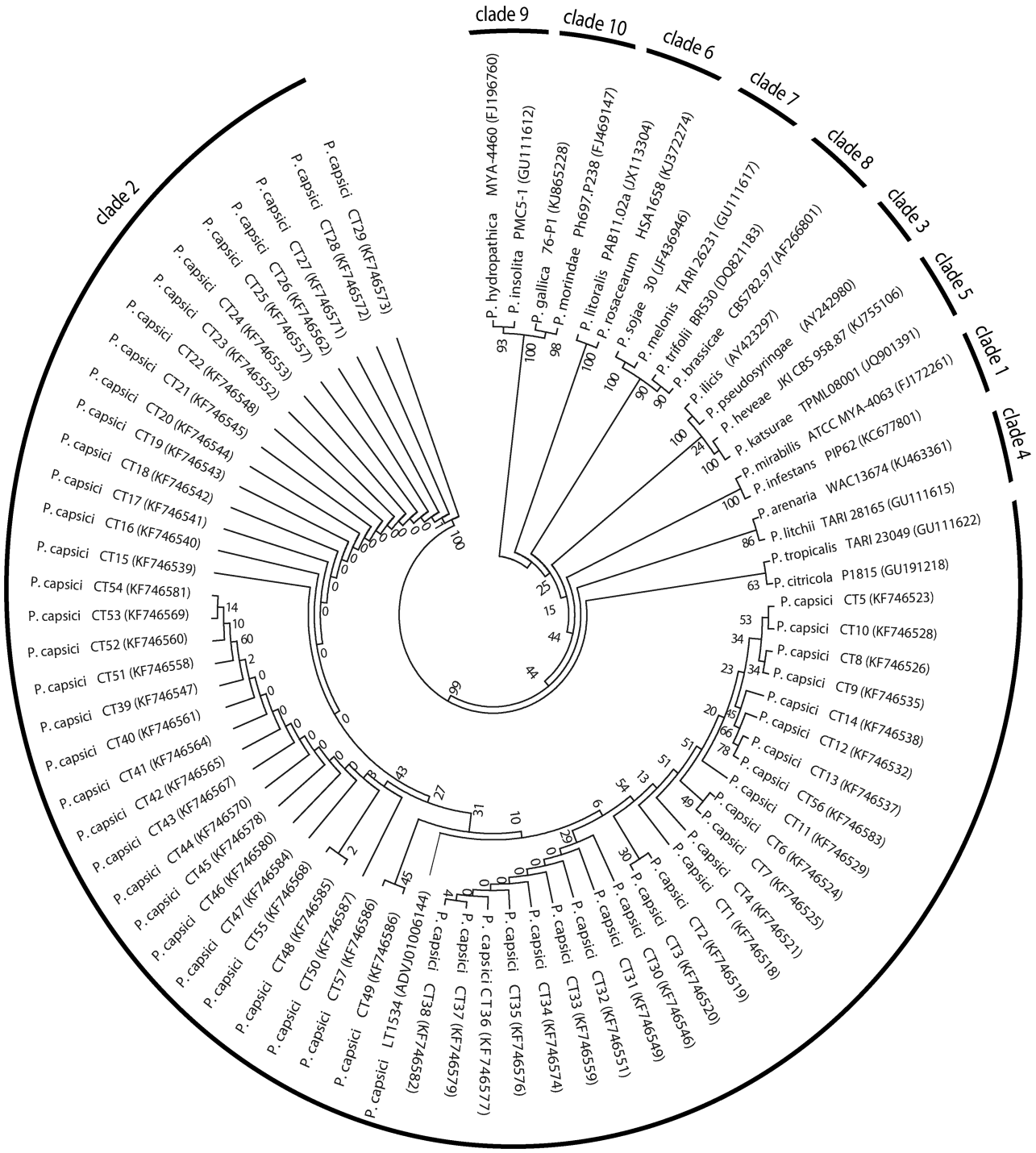

Figure 1. Phylogenetic relationships between representative species of the genus *Phytophthora* environmental *P. capsici* strains based on NJ analysis of the ITS locus. The *Phytophthora* species are divided into 10 clades (Kroon *et al.* 2012). These subclasses are shown. Bootstrap percentages are indicated at the branch points.

PcAVR3a1-GI is functional

Previous studies with the common bean pathogen *Pseudomonas syringae* pv. *phaseolicola* have shown that the exposure to resistance mechanisms acts as the driving force for genome reorganization through the induction of excision of a genomic island containing virulent factors including the effector gene *avrPphB* (Pitman *et al.* 2005). In addition, we have previously shown that a genomic island containing an avirulent factor, the LOV-domain photoreceptor involved in the attenuation of the virulence of *P. syringae* pv. tomato DC3000 in light-exposed leaves, is integrated and excised via an ECI (Moriconi *et al.* 2013). While the function of the genomic island in pathogenicity and its mobilization via ECI have been regularly described in bacteria (Darmon and Leach 2014), the function of these mobile elements in eukaryotic pathogens has not been classically considered and the existence of circularized forms of the excised genomic island DNA in Oomycetes has not been documented.

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                                        **      ****** *      *     ***    *   *
Environmental P. capsici strains        LNGQADGTRFLRAHHESEEESDREERGFTDLFKNEKAAEK
P. capsici strain LT1534 PcAVR3a1       LNGQADGTRFLRAHHESEEESDREERGFTDLFKNEKAAVK
P. sojae strain P7074 PsAvr1b           RNGDIAGGRFLRAHEE-DDAGER-TFSLTDLWN--KVGAK
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Figure 2. Alignment of residues 38–77 of PcAVR3a1 from environmental *P. capsici* strains with the corresponding regions of effector proteins from *P. capsici* strain LT1534 (PcAVR3a1) and *P. sojae* strain P7074 (PsAvr1b) revealing RXLR motif. These four residues are shown in gray.

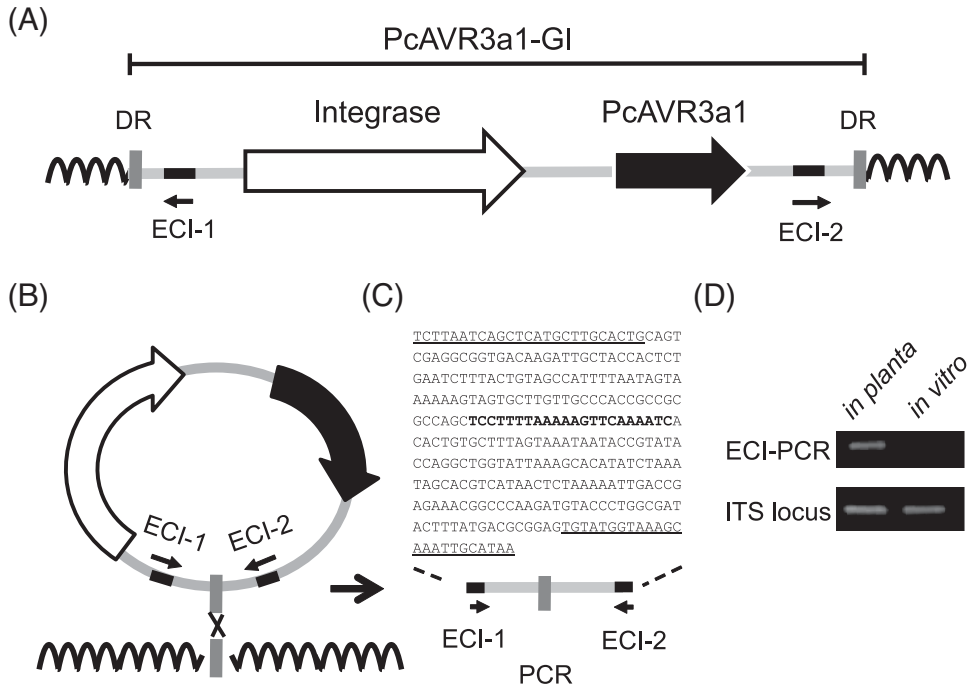

Figure 3. PcAVR3a1 gene is situated within a genomic island (PcAVR3a1-GI). (**A**) Genetic organization of PcAVR3a1-GI; boxes indicate the position of direct repeats (DR); white and black arrows indicate ORFs sharing homology with phage integrases and RXLR effectors, respectively; orientation and localization of ECI-1 and ECI-2 primers using for ECI detection. (**B**) Schematic representation of ECI derived from PcAVR3a1-GI. (**C**) Sequence of the PCR product showing the region containing the 22 bp overlap defining the DR site where circularization occurred (bold) and sequences recognized by primers ECI-1 and ECI-2 (underlined). (**D**) ECI detection *in vitro* and *in planta*; ITS locus-PCR: DNA amplification quality control.

Here, we empirically examined the occurrence of ECI forms of PcAVR3a1-GI in *P. capsici* strain CT21 by using primers ECI-1 and ECI-2 directed outwards from the internal boundaries of PcAVR3a1-GI (Fig. 3B). We obtained a PCR product of 301 bp from tomato-infected plants, but not from *in vitro* cultures of *P. capsici* (Fig. 3B). DNA sequencing of the amplified fragment from *P. capsici in planta* confirmed the presence of a circular molecule (Fig. 3B). These results indicate that PcAVR3a1-GI is a functional genomic island, whereas its mobilization seems to be specifically induced in the plant.

CONCLUSIONS

The function of mobile elements in pathogenic plasticity of *Phytophthora* has been proposed since the discovery of large sets of mobile elements within *Phytophthora* genomes, but the association of these elements with virulence factors has not yet been analyzed in depth. In this work, we showed that the PcAVR3a1 effector of *P. capsici* is situated within a functional genomic island, suggesting that this plant pathogen is able to rapidly change its effector protein repertoire via mobile genetic elements.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

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Conflict of interest. None declared.

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